Acinetobacter baumannii deactivation by atmospheric-pressure cold plasma in the form of "guided streamers"

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Acinetobacter baumannii is a typically short, almost round, rod-shaped (coccobacillus) Gram-negative bacterium. It can be an opportunistic pathogen in humans, affecting people with compromised immune system, and is becoming increasingly important as a hospital-associated (nosocomial) infection. It has also been isolated from environmental soil and water samples. In this work, the influence of atmospheric-pressure cold plasma (in the form of axially propagating streamers in helium/air) on this bacterium is evaluated by means of colony count technique and scanning electron microscopy. The plasma itself is probed with optical emission spectroscopy. Based on the experimental results, post-treatment (delayed) biochemical effects on *A. baumannii* and morphological modifications appear dominant.

1. Introduction

Nosocomial or health-care associated infections, including bacteremia, represent a leading cause of death worldwide [1]. Most importantly, the irrational and excessive consumption of antimicrobial agents has led to multi- or even pan-drug resistant bacterial strains leaving no available treating options in the armamentarium of clinicians. Looking to the twilight of the era of antibiotics, other treatment approaches appear as an appealing as well as imperative alternative. Non-equilibrium atmospheric-pressure plasmas have been shown to present a great promise with superior characteristics for local inactivation of bacteria cells [2].

Unlike to previous works where *B. atrophaeus*, *A. niger*, *B. subtilis*, *E. coli*, *S. aureus*, *B. stearothermophilus*, *B. globigi* or *B. cereus* were inactivated by different plasma setups (for a nice survey in the field see reference [3]), *Acinetobacter baumannii* is here subjected to atmospheric-pressure guided streamers [4-6] and the inactivation efficiency is tested. *A. baumannii* is an aerobic Gram-negative coccobacillus that causes nosocomial human infections, particularly in immunocompromized individuals. These infections can result in septicemia, meningitis, endocarditis, pneumonia, wound infection, and urinary tract infections. *A. baumannii* has the ability to colonize and survive for long periods of time on dry inanimate surfaces, such as hospital equipment. In addition to its intrinsic resistance to antibiotics and desiccation, *A. baumannii* is very prone to accumulating resistance mechanisms especially in environments where antibiotic overconsumption is taking place [7]. Here, colony counting and scanning electron microscopy (SEM) provide insight into the plasma-induced damage of this bacterium and it is shown that post-treatment (delayed) effects are dominant.

2. Experimental setup and Specimens

2.1. Plasma setup

The plasma setup employed here for treatment of bacteria and the relative physics have been discussed in [5] and [6], respectively. Briefly, a thin tungsten wire (diam. 0.125 mm) is inserted in an alumina capillary tube (inner diam. 1.14 mm). A brass hollow cylinder (length of 10 mm) is attached on the outer surface of the alumina in such a way to be extended up to the tip of the wire. The distance between the tip of the wire and the tube orifice is 20 mm. Helium gas (N50) is introduced in the tube with a controllable flow rate. The wire is biased with a home-built high voltage (10 kHz sinusoidal / 0-12 kV) power supply and the cylinder is grounded directly. By this configuration, radial dielectric-barrier discharges (DBDs) are developed and axial "guided streamers" propagate in the atmospheric air where helium diffuses [8]. Here, the voltage and the flow are fixed at 11.5 kV and 2 slm, respectively, and a plasma "plume" of about 35 mm is achieved.

The gas temperature is approximated by UV-visible optical emission spectroscopy (OES), as described in references [5,6,8,9]. The rotational distributions corresponding to the follow band heads are here considered: $OH(A^2\Sigma^+(0)–X^2\Pi(0))$ and $N_2(C^3\Pi_u(0)–B^3\Pi_g(0))$. The probed zone is 20 mm along the plasma axis in respect with the tube orifice.

2.2. Bacteria preparation and treatment

The bacterium under study was *A. baumannii*. All strains were isolated in the Department of Clinical Microbiology, University Hospital of Patras, from the blood of patients treated for bloodstream infections and represent multi-resistant strains. All microbial strains used in this study were stored in TSB (BBL, Microbiology, Cockeysville, MD, USA) supplemented with 20% glycerol at -70°C and, when needed, they were sub-cultured on blood agar plates (BioMérieux S.A., France) for 24 h at 37°C.

