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Edited by V. Medvecká, J. Országh, P. Papp, Š. Matejčík

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ANTIBACTERIAL EFFECTS OF PLASMA ACTIVATED WATER COUPLED WITH ELECTROPORATION

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Transient Spark discharge operated in atmospheric air with water electrospray, with nanosecond high energy pulses, demonstrated strong and fast antimicrobial effects when bacteria are directly exposed to the discharge. It can also activate water which keeps its antibacterial properties for a few hours after plasma treatment. However, the antibacterial effects of plasma activated water are weaker than in direct exposure. Preliminary experiments shown here are focused to understanding the role of strong electric fields in the overall plasma action to bacterial cells and the effect of electroporation induced by high electric field in combination with chemical effect of plasma activated water.

1. Introduction

Operative bacterial infections are still responsible for several thousand deaths per year, requiring the use of disposable medical equipment or more reliable sterilization. Transient Spark (TS) discharge induced by several kilovolts drop during 20 ns and driving a current of several tens of amperes demonstrated a significant antibacterial effect when combined with water electrospray [1]. However, the mechanisms have not been clearly identified yet. Previously we observed significant bacteria reduction after direct exposure of *E. coli* bacterial suspension to TS with electrospray compared to only Plasma Activated Water (PAW) produced by TS with water electrospray. The difference between the results may be due to short lived reactive oxygen and nitrogen species (RONS).., which were clearly identified as having strong antibacterial effects. However, their extremely short life time limits their detection in the liquid and makes their storage impossible, which also limits a precise estimate of their effects. On the other hand, the electric field between the electrode and the grounded metal grid is a good candidate to explain the enhanced antibacterial effects. Cell membrane can be affected by electric field that may open pores in the cell membrane. This is also known as the electroporation or electro-permeabilization and can lead to antibacterial effects [2, 3]. As electroporation also increases the cell porosity, it allows penetration of RONS produced by the TS plasma into the cell interior. This may result in a high cell mortality compared to the separated effects of the electric fields or the PAW.

Applying a high electric field moves the lipid bilayer of the cell membrane; which expands the membrane pore size depending on the length of the pulse and the electric field intensity. Pulses of several millisecond with a voltage of several tens of millivolts across the cell membrane can pass the membrane. For exposures of seconds to several minutes with tens of kV/m the small molecules can pass through the membrane pores and even damage the cell. If the position of lipids, organized in a double layer, is modified a little, the effect is reversible. Otherwise the lipids cannot be rearranged, the effect is then irreversible and leads to cell death.

Electro-sprayed droplet size and evaporation increase the complexity of the investigation because plasma species are created from the air plasma and from the water. The objective of the experiments presented here is to investigate the effect of the increase of the antibacterial effect due to PAW when it is coupled with the electroporation.

2. Materials and methods

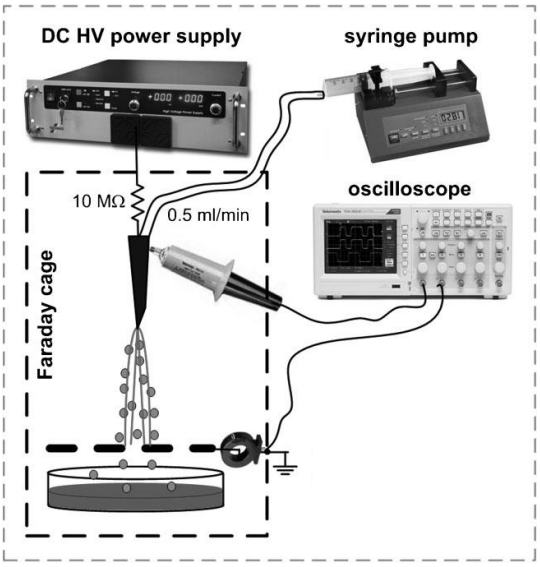

Figure 1. The experimental setup with electro-spray of water. [4]

