HAKONE XV

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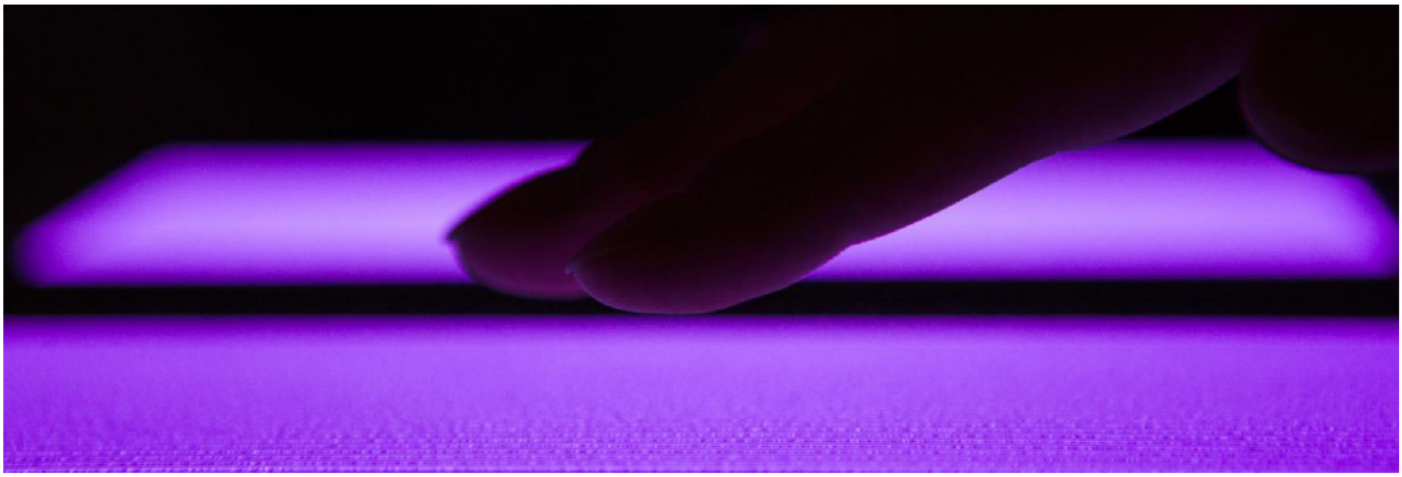
with joint COST TD1208 workshop Non-Equilibrium Plasmas with Liquids for Water and Surface Treatments



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Masaryk University

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DC CORONA DISCHARGE FOR BACTERIAL BIOFILM DECONTAMINATION

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DC corona discharges in air were tested for the eradication of 48-hour Escherichia coli biofilms on glass slides. Thermostatic cultivation and confocal laser scanning microscopy of the biofilm stained with fluorescent dyes were used for biocidal efficiency quantification. Up to 5.7 $\log_{10}$ reduction of bacterial concentration in the biofilm was measured after exposure to DC corona discharges for 15 min. CLSM showed that live/dead ratio in the biofilm was decreasing after plasma treatment and that the biofilm thickness was reduced for both corona polarities. The biofilm biomass loss was supported by crystal violet assay.

Keywords: bacterial biofilm; non-thermal plasma; corona discharge; water electrospray, fluorescence.

1 Introduction

Bacteria on surfaces exist predominantly in the form of biofilms, which are populations of microorganisms concentrated at an interface (usually solid-liquid) and encased in a hydrated matrix of exopolymeric substances (EPS), polysaccharides, and proteins that are produced by the resident microorganisms [1]. EPS protects cells from the outer environment and facilitates cell-to-cell communication (quorum sensing) [2]. Bacteria in the biofilm are protected from harsh conditions (high temperature, low pH, ultraviolet radiation, dehydration, etc.), and therefore are more resistant than their planktonic (suspended cells) counterparts [2]. In order to avoid using toxic chemicals or high concentrations of antibiotics to achieve the desired decontamination efficiency in biofilms, a search for new alternative methods of decontamination is required; one of these is low-temperature plasma.

2 Materials and Methods

2.1 DC corona discharges in air

Corona discharges in atmospheric pressure air were generated in open air in a point to plane geometry. The needle electrode was a sharp or a clipped hypodermic syringe needle connected to a DC high-voltage (HV) power supply. Opposite to the needle HV electrode, a copper plate was grounded through a 50 Ω resistor. Some experiments were performed with sterile distilled water electrosprayed onto the sample, through a hollow clipped HV needle electrode; this was supplied to the needle by pumping with NE-300 SyringePump. Electrical characteristic of the discharge are presented in [3].