Each bacterial isolate was suspended in PBS (pH 7.2) and photo-metrically adjusted to the desired concentration of $3x10^6$ CFUs/mL (CFUs: Colony Forming Units). 1 mL from the suspension was added in each of nine (three for each treatment time) wells of a 24-well flat bottom tissue culture plate (Sarstedt, Newton, USA). Bacterial suspensions were treated, at 4 mm distance between the tube orifice and the surface of the aqueous solution, for 0 (control samples), 10 and 20 min. The content of each of the nine wells was kept at 4°C in separate sterile eppendorfs.

Different dilutions of each suspension were sub-cultured at 2, 6, 24, 48 and 72 h upon plasma treatment on blood agar plates and incubated for 24 h at 37°C. Corresponding bacterial concentration was thus estimated. The effect of the plasma treatment was evaluated depending on the number of live bacteria capable of forming colonies on solid media. Bacterial concentration at the content of the three wells that were not treated with plasma (0 min) was used as control reference. The effect of helium gas itself on the bacterial viability was evaluated by subjecting bacterial suspensions to 2 slm He flow. Negligible effect was measured in respect to control samples.

2.3. Scanning electron microscopy of bacteria

The specimens were prepared for morphological examination by scanning electron microscopy (SEM). The microscope was a LEO-ZEISS Supra 35VP instrument equipped with an energy-dispersive X-ray (EDX) microanalysis unit (Bruker), allowing for elemental semiquantitative analysis of the specimens. The content of each well was filtered on 0.45 μm filters (Sartorius). Withheld bacteria on the filter surface were fixed in a solution of 2.5% glutaraldehyde overnight at 4°C, carefully washed two times for 10 min in PBS and dehydrated sequentially in ethanol solutions 10 %, 30 %, 50 %, 70 %, 80, 90 for 10 min each and finally in ethanol 100% for 2x10 min, as previously described [10,11] but with small alterations. Eventually, a thin film of about 4 nm of gold was coated on the filter-bacteria by means of a BAL-TEC SCD-005 sputter coater.

3. Results and Discussion

Fig. 1 presents the number of survived bacteria in CFUs as a function of the time elapsed starting from the end of 10 and 20 min plasma treatment. The evolution of the untreated sample (control) is shown as well for comparison reasons. Clearly, the present plasma has a profound effect on the *A. baumannii* population which was drastically decreased mainly in the post-treatment time. The first measurement ("zero-time" point) was carried out 2 h after the plasma treatment due to sample transfer and analysis waits. This first point (**Fig. 1**) indicates colony diminution up to 4 orders of magnitude. The effect was more pronounced upon increasing bacteria exposure time to plasma. Cell death could thus be achieved either by increasing the plasma exposure dose or allowing sufficient time for the reactive species to initiate an intracellular signalling response in the cells [12].

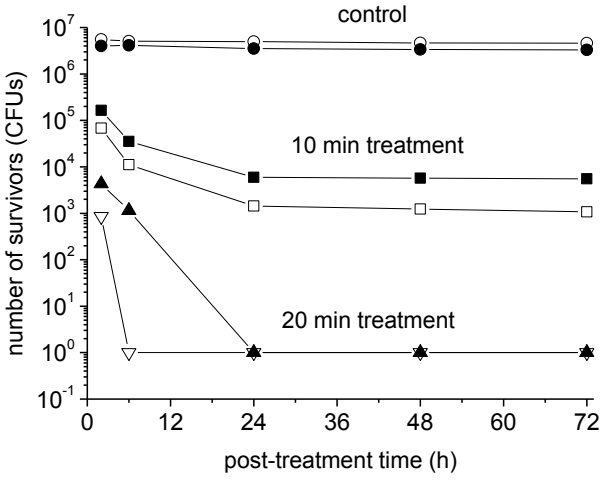

Fig. 1. Post-treatment evolution of the *A. baumannii* formed colonies as a function of the plasma treatment time. Open and closed symbols refer to two individual series of experiments under exactly the same conditions but on two different dates.

