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Bio-decontamination with plasmas

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Classification of plasma approaches (direct and indirect) applied in biomedicine is given. Plasma sources used in each approach as well as advantages and drawbacks of the mentioned methods are discussed shortly. Main attention in this contribution is paid to direct plasma treatment which has higher efficiency as compared to that of indirect method. The benefits of direct plasma method are illustrated experimentally by example of an inactivation of Gram positive (*Staphylococcus aureus*) and Gram negative (*Pseudomonas aeruginosa*) bacteria by positive (PC) and negative (NC) coronas. The splitting of contributions from various active plasma agents was done. Different mechanisms of bio-inactivation by positive and negative charged particles were revealed. Synergy effect of joint action of negatively charged particles and reactive neutral species was found out.

1. Introduction

Methods of plasma inactivation can be divided into two groups - direct and indirect ones. In the first method, bio-inactivation happens inside discharge zone (Fig.1a); in the second method, bio-objects are exposed to afterglow plasma jet, plasma plum, plasma "bullets", etc outside the discharge zone (Fig.1b).

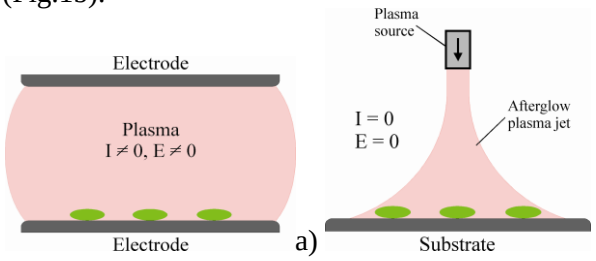

Fig.1. Schemes of plasma inactivation: inside (a) and outside (b) discharge zone. In the first case, cells are influenced not only with reactive species (*R* agents – O, $O_2(^1\Delta)$, O_3, NO, OH, etc) but exposed also to discharge current I and strong electric field E (*E* agents – electric field and ions). Joint action of reactive species and charged particles can give synergy biocidial effect.

2. Experimental setup and methods

Figure 2 shows external view of gas discharge chamber and image of the PC above Petri dish with agar (working regime). Petri dish of 36 mm inner diameter was placed inside the discharge chamber. At the bottom of dish there was a grounded metallic disk of 25 mm in diameter. Six holes on the side of the camera serve for gas inlet; two tubes at the chamber top serve for disposal of gas and reactive species generated by corona discharge. Using of PC and NC allowed us to found out the role of positive and negative ions in plasma inactivation; using of gas blowing and modified Petri dish allowed us to split the contribution of different active agents into inactivation of cells.

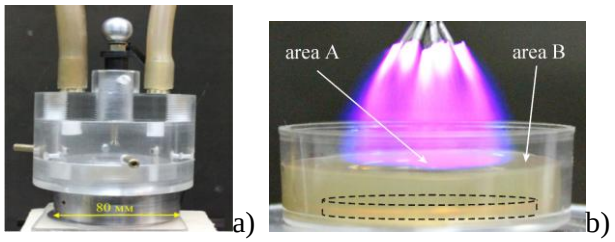

Fig.2. The images of gas discharge chamber (a) and positive corona in ambient air and Petri dish with agar (b) the part of which serves as an electrode collecting the electric current on the grounded metallic disc (drawn in dashed). In the case of absence of gas blowing, cells located in the area with electric current (area A) are exposed to *UV*, *R* and *E* agents; cells located outside that area (area B) are exposed to *UV* and *R* agents. In the case of gas blowing through the chamber, cells in area A are exposed to *UV* and *E* agents; cells in area B are exposed to *UV*.

We used strains of *S. aureus* Sa78 (a clinical isolate) and *P. aeruginosa* Pa103 (type strain). Overnight culture was diluted to an optical density of OD600 1.0 in saline solution (0,15 M NaCl). Ten-fold dilutions were spread on surface of the agar, starting with the second dilution (approximately 10^6 CFU/ml) in the volume of 30 µl. The cell density on the agar surface was approximately $3x10^2$ – $3x10^4$ cells/cm^2. The treatment of pathogens by corona discharge was performed as described above, by varying the processing time. Calculation of the number of grown colonies was performed after 24 hours.