2.2 Biofilm on glass

Escherichia coli strain BW25113 F+ was used to form biofilm on glass cover slides (2×2 cm) for 48 hours. One mL a stationary phase culture was diluted 1/100 in buffered M63 medium was placed into each well of a homemade 6-well plate. The samples were incubated without agitation at 30°C for 48 hours with media refreshment after 24 hours.

The biofilms on glass cover slides were taken out from the 6-well plates after 48 hours. The excessive liquid was removed, the samples were dried for 20 min at 35°C, and then placed onto the grounded electrode 0.5 cm from HV electrode. The control samples were dried using the same procedure but not treated with plasma. Cultivability was determined by repetitive rinsing and scraping of the biofilm into 5 mL of 0.85% NaCl saline solution. The solution with recovered bacterial cells was vortexed, serially diluted and spread over LB agar in petri dishes and incubated at 37°C overnight, then the bacterial colony forming units (CFUs) were counted.

For imaging using confocal laser scanning microscopy (CLSM), the treated biofilms were stained with a solution of three fluorescent dyes: Syto9, Propidium iodide (PI) and DAPI (4',6-diamidino-2-phenylindole) in phosphate buffered saline (PBS, pH 7.4), and incubated for 25 min in the dark. CLSM images were acquired with an inverted confocal laser microscope OLYMPUS IX81.

Biofilm biomass was evaluated using crystal violet (CV) staining following the established microbiology protocol [4]. The control and plasma-treated biofilms were resealed in the 6-well plate after treatment and 200 µL of 0.1% crystal violet was introduced into each well. After 10 min incubation the CV solution was carefully removed by pipetting and biofilms were rinsed with deionized water until the waste liquid was clear. The 6-well plate was dried overnight at room temperature. When fully dried, 200 µL of 33% acetic acid was added to each well to solubilize the CV for 15 min, then recovered and diluted in 1/10 deionized water and the absorbance measured at 550 nm.

3 Results and Discussion

3.1 Biofilm cultivation

The initial bacterial concentration in the biofilm was 3.1±6.6 × 10^7 CFU per mL. In both corona polarities (Fig. 1), the decontamination efficiency increased with exposure time and

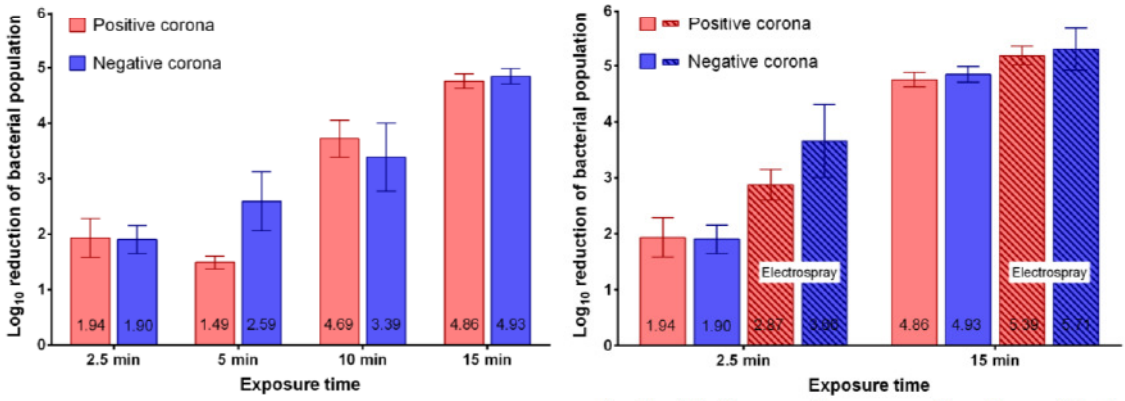

Fig. 1: Logarithmic reduction of bacterial concentration in the biofilm on the cover glass by cold plasma treatment of the corona discharges. Left: Reduction with increasing plasma treatment time. Right: The effect of water electrospray 0.01 mL/min on the sample - hatched bars (mean±SEM, 5–6 repetitions).

reached up to 4.9 $\log_{10}$ reduction for positive and negative corona within 15 min. The electrospray increased bactericidal effect of plasma, for the 2.5 min exposure from 1.9 $\log_{10}$ in both polarities to 2.9 and 3.7 $\log_{10}$ for PC and NC, respectively. For longer exposure time 15 min this increase was from 4.9$\log_{10}$ to 5.4 and 5.7 $\log_{10}$ for PC and NC, respectively.