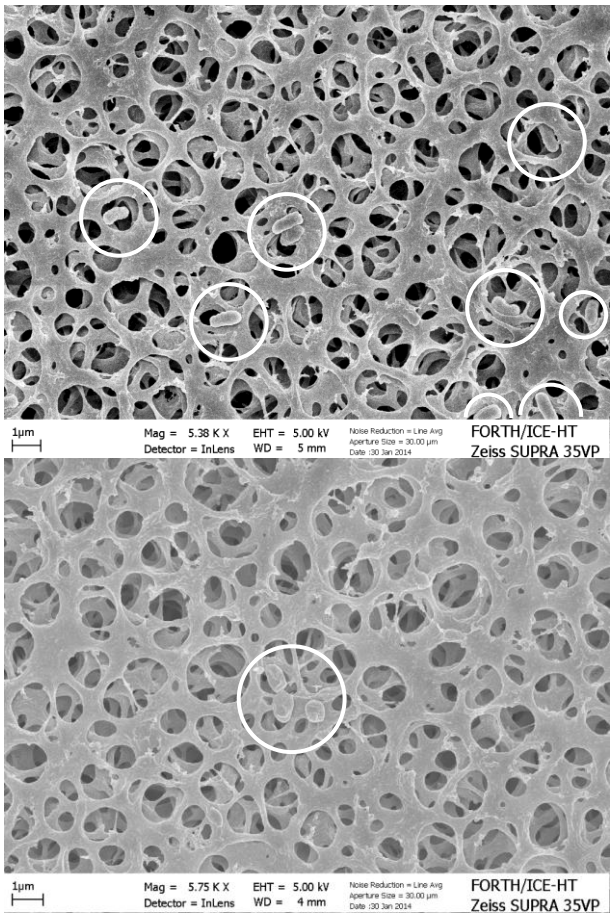

Fig. 2. SEM micro-images of *A. baumannii* bacteria untreated-control **(upper)** and plasma-treated for 20 min (**lower**). Images were taken about 24 h after the plasma treatment of the samples (refer to Fig. 1) and both control and treated samples were stored at 4°C.

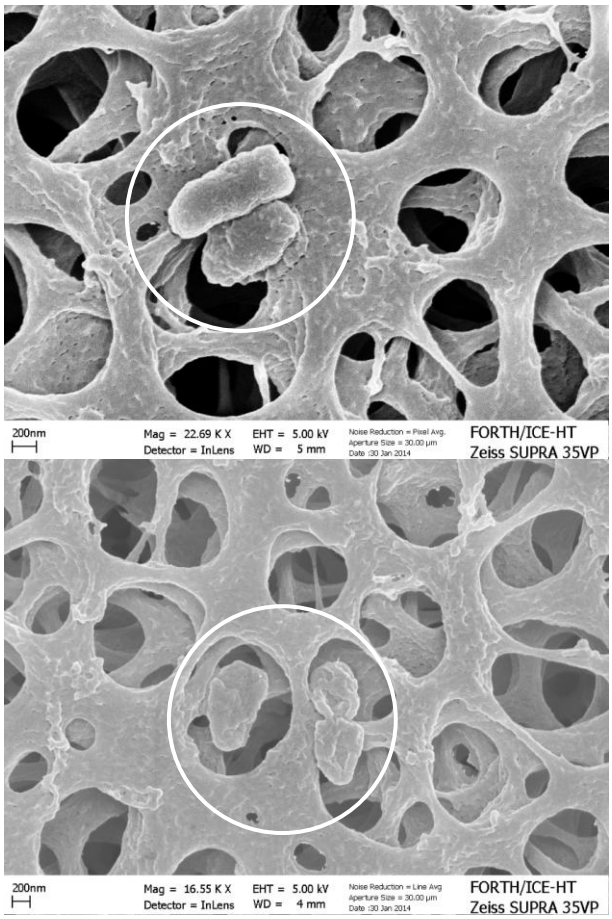

Fig. 3. Zoomed in SEM micro-images of *A. baumannii* bacterial cells untreated-control **(upper)** and upon plasma treatment for 20 min (**lower**). See caption of Fig. 2 for details.

Untreated and plasma treated bacteria are shown in **Figs. 2** and **3**. Treated bacteria were significantly fewer and completely deformed. More specifically, they showed ruptured and shrinked morphology. It cannot be stated whether this morphological degradation was the cause or the result of the bacteria death. In any case, the plasma led to leakage of intracellular ingredients leaving empty cell walls and membranes.

