

*Supplementary Data*

**Towards the understanding of non-thermal air plasma action: effects on  
bacteria and fibroblasts**

Oleg Lunov,<sup>a,\*</sup> Vitalii Zablotskii,<sup>a</sup> Olexander Churpita,<sup>a</sup> Ales Jäger,<sup>a</sup> Leoš  
Polívka,<sup>a</sup> Eva Syková,<sup>b</sup> Natalia Terebova,<sup>c</sup> Andrei Kulikov,<sup>c</sup> Šárka Kubinová,<sup>a,b</sup>  
Alexandr Dejneka<sup>a</sup>

<sup>a</sup>Institute of Physics of the Czech Academy of Sciences, Prague, Czech Republic

<sup>b</sup>Institute of Experimental Medicine AS CR, Prague, Czech Republic

<sup>c</sup>St Petersburg State University of Information Technologies, Mechanics and Optics, St  
Petersburg, Russia.

*Correspondence:* O.Lunov e-mail: lunov@fzu.cz

## **Materials and Methods**

### **1. Physicochemical characterization of the plasma**

To produce uniform non-thermal plasma for biological applications, we utilized the plasma setup published in<sup>1, 2</sup>. The input voltage was about 600 V, electric current 167 mA and the power was 100 W; such a high voltage supply resulted in electron energy of about 0.5 keV. The gas supply was administered through a gas inlet followed by gas ionization in the pores of the ceramic membrane utilizing an electric field between two electrodes. The gas temperature at the tip of the plasma jet was measured using a thermocouple embedded in an optical spectrograph USB 4000 (Ocean Optics Inc.). The temperature remained 37-40 °C during the cell treatment. The optical emission spectrum of the non-thermal plasma was measured using an optical spectrograph USB 4000 (Ocean Optics Inc.). FTIR experiments were carried out using a Nicolet™ iS™5 FT-IR spectrometer (Thermo Scientific). To compare effects of plasma with ozone, cells were exposed to ozone produced by an ozone generator with input voltage 230 V, the power 12 W and ozone gas production rate 400 mg h<sup>-1</sup> (FORTEZON 400, UVC servis). Ozone concentration was measured using the gas detector (GasAlert Extreme, BW Technologies).

### **2. Cultivation of bacteria and inactivation by plasma**

To study the bactericidal effects of plasma we used the Gram-negative bacteria *E. coli* (ATCC 700728) and *P. aeruginosa* (ATCC 27853), and the Gram-positive bacteria *S. aureus* (ATCC 6538) and *B. subtilis* (ATCC 6633) (Czech Collection of Microorganisms (Brno, Czech Republic). The gelatine pellets containing the bacterial strains were incubated in 9 ml of liquid media (Tryptic Soy Broth, Mecrotube®, Merc, NJ, USA) at 35°C for 18 hours, and then diluted in a phosphate buffer (PBS) to a concentration 6 x 10<sup>6</sup> colony forming units (CFU)/ml. A volume of 0.8 ml of diluted bacteria suspension was spread onto the agar plate (Caso-Agar, Mercoplate®, Merc) and exposed to either air plasma or ozone device located 10 mm away, for 15, 30 and 60 s. The plates were incubated overnight and the number of CFU in the inhibition zone was counted using OpenCFU software<sup>3</sup>. The

tested layers were tested in triplicates. The effectiveness of the treatment was calculated as the percentage of CFU observed on treated plates relative to CFU on untreated plates.

### **3. Scanning electron microscopy (SEM)**

The effect of bacteria exposure to either air and helium plasma or ozone on changes in cell morphology was studied by scanning electron microscope (SEM). 3T3 fibroblasts, bacteria strains and *B. subtilis* endospores were placed on glass coverslips and exposed to either air and helium plasma or ozone located 10 mm away, for 15, 30 and 60 s, followed by 4 % paraformaldehyde fixation. Afterwards, cells were post-fixed with 1% OsO<sub>4</sub> at room temperature then dehydrated with a graded ethanol series followed by subsequent chemical drying with hexamethyldisilazane. Micrographs were taken using high resolution SEM FEI Quanta 3D FEG at an acceleration voltage of 2 kV to reveal surface details and prevent charging of the non-conductive samples.