3.2 Confocal laser scanning microscope results

The fluorescence was acquired from four random examined spots on the biofilm. Examples of reconstructed three-dimensional examined spots of the biofilm are presented in Fig. 2. From this representation we can see that the plasma treated biofilms contain more dead cells (red stained by PI) than the controls (green cells stained by Syto9). The structure of the biofilm also changed with plasma treatment: the biofilm seems denser with more compact cellular structure. After plasma treatment with the water electrospray, the biofilm became thinner and patchy.

Fig. 2: Three-dimensional reconstruction of the biofilm *z*-stacks from CLSM. Red indicates dead cells and green live cells. From the left: control dried 20 min at 35°C, DC corona discharge treated samples for 15 min with or without water electrospray (magnification 60×, section size 211.2×211.2 μm).

From the third fluorescent dye – DAPI, which stains all cells, the biofilm thickness was calculated (Fig.3). It was calculated for all *z*-stacks in all biofilms and the resulted mean values are presented in Fig. 3 (left). There is a trend of reduction of the biofilm thickness with the rising exposure time and it is enhanced by adding the water electrospray to the discharge. The only significant difference was found for NC 15 min with electrospray (12.3 μm) in comparison with the control sample (18.1 μm).

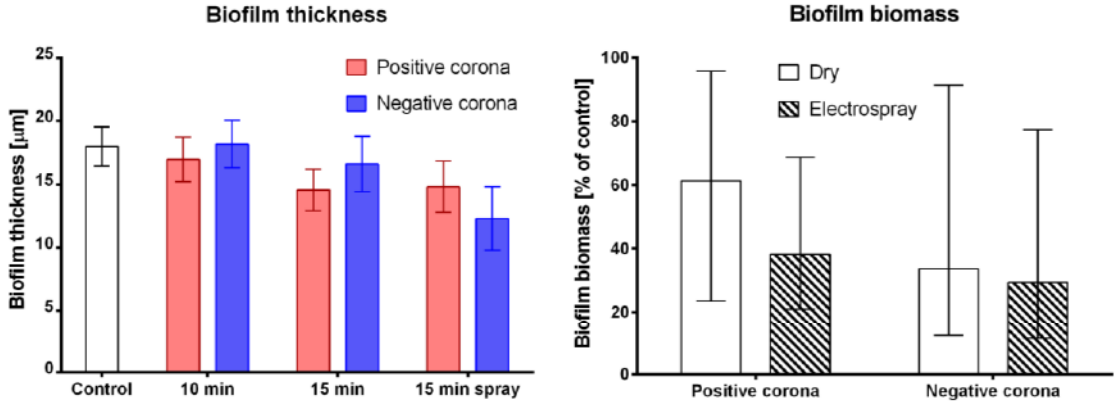

Fig. 3: Left: The biofilm thickness calculated from DAPI fluorescence integrated over the entire biofilm thickness for samples treated (plotted mean ±SEM, 8≤n≤16 in each group). Right: The biofilm biomass calculated as percentage of controls (100%) from crystal violet absorbance after 15 min PC and NC treatment with or without water electrospray. (Median with IQR, n = 10 in each group).

Biofilm biomass after plasma treatment was calculated as the percentage of the control sample absorbance (control sample biomass is equal to 100%), taking into consideration the dispersion in biofilm (controls) thickness over all experiments. Experiments were done for 15

min exposure time, because of the most significant decrease in the attached biomass was expected (Fig.3, right). For PC 61.3% and NC 32.7% of the biomass remained attached to the surface after exposure to those discharges. When the water electrospray was added to the discharge, stronger bacteria detachment occurred and only 38.1% and 29.5% of the biomass remained attached to the cover glass in PC and NC treatment, respectively.

4 Conclusion

Low-temperature (cold) atmospheric pressure plasmas represent a new promising method for surface decontamination from bacterial biofilms. Within 15 min 5.7 $\log_{10}$ reduction in population of the bacterial biofilm was achieved by using DC corona discharges with water electrospray. With CLSM we observed increase in red fluorescence of biofilm after plasma treatment, and the DAPI staining showed thinning of biofilms after plasma treatment (negative corona with electrospray for 15 min), with its thickness decreased from 18.1 μm in the control samples to 12.3 μm after treatment. A substantial biofilm biomass loss was confirmed by the Crystal Violet assay.

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