ISPC 24

24TH INTERNATIONAL SYMPOSIUM ON PLASMA CHEMISTRY

NAPLES (ITALY) JUNE 9-14, 2019

Final Program

ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

www.ispc24.com

Bactericidal effects induced by air transient spark with electrospray and/or PAW linked with RONS chemistry enhanced by other plasma agents

B. Tarabová[1], P. Lukeš[2], E. Doležalová[2], R. Menthéour[1] and Z. Machala[1]

[1]*Faculty of Mathematics, Physics and Informatics, Comenius University, Bratislava, Slovakia*
[2]*Institute of Plasma Physics of the CAS, v.v.i., Prague, Czech Republic*

Abstract: We focused on the bactericidal effects induced by the direct treatment by air transient spark discharge in combination with electrospray, as well as on the bactericidal effects induced by plasma activated water (PAW) solutions. Plasma induced chemical changes in PAW (especially formation of ONOOH) were correlated with the antibacterial effect, sublethal injury of bacteria and peroxidation of membrane lipids. The electroporation induced by TS discharge may enhance the bactericidal effect induced by RONS and chemical changes.

Keywords: bactericidal effect, plasma activated water, plasma agents, plasma-liquid interactions, reactive oxygen and nitrogen species, oxidative damage, electric field.

1. Introduction

Cold air plasmas generated by electrical discharges at gas-liquid interface or directly in liquids, as well as air plasma activated water (PAW) and liquids are nowadays of the great interest. Plasmas generated in air and in contact with liquids generate a number of gaseous reactive species (NO_x, O_3, H_2O_2, $^{\bullet}OH$ and $HO_2^{\bullet}$ radicals, etc.), which induce formation of aqueous reactive oxygen and nitrogen species (RONS) in the liquid phase (NO_2^- /NO_3^-, H_2O_2, O_3, $ONOO^-$/ONOOH, $^{\bullet}OH$, $^{\bullet}NO_2$ radicals, etc.) through the gas-liquid interface. Formation of RONS is linked with chemical changes of such activated liquids. Thanks to the synergistic effects of the plasma agents (electric field, electrons and ions, UV radiation, RONS) and the induced chemical changes in the liquid, cold air plasmas and PAW lead to various biocidal effects on microorganisms and cytotoxic or growth stimuli effect on cells [1-3].

In this work, we investigated and compared the effects of the direct cold air plasma treatment and/or indirect treatment by plasma activated solutions (mainly PAW) on model bacterial cells *E. coli* (cell viability, membrane damage by electric field or RONS, and metabolic activity). The aim is to distinguish the plasma agents responsible for the strong inactivation in the case of direct plasma treatment.

2. Experimental set-up and methods

Transient spark discharge with water electrospray

The experimental set-up of DC-driven transient spark (TS) discharge in positive polarity in ambient air at atmospheric pressure operated in combination with water electrospray (ES) is depicted in **Fig. 1**. TS discharge was typically generated in point-to-plane geometry between the high voltage needle anode and grounded metallic mesh cathode with inter-electrode spacing kept at 1 cm. A positive high voltage (HV) was applied through the ballast resistor *R* (10 MΩ), and discharge voltage and current were measured by HV probe *Tektronix P6015A* and by Rogowski current monitor *Pearson Electronics 2877*. The self-pulsing TS discharge can be characterized as a repetitive streamer-to-spark transition discharge with strong spark pulses (~ 25 A) with repetitive frequency ~ 1 kHz. Thanks to very short pulse duration (< 50 ns), the TS plasma remains in nonequlibrium at relatively low gas temperature (~400 K). When the HV is applied (~12 kV), the effect of electrospraying of solutions occurrs. The hollow anode enables the solutions flow (0.5 mL/min) directly through the discharge, where interactions of micrometric size droplets with the plasma of repetitive spark channels happen. ES represents an efficient way of water activation due to enhanced mass transfer of gaseous reactive species into the liquid droplets [4-5].