3. Experimental results

Experiments on the inactivation of *P. aeruginosa* and *S. aeruginosa* by NC and PC were carried out at just about the same electrical current (I=225±25 μA). The results on separate and joint biocidial effects of different active agents (*UV*, *R* and *E*) on cell survival are shown in figure 3.

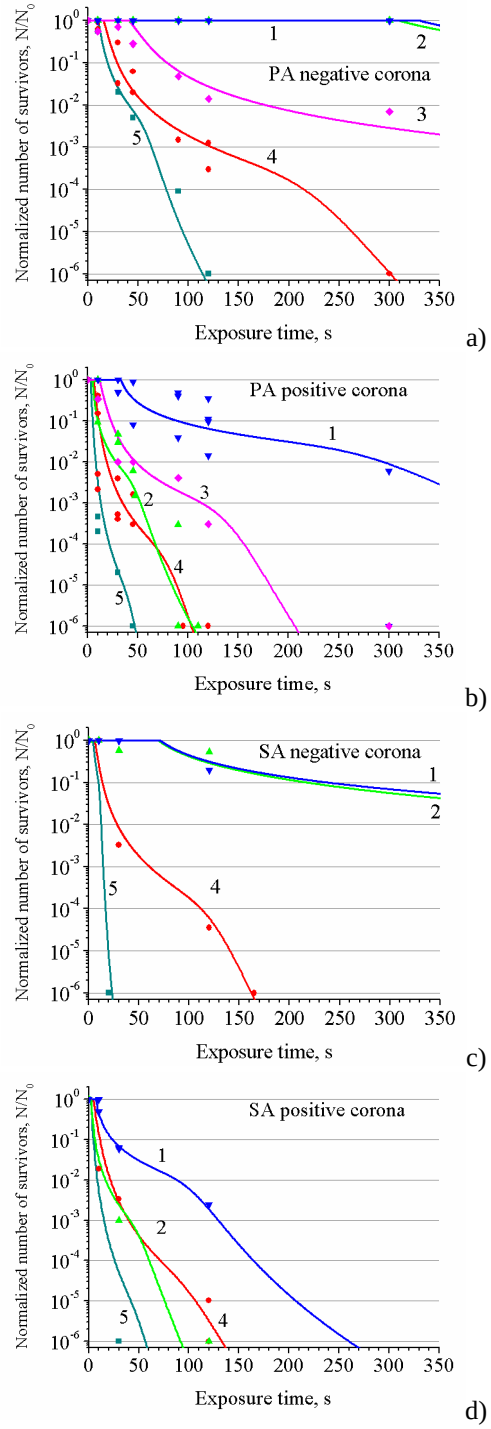

Fig.3. Survival curves for cells of *P. aeruginosa* (a, b) and *S. aeruginosa* (c, d) exposed to different active agents of negative (a, c) and positive (b, d) corona. Figures 1-5 denote the combinations of active agents influencing cells as follows: 1 ▼ – *UV*; 2 ▲ – *UV*+*E*; 3 ♦ – *R* (O_3); 4 ● – *UV*+*R*; 5 ■ – *UV*+*E*+*R*.

4. Discussion

In most cases the survival curves characterizing the inactivation kinetics of cells exposed to plasma exhibits one or several phases. Due to that, survival curves plotted on semi-logarithmic scale (Fig.4) can be approximated by line graph of several legs [1]. At present, bio-chemical and bio-physical mechanisms responsible for cell inactivation at each phase are still under study and discussion.

