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Cell exposure to atmospheric pressure plasmas: modification of cell cycle and molecular structure

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Atmospheric pressure DC-driven self-pulsing transient spark (TS) discharge operated in air and pulse-driven dielectric barrier discharge (DBD) plasma jet (PJ) operated in helium in contact with water solutions were used for inducing chemical effects in water solutions, and fthe treatment of bacteria (Escherichia coli), mammalian cells (Vero line normal cells, HeLa line cancerous cells), deoxyribonucleic acid (dsDNA) and protein (Bovine Serum Albumin). The effects of the TS and the PJ systems were compared, as well as a direct exposure to the discharge with an indirect exposure to the discharge activated gas flow. The key finding is that all investigated bio-effects were stronger with the air TS discharge than with the He PJ, even in indirect exposure.

1. Introduction

A large number of recent publications on various biomedical applications of non-thermal atmospheric pressure plasmas demonstrate a great potential of this new interdisciplinary field. Various cold plasma sources have been proven to induce interesting phenomena in the cells of higher organisms; often leading to promising therapeutic effects when carefully set and dosed. However, despite numerous reported positive effects of plasma disinfection and therapy, plasma interaction with living cells and microorganisms still remains not well understood and the plasma treatment of biomolecules, cells and tissues is still a subject of debate [1, 2].

The objective of this paper is to demonstrate some effects of cold plasmas on bacteria, eukaryotic cells, and selected biomolecules that are related to bio-decontamination and potential cancer therapies. We compare the direct and indirect effects of two different cold atmospheric plasma sources: the self-pulsing transient spark (TS) discharge generated in air as an efficient producer of RONS applicable for water solutions, and the pulsed DBD plasma jet (PJ) generated in helium as a representative of the most convenient plasma sources for direct cell/tissue treatments and medical in-vivo applications. We investigate the interaction of the discharges with solutions containing bacteria (*E. coli*), cells (normal Vero and cancerous HeLa) and biomolecules (DNA and protein) and investigate their response linked to the chemical effects induced by the discharges. Direct exposure of the water solutions to the plasma is compared with an indirect exposure of the solutions to the plasma activated gas flow.

2. Experimental

2.1. Discharge systems

The first type of the discharge we used in the experiments is the positive DC-driven transient spark (TS) discharge, a repetitive streamer-to-spark transition discharge generated in ambient air, described in detail in [3, 4]. The discharge was generated in two different configurations: the water electrode systems and the water spray systems (Fig. 1). The water electrode (WE) system consisted of high voltage hollow needle electrode placed above the inclined grounded plane electrode in point-to-plane geometry. In this system, the water solution was supplied via a narrow channel in the plane electrode and circulated by a peristaltic pump with various flow rates. The water spray (WS) system described in detail in [5] consisted of high voltage hollow needle electrode placed above the grounded mesh electrode also in point-to-plane geometry. The water solution was supplied via the needle with a constant flow rate (0.5 mL/min). Thanks to the applied high voltage the solution was electrosprayed through the active zone of the discharge and then collected under the metallic mesh. The direct discharge action on water solutions were compared to the indirect exposure to discharge activated gas flow, where the hollow needle electrode was used to supply additional gas flow to drive active species generated by the discharge toward the sample. Positive DC high voltage was applied through the ballast resistor R to maintain the TS mode operating in the typical conditions: applied voltage ~ 13–18 kV, spark pulse frequency ~ 1–4 kHz, exposure time ~ 10 or 20 min, and volume of the solution 2–5 mL (for direct) or 100

μL (for indirect exposure). All TS systems were operated in ambient air with the electrode distance of 1 cm.