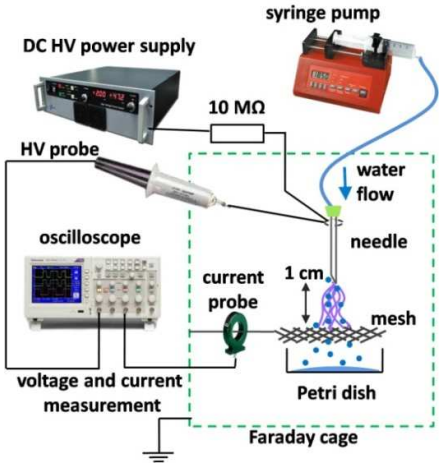

Fig. 1. Experimental set-up of air transient spark with water electrospray.

Chemical and microbiological procedures

The liquid samples in our experiments were represented by two aqueous phosphate solutions with similar composition and electrical conductivities, but different buffering capacity:

- **PB** - phosphate buffer (2 mM Na_2HPO_4/KH_2PO_4) with initial pH 6.9 and conductivity ~560 μS/cm;
- **W** - weak phosphate aqueous solution (8.5 mM NaH_2PO_4) with initial pH 5.5 and conductivity ~600 μS/cm.

These solutions were also used for preparation of the bacterial suspension of planktonic model bacteria *Escherichia coli ATCC25922* with initial concentration ~ 10^7 CFU/mL. We treated either directly bacterial suspension by TS with water ES, or just aqueous solutions, which were used for indirect treatment of bacteria with different incubation times post plasma tretment. The bacterial inactivation was evaluated by classic thermostatic cultivation on Petri dishes (M-FC agar supplemented with Rosolic acid). Further effects induced by plasma and PAW on the bacterial cells were detected - sublethally injured cells with damage of outer and cytoplasmic membrane, the peroxidation of membrane lipids, the loss of membrane integrity and the metabolic activity. To study the presence of sublethally injured cells [6], bacteria were plated on non-selective (tryptone soya agar enriched with yeast extract) and two selective media: with 3.1% of sodium chloride or with 4% of bile salts. Sublethally injured cells become sensitized and unable to grow on the selective media containing bile salts if their outer membranes are damaged, whether the loss of tolerance to the presence of sodium chloride is linked with the loss of the functionality and/or integrity of cytoplasmic membrane. The concentrations of bile salts and NaCl do not inhibit growth of healthy bacterial cells and were determined experimentally. Reactive species forming during the plasma treatment or in PAW cause the peroxidation of cell membrane lipids, which was detected by the thiobarbituric acid test (TBA). Thiobarbituric acid reacts with secondary product of lipid peroxidation – malondialdehyde forming the pink colored MDA-TBA_2 complex [7]. The metabolic activity of treated bacteria cells was evaluated by fluorescent resazurin assay. Resazurin is an oxidation-reduction indicator used for evaluation of cell growth. This blue non-fluorescent dye becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells [8].

Chemical changes (pH, conductivity) and RONS formed in PAW were detected by following methods:

- H_2O_2 – colorimetric method where titanyl ions Ti^{4+} react with H_2O_2 in the presence of NaN_3 and create pertitanic acid with the absorption maximum at 407 nm;
- NO_2^-/NO_3^- detected by ion chromatography – samples were stabilized by phosphate buffer to stop the acidic decomposition of NO_2^- before injection into chromatography column;
- $O_2^{\bullet-}$ - detected indirectly as the increase of H_2O_2 concentration after superoxide dismutation by the superoxide dismutase enzyme;
- ONOOH – the rate of formation of peroxynitrous acid r_{ONOOH} in PAW was calculated from the kinetic analysis of post-plasma treatment processes in PAW (i.e. reaction between H_2O_2 and NO_2^-) [9, 10].