According to the wide scan optical emission spectrum of the present plasma (as presented elsewhere [5,13]), the aqueous solution with the bacteria is subjected to a flow of (re)active species including: $N_2(C^3\Pi_u - B^3\Pi_g)$, $N_2^+(B^2\Sigma^+_u - X^2\Sigma^+_u)$, $OH(A^2\Sigma^+ - X^2\Pi)$, $He(3^1P - 2^1S; 3^3D - 2^3P; 3^1D - 2^1P; 3^3S - 2^3P)$, and $NO_\beta(B^2\Pi - X^2\Pi)$ of much weaker OES intensity. At the same time, the gas temperature (**Fig. 4**) remained rather low (<65°C). In reality, when the rotational distributions of **Fig. 4** were recorded at higher resolution (i.e. 0.001 instead

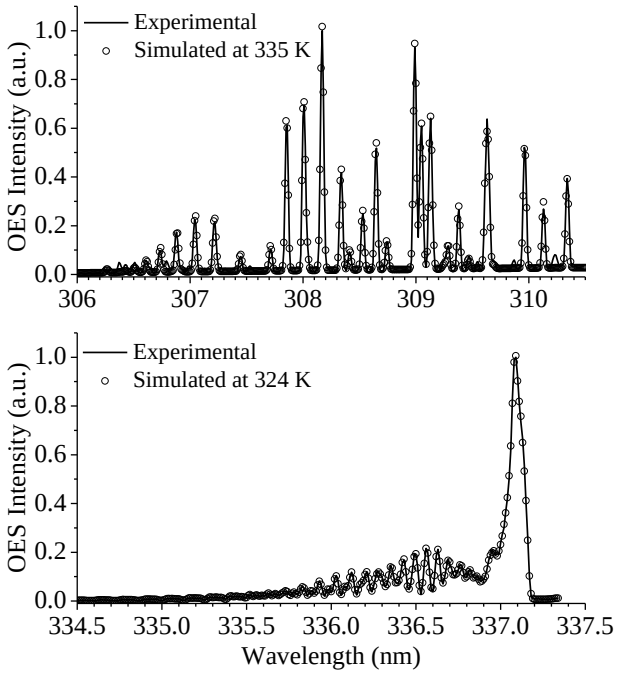

Fig. 4. Theoretical rotational distributions fitted on experimentally determined ones for estimation of the gas temperature. OH **(upper)** and N_2 **(lower)** molecules.

of 0.01 nm here) a better approximation of the temperature (close to 36°C) was found [5]. Thus, the role of heating in *A. baumannii* inactivation with the present setup should not be considered principal.

On the contrary, reactive oxygen and nitrogen species (ROS and RNS, respectively) [14], UV photons [3], and ozone [15] have been widely recognized as main actors in the action of plasma on bacteria inactivation. This action may be direct on the bacteria or indirect by altering the chemical properties of the aqueous solution where the bacteria are suspended. For instance, ozone decomposition in water is complex and many transient oxidizing species ($^{\cdot}OH$, $^{\cdot}HO_2$, O^-, O_3^-, $^{\cdot}O$ and singlet $^1O_2$) can be formed [16]. Finally, recent experiments on cell models (liposomes) suggest that charged particles like ions may participate to membrane disruption [13]. Indeed, in another work [15], it has been reported, that inactivation is due to both chemical damage (ozone and atomic oxygen) and physical damage (by ions in the streamer discharge). The latter could possibly explain the images shown in **Figs. 2** and **3**.

4. Conclusions

According to the present work, *A. baumannii* may efficiently be deactivated by atmospheric-pressure cold plasma produced in the form of "guided streamers" which propagate into helium/air. In the first 24 h after a 20 min treatment, the formation of *A. baumannii* colonies is interrupted completely and the bacteria are morphologically deformed. Aforementioned results demonstrate that external application of this plasma-based method (e.g. around surgical sites) could contribute to prevention of hospital-associated infections and elimination of antibiotic use. This alternative does not involve high temperature and the inactivation is triggered by the plasma reactive species.

5. References

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