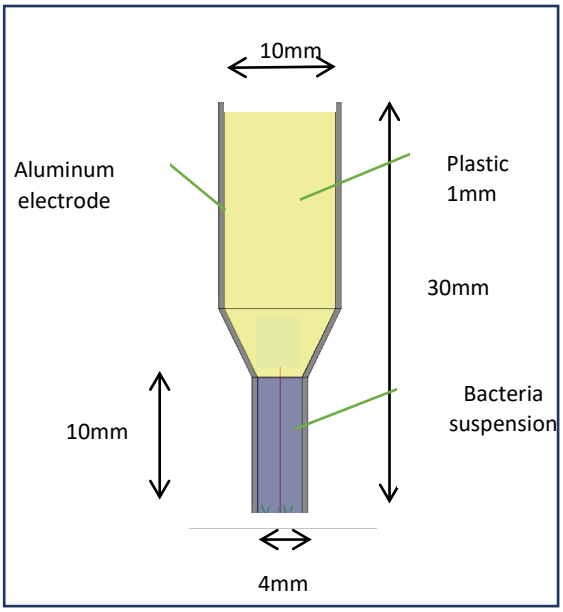

Figure 2. Electroporation cuvette

Plasma Activated Water (PAW) is created by electrospraying of deionized water (DW) through the cold plasma produced by TS discharge. DW is injected by a syringe pump with the flow rate 0.5 ml/min through a high voltage (HV) needle placed 1 cm from a metal grid grounded through a 1Ω resistor. We trigger the discharge only when DW drops from the needle. The PAW is collected in a small Petri dish placed below the grid. When HV is applied on the needle, it induces the TS discharge between the needle and the grounded grid. Voltage is measured by a HV probe Tektronix P6015A connected to the needle. Maximum voltage was 15 kV (experiment A-B) and 17 kV (exp C. Current is measured as the voltage drop across 1 Ω resistance between the grid and the ground, typically with pulses of 27-30 A (experiment A-B) or 40 A (experiment C). The electrical parameters were recorded by oscilloscope *Tektronix TDS 2024C* that measured the frequency of the discharge pulses, which is related to the applied voltage. This frequency is maintained at 1 kHz (+/-200 Hz). We used a 10 MΩ protection resistor on the output of the HV generator.

experiment	Pulse Voltage (kV)	Pulse Current (A)	Pulse Length	Pulse Frequency	EP time treatment	Number of Pulses
A	7.8(DW) 4.2(PAW)	-	1µs	100Hz	1s	100
B	2.0	5.0	200ns	1kHz	90s	90 000
C	1.0	4.5	200ns	1kHz	90s	90 000

Table 1 : Electroporation experiment parameters

Electroporation (EP) experiment: 1ml of bacterial suspension (300µl) in DW (3ml) or PAW is collected and is placed into the sterile electroporation cuvette. It is a 30 mm high plastic cuvette filled only in the bottom narrowed area (10 mm high and 10 mm wide),where is a 4 mm gap between two aluminum tape electrodes. The cuvette is decontaminated before use by being fully filled by isopropyl alcohol and a overnight UV exposure. Cuvette was flushed off thrice by sterilized DW, a pipette

mixing was done. Pulses with 100Hz frequency and 1µs duration are applied from *Behlke HTS 301-03GSM* pulser driven by a 5V pulse signal from Textronix AFG2021 generator. The pulsed voltage was measured by a HV probe Tektronix P6015A.
For the electroporation alone, Table 1 the output voltage of the HV power supply was fixed at 10kV but due to the certain conductivity of the suspension we measured 7. V
max value and 300ns time constant of the voltage decay for the suspension bacteria-DW,
For the combined effect at 7 min after mixing PAW with bacteria, the pulses are applied on the cuvette electrodes. and 4.2kV and 100 ns time constant for the mixture PAW-bacteria, respectively.

Three experimental settings were tested:
A-experiment was carried out with a 1 µs pulses on the cuvette input electrode: we applied 100 pulses at a 100 Hz frequency. However, considering the experiment duration we fixed only the generator voltage at 10 kV and the voltage measured on the cuvette depended of the media, 4.5 kV for PAW and 8 kV for DW.
The B-experiment was carried out with a 200 ns, 2 kV pulses (E=5kV/cm) and 11 A on the cuvette input electrode applied during 90 s at a 1 kHz frequency. Pulse duration is limited by water conductivity that limits the voltage drop at 200-500 ns exponential decay time constant and the minimum length of pulses 200 ns allowed by the pulser.
C-experiment:200 ns, 1 kV pulses, 5 A, 1 kHz. The only difference between C and B-experiment was the exposure time on PAW and the pulses were only 1 kV in C. PAW is produced at a frequency of 1 kHz but for a higher voltage of 17 kV and current 40 A. The gap between electrodes for preparing PAW was still around of 1 cm, nevertheless a little difference can explain these different characteristics.

Bacteria decontamination. Gram-negative bacteria *Escherichia coli* (ATCC 25922) of initial population 10^7 colony forming units (CFU) per ml were growing in the overnight culture in a sterile liquid nutrient (Lauria-Bertani broth, Biolab) at 37°C. Bactericidal effects were observed on bacterial suspension prepared from overnight culture diluted in DW or PAW (i.e. DW treated by TS discharge with electrospray). Immediately after treatment by PAW or EP bacteria were diluted by serial 10-fold dilutions and cultivated on agar plates (Lauria-Bertani agar, Biolab) during 22-24h at 37°C. Then CFUs were counted on the Petri dishes. Usually 4-6 agar plates from each sample was used to be statistically relevant. Replication of the experiments must be done to confirm our results to be statistically stronger.