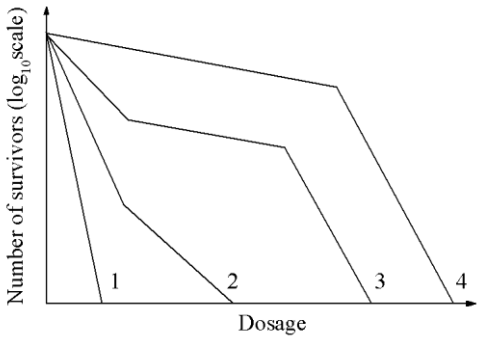

Fig.4. Sketch of four commonly observed survival curves plotted on semi-logarithmic scale (CFU/ml vs Dosage).

Total duration of these phases depends mainly on the specific type of microorganism and sort and intensity of active agents. As a rule, full plasma inactivation by indirect method takes about several minutes for cells and several tens of minutes for spores. One can see in Fig.3 that plasma inactivation by PC and NC (direct method) takes much shorter time (see curves 5 in graphs a-d).

In fact, bacteria are quite transparent objects for UV radiation. It means that, first, UV has low inactivation efficiency (approximately only one of 10^6 absorbed photons leads to the cell injury) and, second, UV interacts simultaneously with different extracellular and intracellular components independently on presence or absence of other active agents (*R*, *E*) outside or inside cell.

Because of small sizes, neutral reactive species (besides of extremely reactive OH radical) can also penetrate through extracellular components and cell membranes mainly due to porins (hydrophilic pores of about 1 nm) and ion channels. However penetration ability of *R* agents depends on the state of membranes - an increase in the membrane permittivity induced by *UV*, *E* and *R* agents leads to increase of *R*-inactivation. It means that *UV* and *E* agents can provide synergy effect with *R* agents.

Close examination of the curves in Fig.3 showed that positive and negative ions play essential role in bacteria inactivation by PC and NC. In our opinion, biocidial mechanisms exhibited by these ions are different. Moreover, we suppose these mechanisms

are not bio-chemical ones offered by [1] for quasi-neutral plasma conditions (indirect method) when total charge Q_{cell} falling to cell equals to zero. In the case of PC and NC (direct method), the Q_{cell} is not zero. It means that electrical effects associated with Q_{cell} are dominant, and biocidial mechanisms of positive and negative ions have rather bio-physical nature. Let us discuss this issue in more details.

Important thing which has to be taken into account is that cell located on the agar surface is surrounded by thin mucous capsule with salt water having a good electric conductivity σ. An existence of the conductive medium around cell leads to slow run-off (leakage) of the charge quickly deposited onto cell. In fact, the deposited charge could be kept on cell over time $\tau \cong \varepsilon\varepsilon_0/\sigma$ (several μs) before its flowing through capsule down the ground (ε_0 and ε are dielectric permittivity of vacuum and liquid).

Positive ions of PC. Electric current in PC is carried with transient streamers that are bright and thin (150 μm in diameter) plasma filaments having high current density. Each streamer transfers onto agar huge positive charge ≈ 50 nC. Once streamer strikes cell, great positive charge $Q_{cell} \approx 3\text{x}10^{-13}$ C very quickly ($t<\tau$) is deposited onto cell (Fig.5).

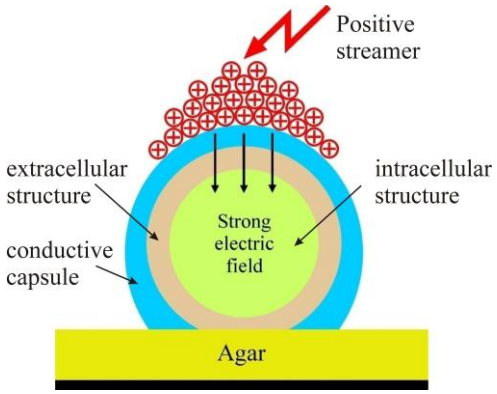

Fig.5. Transient distribution of a positive charge quickly deposited on cell by PC positive streamer.

Such amount of the deposited charge exceeds own positive charge located on the external side of the cell membrane by three order of magnitude. Huge charge Q_{cell} induces very strong electric field, providing instantly heavy electrical breakdown of cell as whole that results in non-reversible damages of extracellular and intracellular components and eventually leads to instant death of cell. Speaking in images and keeping in mind a difference in scales of phenomena, one can say that streamer strike at cell is similar to a strike of lightning at macro-object. The same electrical effect can happen with cells deposited on a dielectric barrier in DBD due to existence of microdischarges similar to streamers.