### **4. Cell culture**

3T3 fibroblasts (American Type Culture Collection) were grown in culture medium containing Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories, Pasching, Austria) with 10 % fetal bovine serum (FBS; PAA Laboratories) and Primocin TM (100 µg/ml; Lonza, Cologne, Germany). Cells were cultured in a humidified 5 % CO<sub>2</sub> atmosphere at 37 °C.

### **5. Measurement of cellular viability**

Cell viability was analyzed by WST-1 assay (Roche Diagnostics), which is based on the cleavage of tetrazolium salt WST-1 by cellular mitochondrial dehydrogenases, producing a soluble formazan salt; this conversion only occurs in viable cells, thus allowing accurate the spectrophotometric quantification of the number of metabolically active cells in the culture. 3T3 fibroblasts were seeded onto 96-well plates at a density of 8000 cells per well and treated with plasma or ozone. Immediately after the treatment, WST-1 reagent was added to each dish and incubated for 2 h at 37 °C to form formazan. The absorbance was measured using a

Tecan-Spectra ELISA plate reader (Mannedorf, Switzerland) at 450 nm. Readings were done in quadruplicates; three independent experiments were performed for each measurement.

## **6. Detection of intracellular ROS and RNS**

ROS and RNS levels were measured using Cellular ROS/Superoxide Detection Assay Kit (Abcam). Briefly, cells were seeded onto 96-well black/clear bottom plates (Corning, BD Biosciences) at a density of 8000 cells per well. Following this, plasma treatment cells were labeled with Oxidative Stress Detection Reagent according to the manufacturer's instruction (Abcam). Fluorescence was then measured using a fluorescent microplate reader (Tecan Infinite® 200 PRO). Readings were done in quadruplicates. Quantification of ROS levels was done using methods published earlier<sup>4, 5</sup>.

## **7. Stopping and Range of Ions in Matter (SRIM) simulations**

Stopping and Range of Ions in Matter (SRIM) simulations<sup>6</sup> are the most common way of obtaining a rough view of the in-depth composition and the amount of implanted ions in the layered structures. Simulations were performed for helium and atmospheric plasmas with an ion energy of 0.5 keV and typical fluences on the three-layers H<sub>2</sub>O (20 nm) / cell wall / H<sub>2</sub>O (200 nm). We utilized the typical structure of Gram-positive or Gram-negative cell walls described as follows: Gram-positive walls consists of a peptidoglycan layer (90 nm) and cell membrane (10 nm), whereas Gram-negative walls are composed of a cell membrane (10 nm), peptidoglycan layer (20 nm) and another cell membrane (10 nm)<sup>7</sup>.

## **8. Histological studies of wound healing**

Male Wistar rats (350-400 g) were placed under isoflurane anesthesia (2–4%, Forane, Abbott Laboratories, Abbott Park, IL), and the hairs on their back were removed using an electric shaver and depilatory cream. Two 8 mm full-thickness skin excisions were created by punch biopsy on the upper back of the rat. The wounds were treated for 1 and 2 min with air plasma