3. Results and discussion

Fig. 3 shows the survival of *E. coli* bacteria treated by electroporation, PAW or their combined effect, , for three experiments A, B and C with different pulse parameters described above. All three experiments are processed with 4 different sample types : control, electroporation (EP) only, PAW only, and PAW combined with EP. EP only slightly reduced the bacterial population (52% survived in A, all bacteria dead in B so it is close to 0%, and 33% in C) against respectively 83% in A 49% in B and 73% in C. PAW effect is even stronger - in A incubation time was 15 min and only 1.4% of bacteria survived, i.e. 1.9 log reduction, in B this effect was the strongest : 0.06% survived (3.2 log reduction) due to longer incubation time (30 min) and a probable overheated sample of the electroporation with PAW due to the high current. In C we observed 15% of survived (0.8 log reduction). The combined effect was the strongest, as we expected: combination of EP and PAW seems to facilitate penetration of PAW RONS into the cells through cell membrane pores induced by EP. The effect correlates with the PAW only effect (in all A, B, C - please note that in B, the combined EP+PAW effect resulted in complete bacteria sterilization, confirming the dominant effect of RONS in bacterial killing by air plasmas, in agreement with [1]-[2]-[3] and other authors. Moreover, electroporation has an effect on the bacteria survival and it is increased by the effect of PAW. Pulse duration, electric fields magnitude, number of pulses are known to increase the number of cells pores

and pore size and consequently increase the effect of antibacterial agent RONS molecules present in PAW.

Temperature may have increased in all experiments, but we measured it only for the C experiment by EU 620-2343 thermometer. Temperature 52 °C was measured in PAW+EP case and 34°C in EP+DW case. Temperature 52 °C might have negatively affected the bacterial population. Viscosity of the bilayer is already changing at such temperatures, which may influence the reaction of the bacteria with PAW and the electric field, making them more fragile. A cooling system should be added in the future. Nevertheless, a more precise temperature measurement, with a thermal camera, will be done to be sure of the temperature during the experiments

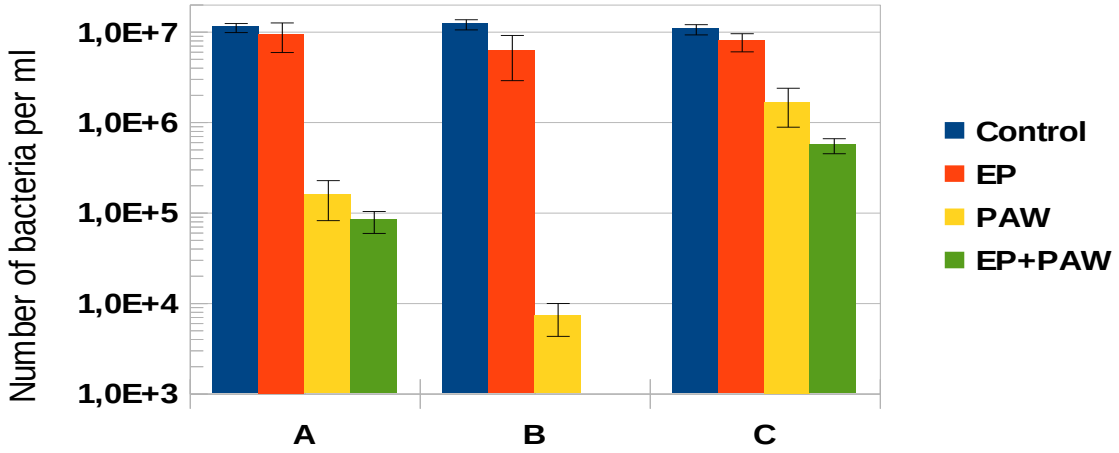

Figure 3: Number of bacteria per ml after treatment. (A) 100 pulses of 10 kV at 100Hz. PAW prepared by TS 15kV, 1 kHz, 27 A, 15min incubation; (B) 2kV, 5A, 200ns pulses at 1kHz operated during 90s, PAW prepared by TS 15 kV, 1 kHz, 27A, 30min incubation;(C)1 kV,4.5 A, 200ns pulses at 1kHz during 90s,PAW 17 kV, 1 kHz, 40 A,15min incubation. Error bars are expressed as the standard deviation of bacteria colonies grown on multiple petri dishes.

We used DW for preparation of bacterial suspensions to increase the pulse length, limit the voltage drops which trigger high currents that increase temperature. However, since DW has very low osmotic pressure, it affects the bacteria cells to increase their volume to balance the low osmotic pressure of their environment. This phenomenon could make the cell membranes more fragile and could influence bacterial survival, especially after combination of EP and PAW.

4. Conclusion

In simple preliminary experiments, we tested the effects of electroporation, PAW and their combination on *E. coli* bacterial cells. The electroporation seems to contribute to the antibacterial effect induced by PAW: the combined EP+PAW effect resulted in the strongest bacterial reduction during our experiments. The issue of short-lived species also remains to be discussed for plasma application. However, local temperature increases in some experiments could be in part responsible of antibacterial effect. Further experiments and/or simulations are needed to set up better experiments to identify the dominant effects of plasma induced bacterial inactivation, as well as the coupling of different agents in bacterial decontamination.

5. Acknowledgment

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