3. Results

Positive TS discharge was operated in ambient air in the direct contact with aqueous solutions electrosprayed directly through the discharge. The dominant stable gas phase products of TS were nitrogen oxides NO_x. Furthermore, thanks to the presence of water vapours other abundant gaseous reactive species were formed (e.g. H_2O_2, $^{\bullet}OH$ and $HO_2^{\bullet}$ radicals, HNO_2/HNO_3). This cold air plasma activation resulted in chemical effects induced in aqueous solutions. Increase of conductivity in both solutions and decrease of pH in PAW (5.5→3.2) unlike in plasma activated buffer solution (PAPB) due to dissolution of gaseous NO_x were observed. Formation of NO_2^-, NO_3^-, H_2O_2, $O_2^{\bullet-}/HO_2^{\bullet}$ was measured in PAW and PAPB directly after plasma treatment or as evolution of post-discharge processes in post-plasma treatment time (results not shown here) [4-5]. From the kinetics of H_2O_2 and NO_2^- (considering the reaction (1)) in post-treatment time (PAW, up to 90 min at room temperature) the rate constant of the formation of peroxynitrous acid r_{ONOOH} was calculated.

$$NO_2^- + H_2O_2 + H^+ \rightarrow ONOOH + H_2O \qquad (1)$$

The initial rate when PAW was withdrawn from plasma was ~180 nM/s and it decayed exponentially in post-treatment time (discussed later).

Fig. 2 shows the bactericidal effects obtained by the direct treatment of *E.coli* bacterial suspensions by TS with ES and by indirect treatment when bacteria were incubated in fresh activated solutions. A significantly higher bactericidal effect (up to 7 logs, i.e. complete sterilisation) was obtained by direct treatment of bacteria in W in comparison with buffered PB (< 2 logs). Similarly, strong bactericidal effect was observed after indirect treatment when bacteria were incubated in PAW where the inactivation efficiency was increasing with the increasing incubation time (from 1.5 up to 5 logs).

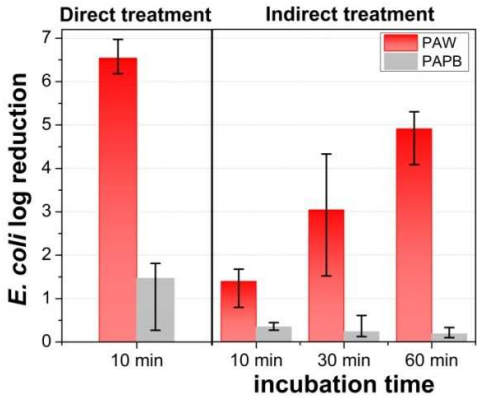

Fig. 2. Comparison of bactericidal effects of direct treatment (TS with ES) and indirect treatment by plasma activated solutions (mean, 1st and 3rd quartile).

The higher efficiency was linked with the acidification (pH 3.2), formation of RONS and different RONS

chemistry associated with decreased pH. Following chemical processes enhanced by the low pH were responsible for the strong bactericidal effects in PAW:

1. production of peroxynitrous acid (reaction (1) followed by its decay at acidic pH (2):

$$ONOOH \leftrightarrow {}^{\bullet}OH + {}^{\bullet}NO_2 \quad (2)$$

2. acidic decomposition of NO_2^- via formation of $^{\bullet}NO$ and $^{\bullet}NO_2$ radicals (3) into NO_3^- as a final product (4) if pH < 3.5:

$$2\,NO_2^- + 2\,H^+ \leftrightarrow 2\,HNO_2 \rightarrow {}^{\bullet}NO + {}^{\bullet}NO_2 + H_2O \quad (3)$$

$$2\,{}^{\bullet}NO_2 + 2\,H_2O \rightarrow NO_2^- + NO_3^- + H^+ \quad (4)$$

Radicals ($^{\bullet}OH$, $^{\bullet}NO$, $^{\bullet}NO_2$) produced by these reactions posses strong cytotoxic effects on cells. Besides these, also hydroperoxyl radical $HO_2^{\bullet}$ may significantly contribute to the bactericidal effect. We detected indirectly its anionic form $O_2^{\bullet-}$ in buffered PB (pK_a ($HO_2^{\bullet}/O_2^{\bullet-}$) is 4.8), therefore we can expected its presence in PAW.