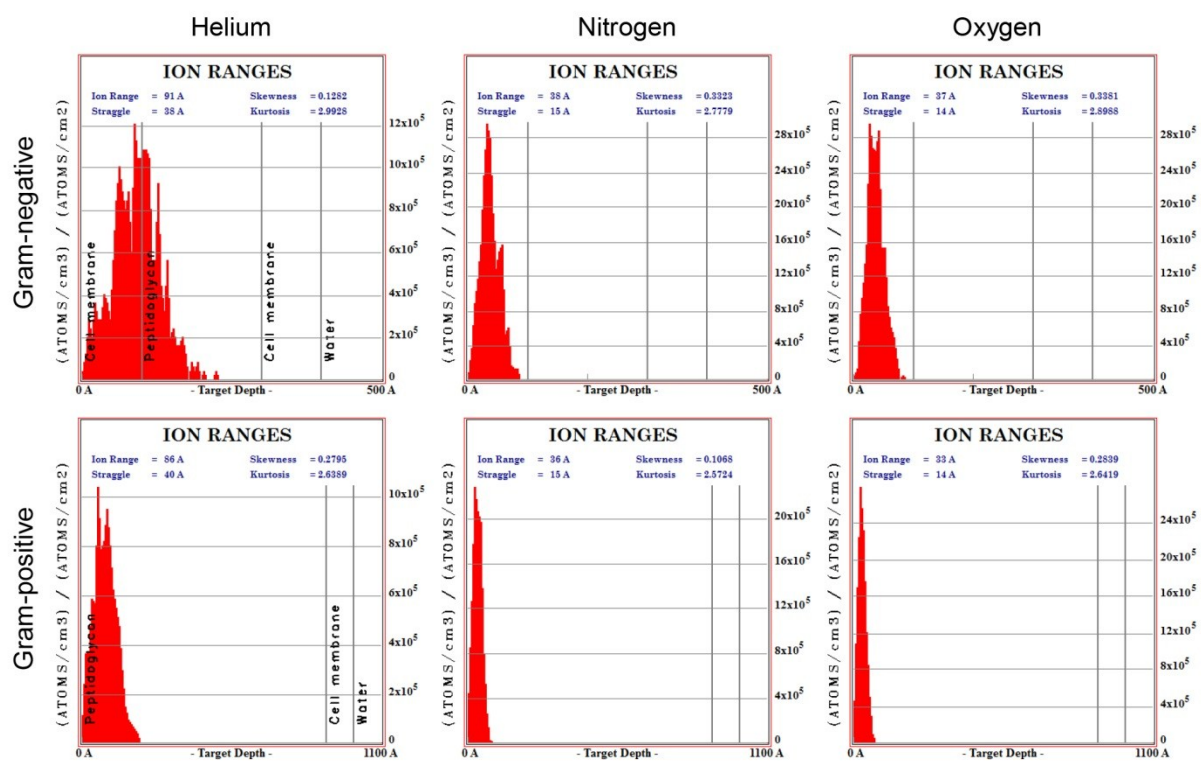
for 3 days. The analysis was done on the 3rd and on the 7th day. Afterwards, the skin was dissected, fixed in 4% paraformaldehyde (in 0.1 M PBS, pH 7.4) for 24h and histologically processed. Fixed skin was paraffin-embedded, sectioned, and stained with a standard Masson's trichrome stain. All experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), regarding the use of animals in research and were approved by the Ethics Committee of the Institute of Experimental Medicine, Academy of Sciences Czech Republic, Prague, Czech Republic.