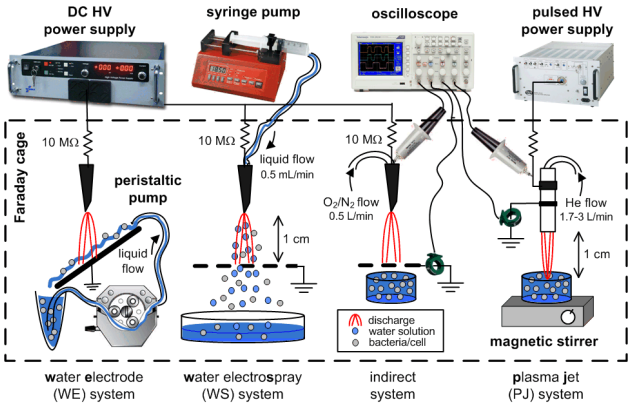

Figure 1. *Experimental setup of the TS and the PJ systems.*

The second discharge type used is positive pulse-driven DBD plasma jet (PJ) generated in atmospheric pressure helium. The device consisted of a quartz cylindrical tube with two aluminium tape electrodes wrapped around the surface of the tube in mutual distance of 10 mm. The system was operated with helium flow rate of 1.7–3 L/min. The PJ was excited using positive high voltage pulses with amplitudes ~ 4–9 kV and frequencies ~ 1–8 kHz. The plasma generated between the electrodes expanded into the open air atmosphere forming a jet with a typical length of 2–3 cm. The distance between the jet nozzle and the treated sample was kept at 1 cm.

2.2. Chemical effects in water solutions

Water solutions with different initial pH and electrolytic conductivities were used for plasma exposure. The solutions were prepared by the dissolution of different salts in deionized water. Reactive oxygen and nitrogen species (RONS) generated by the discharge plasma in treated water solutions were detected mostly by colorimetric methods using UV/VIS absorption spectrometry and fluorescence spectroscopy [5].

2.3. Bactericidal effects

Bactericidal effects of the discharges were tested on *Escherichia coli* (CCM3954) suspended in deionized water with initial populations of 10^7–10^8 CFU.mL^{-1}. The number of bacteria in the solution was evaluated by counting colony forming units (CFU) cultivated on agar during 24 hours at 37°C.

2.4. Viability and apoptosis of mammalian cells

The HeLa (human cervix epithelioid carcinoma) and Vero (monkey kidney epithelial cells) cells were seeded at a density of 5 x 10^4 cells/well in 24 well plates. Viability of the cells was determined by trypan blue exclusion, while apoptosis was assessed with Annexin V-FITC / propidium iodide kit. For cell cycle analysis, the cell suspension was analysed on a flow cytometer.

2.5. Effects on biomolecules

Tests of the plasma exposure on selected biomolecules: dsDNA (deoxyribonucleic acid) and BSA (Bovine Serum Albumin) were performed. UV absorption spectroscopy and fluorescence spectroscopy were used to quantify the DNA damage and the BSA inactivation caused by the plasma. DNA samples were quantified with ACTGene absorption spectrophotometer in order to estimate the degree of DNA integrity. A part of tDNA previously isolated, was subjected to Polymerase Chain Reaction (PCR) using a specific primers pair for cytochrome b gene.

3. Results and discussion

3.1. Discharge systems

Two different plasma sources were used and tested – the self-pulsing TS generated in air and the PJ generated in helium. Electrical and optical characteristics of the TS operating in atmospheric air were published in detail in [4, 5]. The TS is a self-pulsing repetitive streamer-to-spark transition discharge with spark pulses (I_{MAX} ~ 1 A, Δt ~ 50 ns, f ~ 1–10 kHz) that can be well controlled by the applied voltage, typical power of 1–4 W and energy 1–4 mJ/pulse. Electrical parameters of the PJ were published in detail in [6, 7]. It is typical with current pulses of mA amplitude, μs duration, kHz frequency, the average power of 1–3 W and energy 25–50 μJ/pulse.

3.2. Chemical effects in water solutions

The direct TS treatment of water solutions showed that the bactericidal effects were accompanied with the decrease of pH, increase of solution conductivity and the chemical changes in water solutions, i.e. formation of various RONS. The aqueous species measured in WS and WE systems showed very similar concentrations. Gas-phase plasma generated by the TS in contact with water efficiently generates NOx and OH radicals, which upon entering the water solution produce H_2O_2, and NO_2^-, NO_3^-. These are responsible for the acidification of the solution and eventual formation of peroxynitrous acid O=NOOH or peroxynitrite O=NOO$^-$ [3, 8], which are the oxidants with a strong bactericidal effect and may significantly contribute to the cytotoxic effect induced by air plasma in water [8,

9]. The comparison of the concentrations of selected RONS generated by the TS and the PJ (Fig. 2) shows that the concentrations generated by the TS were far much higher than those in the PJ system. Low concentrations of the species produced by the PJ subsequently resulted in its limited bactericidal and cytotoxic effects.