Plasma induced chemical changes and RONS are responsible for the bactericidal properties of plasma activated solutions, which can remain from several minutes up to several hours. However, due to instability of RONS, these properties exponentially decayed in post-plasma treatment time. In **Fig. 3** the effect of the storage temperature of plasma activated solutions on their bactericidal properties in post-plasma treatment time is shown. The incubation with bacteria in certain post-treatment time was 10 minutes. At first, in PAW, unlike in PAPB, there is a fast exponential decay of its bactericidal properties over the first 60 min post-plasma treatment. This can be attributed to the parallel formation and decay of ONOOH in PAW.

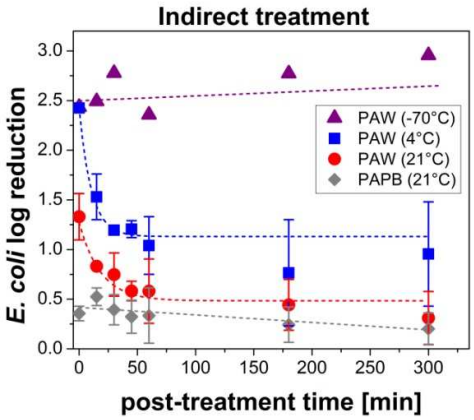

Fig. 3. Post-plasma treatment evolution of bactericidal properties of plasma activated solutions stored at different temperatures (indirect treatment - each point represents 10 min incubation; mean, 1st and 3rd quartile).

In **Fig. 4** the correlation of the bactericidal properties of PAW and of the calculated rate constant r_{ONOOH} is shown. Despite that the life-time of ONOOH is very short (~ ms), the rate constants of both processes are dependent on the temperature of the solution. As can be seen in **Fig. 3** it was possible to preserve the bactericidal properties of plasma activated solutions by their storage at decreased temperatures, especially when PAW was deep frozen immediately at -70°C.

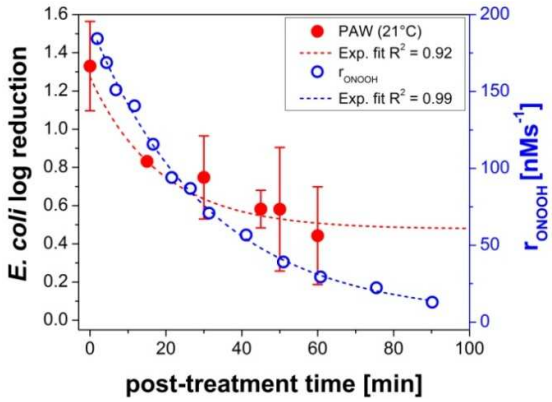

Fig. 4. Correlation of the exponential decay of bactericidal effect of PAW and the rate constant of ONOOH formation (r_{ONOOH}) in PAW in post-plasma treatment (at room temperature).

One of the possible interactions of RONS forming during the plasma treatment in PAW with the bacterial cell membranes is the peroxidation of membrane lipids. Mostly radicals $^{\bullet}OH$, $^{\bullet}NO$, $^{\bullet}NO_2$ and $HO_2^{\bullet}/O_2^{\bullet-}$) were responsible for the oxidative damage of the cell membrane. The significant increase of the MDA-TBA_2 complex was measured on bacteria in PAW after plasma treatment (results not shown here) [11].

Sublethal injury typically appears when cells are subjected to the physical or chemical agents insufficient to kill them. However, the cells become sensitized and less resistant. The sublethal injury is associated with the membrane damage. Since *E. coli* are Gram-negative bacteria, we plated the bacteria on selective media, which distinguished the damage of the outer membrane (OM) or cytoplasmatic membrane (CM). We only worked with the bacteria treated indirectly by PAW here.

In **Fig. 5** the numbers of live bacterial cells for each incubation time are shown, and how many of them had damaged outer or cytoplasmatic membrane. We can see that already after 10 min incubation in PAW resulted in damage of outer membranes of all cells. The number of cells with damaged cytoplasmatic membrane increased with increasing incubation time in PAW, which was also linked with the lowered number of the live cells. The observed sublethal injury was caused only by chemical agents (RONS and acidic pH) in PAW, which are also responsible for the oxidative damage of the cell membranes.