## 9. Statistical Analysis

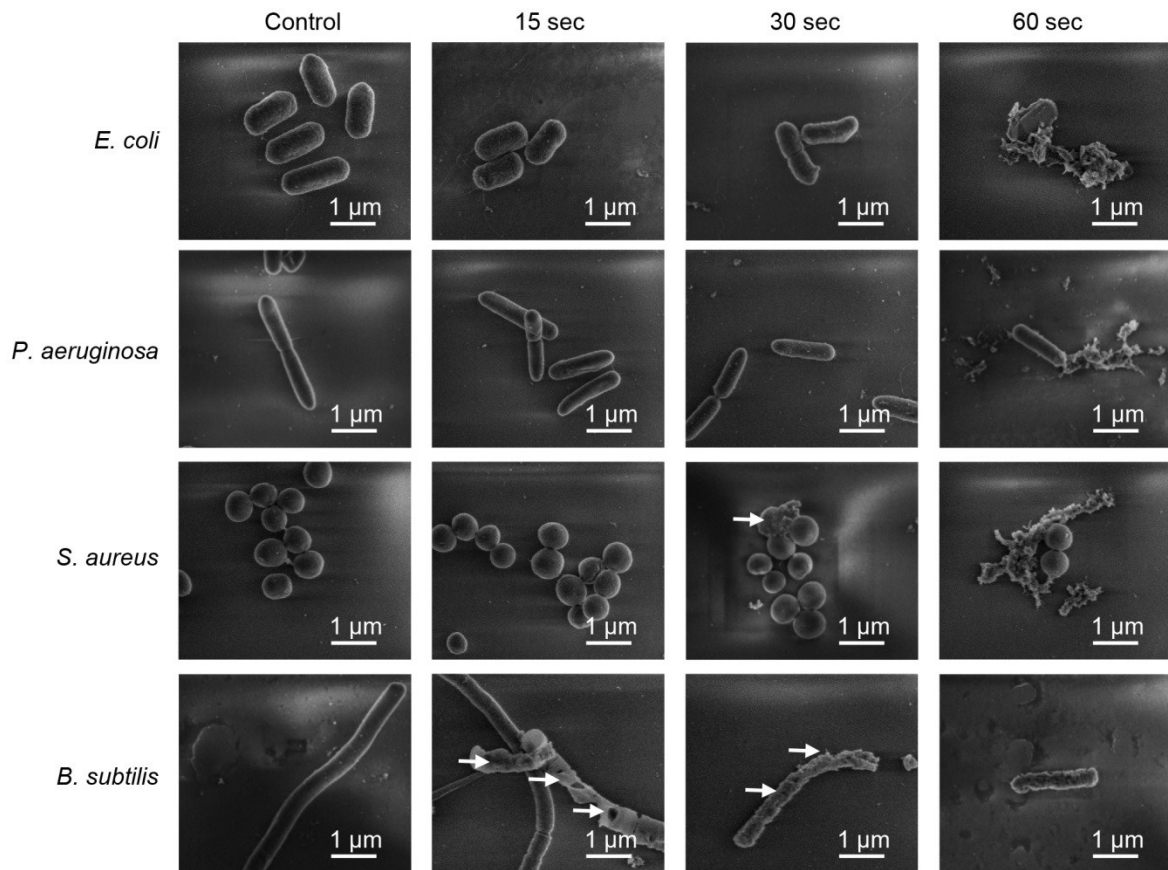
The statistical significance of differences in cell counts in the spinal cord lesions between the groups was determined using ANOVA Fisher's LSD and Newman-Keuls tests. Differences were considered statistically significant statistically at  $*p < 0.05$ .

## References

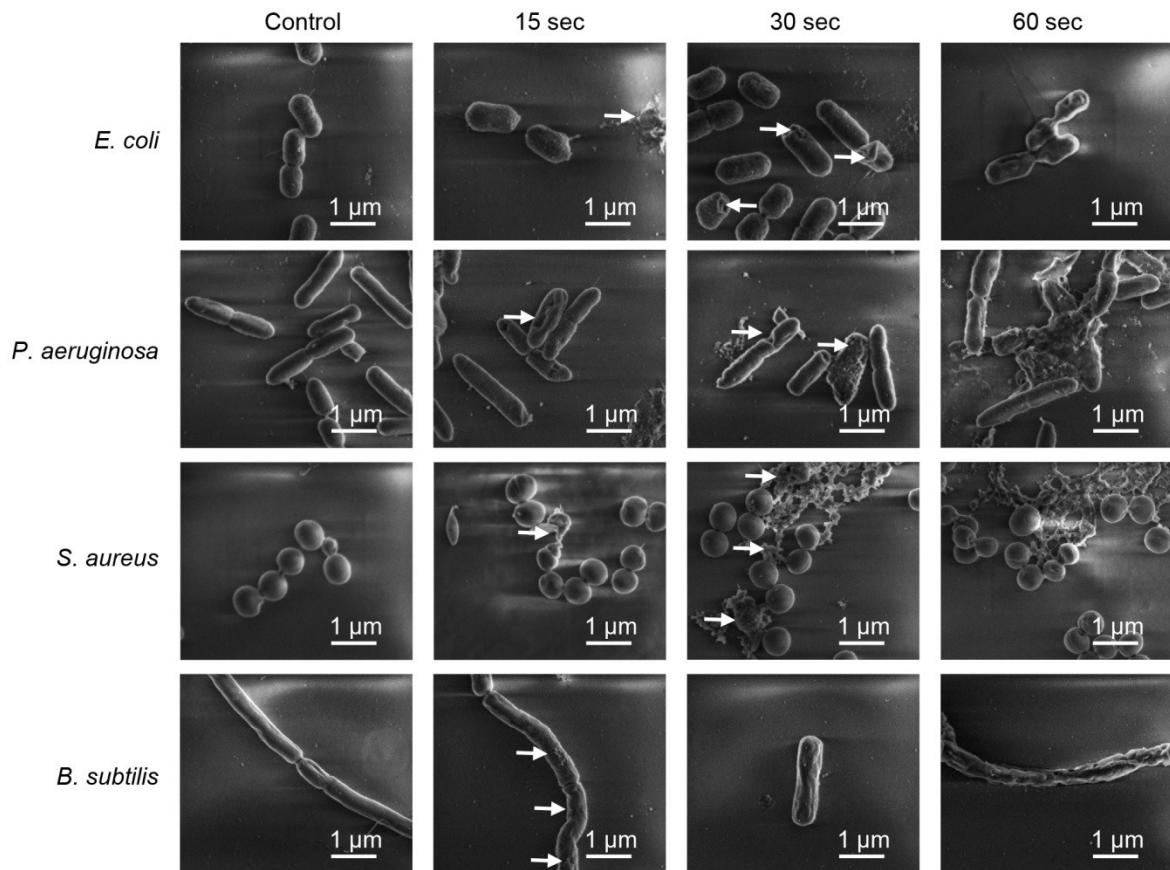
1. O. Lunov, V. Zablotskii, O. Churpita, E. Chanova, E. Sykova, A. Dejneka and S. Kubinova, *Sci. Rep.*, 2014, **4**, 7129.
2. O. Lunov, V. Zablotskii, O. Churpita, A. Jäger, L. Polívka, E. Syková, A. Dejneka and Š. Kubinová, *Biomaterials*, 2016, **82**, 71-83.
3. Q. Geissmann, *PLoS One*, 2013, **8**, e54072.
4. M. A. Kang, E. Y. So, A. L. Simons, D. R. Spitz and T. Ouchi, *Cell Death Dis.*, 2012, **3**, e249.
5. H. Wang and J. A. Joseph, *Free Radic. Biol. Med.*, 1999, **27**, 612-616.
6. J. F. Ziegler, M. D. Ziegler and J. P. Biersack, *Nucl. Instrum. Meth. B*, 2010, **268**, 1818-1823.
7. J. Slonczewski and J. W. Foster, *Microbiology : an evolving science*, W.W. Norton & Co., New York, 2009.