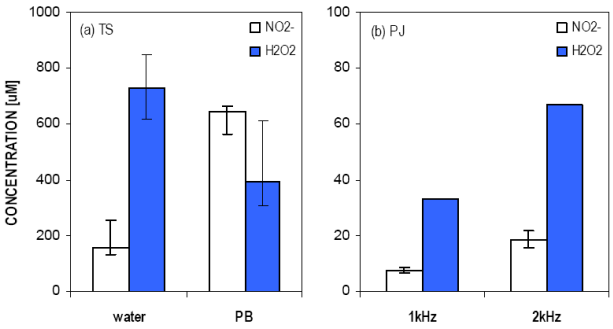

Figure 2. *Concentration of H_2O_2 and NO_2^- generated in water and PB solutions by the TS and the PJ systems: a) TS WS system [V = 5 mL, U ~ 10 kV, f ~ 1 kHz, Δt = 10 min], b) PJ system [water, V = 2 mL, Q_{He} = 1.7 L/min, U ~ 9 kV, Δt = 5 min].*

3.3. Bactericidal effects

Escherichia coli suspended in non-buffered and buffered water solutions with different initial pH and electrolytic conductivity were treated by TS discharge. In both TS WE and WS systems, the stronger bactericidal effect was observed in non-buffered water solution (3–5 log) than in phosphate buffer (PB) solutions (1–2 log). In the WS system, the efficiency was slightly higher due to the effect of the electro spray, which enhances the mass transfer of RONS formed in the gas phase into the solution. The higher efficiency observed in non-buffered solutions was linked with the different RONS chemistry associated with acidification. In PB and PBS solutions, the bactericidal efficiency was lower with almost no decrease of pH [3]. The bactericidal effect of the PJ was tested too. In the same conditions as those in Fig. 2b, only a fair log reduction < 1 was the result of a small chemical activity of the PJ and much lower concentrations of the active species compared to the TS.

3.4. Viability and apoptosis of mammalian cells

Viability, apoptosis and cell cycle of both normal and cancerous cells treated by direct and indirect plasma exposure to the air TS discharge and the He PJ were assessed. The WE and WS systems shows similar cytotoxic effect that increased with exposure time and discharge power. Figure 3a shows the results of the direct TS treatment of HeLa cells. The maximal cytotoxicity of 94% was observed with frequency of 4 kHz and 10 min exposure. Direct TS exposure was compared with the indirect exposure of the cells to the TS activated gas flow (30 % O_2 in N_2, 0.5 L/min) and the effect of the PJ. The cytotoxic effect of indirect exposure was much smaller compared to the direct exposure, with 25% of both HeLa and Vero cells found dead after 4 min of plasma exposure. In case of the PJ system, the cytotoxic effect was < 10%, i.e. even smaller compared with the indirect TS exposure (Fig. 3b).

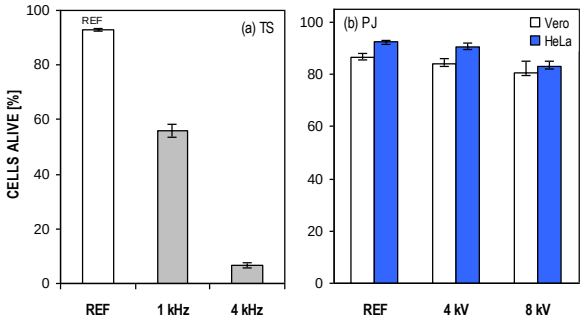

Figure 3. *Viability of cells: a) the WS TS system [HeLa cells, V = 5 mL, Δt = 10 min], b) the PJ system [HeLa and Vero cells, V = 100 μL, f = 4 kHz, Δt = 4 min].*

In order to understand the cell behaviour before their dead, the tests of cell apoptosis were performed. The tests showed that the while apoptotic and cytotoxic effects on (cancerous) HeLa cells observed after 4 and 24 hours of incubation were relatively stable, in the case of (normal) Vero cells the apoptotic and cytotoxic effects significantly vanished with prolonged incubation. It suggests implication of reparation mechanism in Vero cells and decrease of the negative impact of the plasma exposure on normal cells. On the other hand, a lack of recovery observed in HeLa cells indicates that cancerous cells are more sensitive and can be selectively targeted and efficiently killed by the plasma.