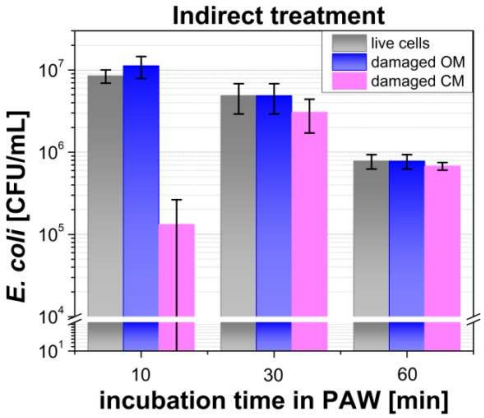

Fig. 5. Sublethal injury of outer (OM) and/or cytoplasmatic (CM) membrane of bacterial cells caused by indirect treatment by PAW.

However, in **Fig. 2** is clearly remarkable difference in bactericidal efficiency of the direct treatment of bacteria in W (~6.5 log) compared to the 10 min incubation indirect treatment by PAW (~1.5 log). Moreover performing the metabolic resazurin assay has shown that the bacteria in W treated directly by TS with ES showed no metabolic activity unlike the bacteria in buffered solution, which metabolic activity was just slowed down in comparison with non-treated bacteria (results not shown here). In the case of direct treatment another active agents of cold plasma beside the RONS need to be considered, e.g. UV radiation, thermal radiation, electric field and charged particles. We can exclude the effect of increased temperature. Even though shortly after the spark pulse the temperature temporarily exceeds 1000 K inside the plasma channel, it is only for a very short time (~ns) and plasma remains in nonequlibrium at relatively low gas temperature (~400 K) [4]. Previously it was shown that the UV emitted from the TS discharge alone did not cause any significant bactericidal effect on bacteria on agar surface. However, the UV emitted by the TS discharge with ES has to be examined in our future experiments [12]. Exposure of biological membranes to a sufficiently high external electric field can lead to a rapid and large increase in their electric conductivity and permeability. This effect is in generally referred to as membrane poration or electropermeabilization. Applying the high pulsed electric field may result in either reversible or permanent membrane poration. Although the TS discharge is generated by DC HV, thanks to its electrical circuit it behaves like a self-pulsing discharge with short (<50 ns) and strong spark pulses (~ 25A, ~ 12 kV) with repetitive frequency ~ 1 kHz. We hypothesize that we can induce the electroporation for a short time when the bacteria cells pass through the discharge in charged microdroplets. This electroporation may act in synergy and enhance the bactericidal effects induced mainly by chemical changes and RONS in PAW. In our future research we plan to investigate the combination of indirect treatment by PAW and by pulsed electric field, as well as to use the fluorescent staining of cells to detect the possible electroporation.

4. Acknowledgement

This work was supported by the Slovak Research and Development Agency APVV-17-0382, Slovak Grant Agency VEGA 1/0419/18, and the Czech Science Foundation (GACR) - project No. 19-25026S.

5. References

[1] H. Tanaka *et al.*, Rev. Mod. Plasma Phys., **1** (2017).
[2] Z. Machala and D.B. Graves, Trends Biotechnol. **36** (2017).
[3] V. Scholtz *et al.*, Biotechnol. Adv. **33** (2015).
[4] B. Tarabová *et al.*, Plasma Process. Polym. **15** (2018).
[5] Z. Machala *et al.*, J. Phys. D: Appl. Phys. **52** (2019).
[6] C. Arroyo *et al.*, Lett. Appl. Microbiol. **51** (2010).
[7] E. Dolezalova and P. Lukes, Bioelectrochem. **103** (2015).
[8] S. D. Sarker *et al.*, Methods **42** (2007).
[9] P. Lukes *et al.*, Plasma Sources Sci, Technol. **23** (2014).
[10] B. Tarabová *et al.*, Phys. Chem. Chem. Phys., submitted.
[11] Z. Machala *et al.*, Plasma Process. Polym. **10** (2013).
[12] Z. Machala *et al.*, J. Phys. D: Appl. Phys. **43** (2010).