Negative ions of NC. In the case of diffusive steady-state NC with low electric current density, maximum negative charge that can be collected on cell is not exceed $3\cdot10^{-18}$ C. This amount of Q_{cell} is too small to induce a strong electric field providing an electrical breakdown of cell or membrane. However in this case there is a steady flow of negative ions falling down each cell ($J^- \approx 3\cdot10^7$ negative ions/s per cell) and slowly running-off through capsule down the ground (Fig.6). We suppose just this current of negative ions plays the crucial role in bacteria inactivation by NC.

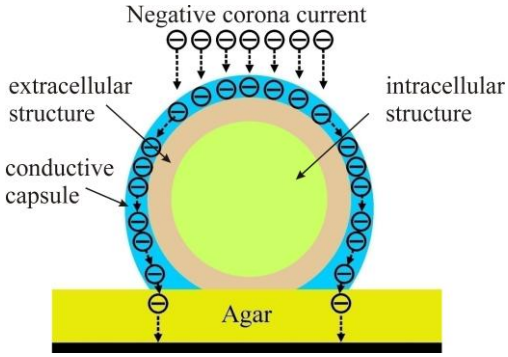

Fig.6. Steady distribution of a negative charge around cell due to flow of NC negative ions **Θ** falling down the cell.

To understand our hypothesis how external negative ions surrounding the extracellular structure can interact bio-physically with cell, let us consider this structure for Gram (-) and Gram (+) bacteria in more details (Fig.7).

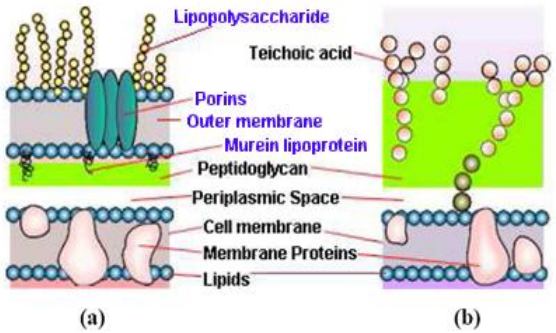

Fig.7. Diagram of the cell envelope arrangement of Gram (-) (a) and Gram (+) (b) bacteria (the cell envelope is defined as the cell membrane and cell wall plus an outer membrane, if one is present). Basic components of the extracellular structure are pointed in the legend.

The Gram (-) bacteria have an outer membrane composed of phospholipids and Lipopoly-saccharides (LPS) (Fig.8a). The LPSs impart a strongly negative charge to surface of Gram (-) bacterial cells. The Gram (+) bacteria cell wall is rich in Teichoic acids (TA) (Fig.8b). The TAs are also highly negatively charged. Bacteria cell surface

charge originates mainly from dissociation of carboxyl and phosphate groups and depends on pH: -COOH ↔ $-COO^- + H^+$; $-HPO_4 \leftrightarrow PO^-_4 + H^+$; $-H_2PO_4 \leftrightarrow HPO_4^- + H^+$.