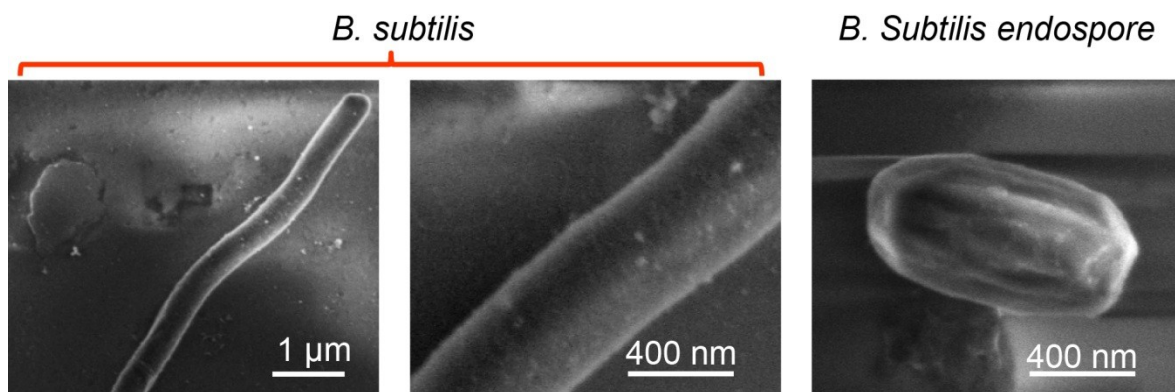
**Fig. S1** Results of SRIM simulations: In-depth profiles of He (green panel), N (red panel) and O (blue panel) ions penetration through either Gram-positive or Gram-negative bacterial wall models. The total number of ions is 10000.



**Fig. S2** SEM analysis of morphological changes of different bacterial strains after treatment with air plasma. Damages are shown by arrows.



**Fig. S3** SEM analysis of morphological changes of different bacterial strains after treatment with ozone. Damages are shown by arrows.



**Fig. S4** Scanning electron micrographs of untreated (control) and *B. subtilis* endospores and *B. subtilis* bacteria.