The analysis of the cell cycle distribution showed that in the case of Vero cells the plasma induced a block in G2/M stage and perturbation of the cell cycle progression indicating the induction of DNA lesions that have to be repaired before the cells pass to mitosis. The results on apoptosis suggested that a fraction of cells damaged by plasma were eliminated while a fraction of cells that were in G0 phase and few other fractions followed reparatory mechanism. The unrepairable cells were directed to apoptosis and finally to death. On the other hand, the impact of the plasma on cell cycle in HeLa cells has determined a block in G0/G1 stage and of frequency in G2/M stage, explained by the occurrence of G0/G1 block.

3.5. Effects on biomolecules

The TS WE and WS systems and the PJ were also applied for the treatment of DNA and protein. The analysis by UV spectrometry was supplemented by fluorometric analysis of 1140 bp amplicons and confirmed the fragmentation of DNA molecules. Figure 4 shows the corresponding results for TS WE system, where the decrease in fluorescence can be linked to the DNA denaturation with the occurrence of single stranded fragments or to the DNA degradation leading to decrease the number of fluorochrome binding sites. Figure 4b shows the concentration of BSA in the TS WS system. The voltage 0 kV refers to the solution supplied through the system without plasma. The results show that after 6 min with 18 kV the concentration of BSA decreased by 47% exposure. The identical test was also performed in the WE system, with a slightly stronger effect observed. In the PJ system, with plasma exposure of 10 min, the concentration of BSA decreased only within a few %.

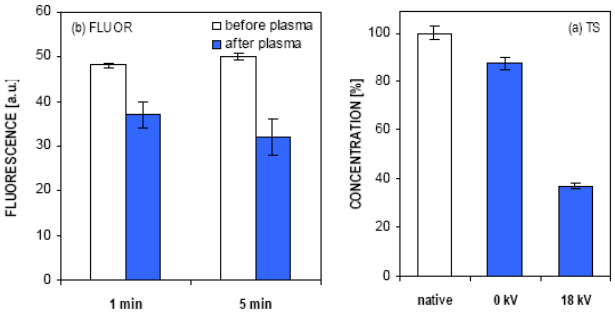

Figure 4. *Analysis of biomolecules after TS discharge: a) DNA [WE system, 1140 bp amplicon fluorescence, 9 ng/μL of DNA in 2 mL of DI water, Q_{liq} = 5 mL/min], b) BSA [WS system, 1 mg/mL of BSA in 3 mL of DI, Q_{liq} = 0.5 mL/min, Δt = 6 min].*

4. Summary

Self-pulsing DC transient spark (TS) discharge operated in air and pulsed DBD plasma jet (PJ) operated in helium in contact with water solutions were used for the treatment of bacteria, mammalian cells and selected biomolecules. Direct exposure to the TS was compared with indirect exposure to the TS activated gas flow and to the effect of pulsed He DBD plasma jet. The two TS systems of direct exposure showed comparable chemical effects, water solution acidification and concentrations of RONS generated in water solutions, which were by one order of magnitude higher than concentrations of species generated by the PJ. The TS systems showed strong bactericidal effects, both in non-buffered (3–5 log) and buffered solutions (1–2 log), as well as cytotoxic effects on cells. The maximum of 94% was found with frequency of 4 kHz and 10 min exposure time in the TS WS system. Small concentrations of active species generated in water solutions by the PJ resulted in limited bactericidal activity (< 1 log reduction) and cytotoxic effects on cells (< 10%). The effect of the PJ was also smaller when compared with the indirect exposure to the TS plasma. The cell cycle analysis showed cell cycle block in G2/M stage for normal cells, and in G0/G1 for cancerous ones. The results of viability, apoptosis and cell cycle show the plasma can selectively target cancerous cells, which is very important for possible future development of new plasma therapeutic strategies in biomedicine. The treatment of biomolecules demonstrated the potential of plasma for successful fragmentation of DNA and denaturation of protein. Our comparisons of the air TS discharge and the He PJ clearly show that the chemical, bactericidal, and cytotoxic effects are stronger in the air plasma of the TS than in the He plasma jet. In our comparison, even indirect exposures to TS air plasma activated gas flow resulted in stronger effects than direct treatment by the PJ, which further proves the dominant role of RONS as key plasma agents. These results successfully demonstrated a great potential of the air self-pulsing TS discharge as an efficient tool for biomedical applications applicable in most settings, perhaps except direct in vivo tissue treatment.

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