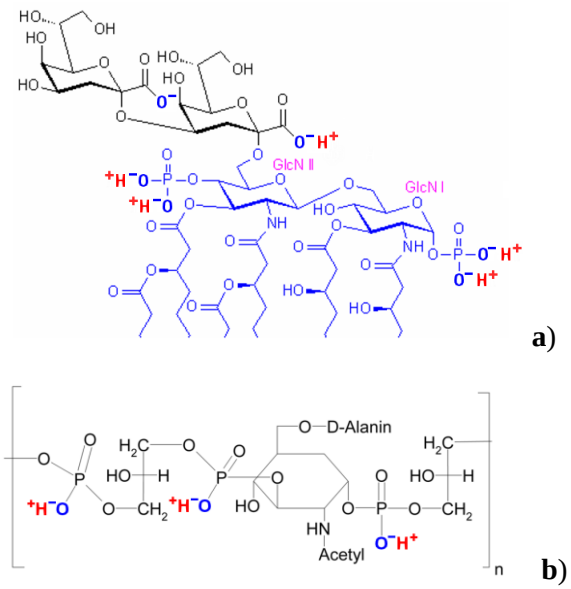

Fig.8. (a) Basic structure of Lipopolysaccharide (LPS), blue - Lipid A, black – Polysaccharide; (b) Basic structure of a teichoic acid (TA).

It means the unsubstituted oxygen anions O^- are bound to the surface but cations H^+ are free and form thin electric double layer (Fig.9a) screening strong electric field of the surface negative charge.

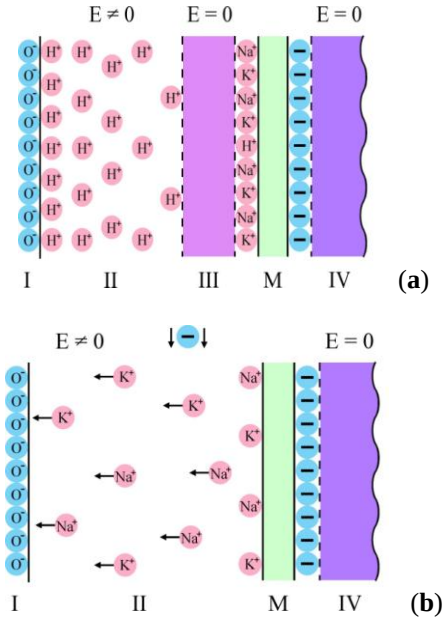

Fig.9. Sketch on distribution of negative and positive charges in the cell envelope: (a) – normal situation; (b) – situation after recombination of cations H^+ with negative ions of NC. I-II – double layer around TA or LPS with E≠0; III and IV – the rest of the cell envelope and intracellular space with E=0; M – polarized membrane.

Thickness δ of this Debye layer is determined by concentration of H^+ at the surface. In normal conditions local pH ≈ 2.1-2.8, and $\delta << \Delta$, where Δ is thickness of the cell envelope containing TA or LPS. Due to that, strong electric field of negatively charged TA or LPS does not penetrate to the outer surface of membrane as it shown in Fig.9a.

Another situation happens if there is external steady-state flow of negative ions falling down the cell surface. These ions will intensively recombinate predominantly with free cations H^+ because of their high concentration in comparison with other positive ions. Due to that the screening layer of H^+ surrounding the TA or LPS disappears, and strong electric field of the negatively charged cell surface will penetrate up to the outer surface of membrane.

The membrane outer surface is enriched with free positive ions of sodium Na^+ and potassium K^+, the presence of which is vital to normal functions of the cell membrane. However strong negative electric field will pull out these ions from the membrane surface to the cell surface (Fig.9b). Heavy lowering the amount of these ions at the outer membrane surface leads to strong disturbance of the membrane functions, in particular, to increase of the membrane permittivity. After that reactive agents *R* (if they are present) obtain practically free entrance inside the cell, and their biocidial effect drastically increases. The comparison of curves 1 and 5 in Figs. 3a and 3c confirms the hypothesis described above.

5. Conclusions

1) The impact of positive ions of PC is due to huge electric charge quickly deposited by streamer onto cell. This charge induces strong electric field across whole cell leading to its full electrical breakdown.

2) In NC there is strong synergy effect of joint action of *E+R* agents which is associated with recombination of cations H^+ surrounding LPS or TA with negative ions. Eventually it leads to high increase in the membrane permittivity that, in its turn, results in higher inactivation by *R* agents.

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5. References

[1] Plasma Processes and Polymers**,** Special Issue: Plasma Sterilization and Decontamination; June 2012 Volume 9, Issue 6 Pages 555–629, Ed by: Michael Kong, Mounir Laroussi, Michel Moisan, François Rossi