The effect of low-temperature plasma on bacteria as observed by repeated AFM imaging

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Abstract. Research on low-temperature atmospheric plasma sources (LTAPS) has grown strongly over the last few years, in part driven by possible medical ‘in vivo’ applications. LTAPS offer new technology for medicine and biomedical engineering. Important application examples include in situ production of reactive molecules and ions, delivery at the molecular level, contact-free and self-sterilizing devices. An important issue is the efficient bactericidal effect of LTAPS, which has already been studied widely in vitro. In spite of the many investigations, details of the plasma effect on bacteria are still largely unknown. To contribute to a better understanding of the sterilization process, we investigated the morphological changes of bacteria using atomic force microscopy before and after plasma treatment at high resolution. We examined both gram-positive and gram-negative bacteria at different plasma exposure times. Additionally, the effect of UV radiation as one agent in the plasma was...
investigated separately. Our results suggest that several sterilizing mechanisms exist and they proceed at different timescales.

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1. Introduction

The bactericidal effect of gas plasmas is routinely used in sterilizing the surfaces of e.g. medical instruments or biomedical materials [1]. The quality and speed of sterilization, the ability of contact-free treatment and penetration of small cavities make this technique highly attractive for medical in vivo applications. A variety of low-temperature atmospheric plasma sources (LTAPS) has been developed recently, such as the plasma needle [2], plasma pencil [3], dielectric barrier discharge (DBD) devices [4] or the plasma torch [5]. A comparative overview of various devices is given in [6, 7].

LTAPS produce a strong bactericidal effect in a very short time, as has already been shown in various in vitro studies (e.g. [8] and references therein). Bacterial cultures are plasma treated for a given (short) time and then incubated for 1 or 2 days. Since bacteria multiply rapidly, the absence of bacteria in the treated region is a direct measure of the bactericidal effect of LTAP. Figure 1 shows two agar plates covered with Escherichia coli that received plasma treatment of 1 min (a) and 2 min (b). In the region where the plasma torch was centred, a zone of inhibition of growth (dark region) is clearly visible. By counting the colony-forming units and comparing with diluted samples, we see a bacterial load reduction of $\sim 10^6$ in the zone of inhibition after a plasma treatment for 1–2 min with 85 W of microwave power. While the bactericidal effect is clearly demonstrated, the exact sterilizing mechanisms [5] of plasma treatment are still unknown. In the literature, several processes such as chemical reactions with reactive species and ions, charging, UV radiation and heat are discussed (see e.g. [9]).

To quantify the effect of plasma treatment on bacteria, we imaged bacteria at high resolution before and after plasma treatment using atomic force microscopy (AFM). Due to its three-dimensional imaging capabilities, the AFM is a well studied technique to study morphological changes caused by the treatment. Both gram positive and gram negative bacteria were plasma treated for different periods of time and imaged with an atomic force microscope. This allows a systematic investigation of the change in the bacteria’s morphology, information that can help us to identify the bactericidal agents. To clarify the role of ultraviolet (UV) radiation in the sterilization process, we additionally imaged bacteria before and after exposure to UV radiation by an UV light-emitting diode.
Figure 1. Bacterial cultures after plasma treatment. (a) Already after 1 min of plasma treatment, an area at the centre of the agar plate is decontaminated from *E. coli* bacteria. (b) This area increases considerably after one additional minute of plasma exposure.

2. Material and methods

2.1. Plasma source

Our ‘plasma torch’ has six hexagonally arranged electrodes of 4 mm diameter placed inside an aluminium tube (torch body) of 135 mm length and 35 mm inner diameter. These electrodes have serrated surfaces and are placed 4 mm from the inner surface. Extensive tests have shown that this arrangement gives the highest efficiency in plasma production. Six plasma clouds are thus produced with argon gas (2.2 slm) at a microwave power of ~85 W and at 2.45 GHz. Argon is applied from the upper part of the torch through an insulator shower plate and flows along the electrodes until it merges into the air. In order to keep the plasma condition stable, the torch body is cooled and maintained at 320 K. For more details and a schematic view of the device, see [5].

2.2. AFM

We chose the atomic force microscope [10] for imaging, since bacteria do not need any special, and potentially destructive, preparation for this microscope. AFM measurements were conducted using a Dimension 3100 (Veeco, Santa Barbara, CA) in tapping mode. For imaging, silicon cantilevers with a nominal resonance frequency of 300 kHz (BS-TAP300, BudgetSensors, Sofia, Bulgaria) were used. Image processing was done using SPIP 4.5 (Image Metrology A/S, Lyngby, Denmark).

An advantage of AFM over conventional light and electron microscopy is the precise three-dimensional measurement of the surface topography, especially of the height of selected objects without special preparation. The measurement of lateral distances and widths is affected by the geometry of the AFM tip. Even with a tip radius of about 10 nm, significant errors can be introduced into the measurement of bacteria width due to the convolution between tip geometry and bacterial shape [11]. The height, however, can be measured with high precision. To ensure accuracy, the instrument was calibrated prior to the experiments with a calibration grating (TGZ02, Micromasch, Tallinn, Estonia).

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In order not to rely on height measurements that are based on only a single point, we developed the following image analysis procedure. Firstly, the mean height of the inner region of a bacterium is determined. Secondly, the mean height of a region in a defined vicinity around the bacterium is computed. The difference of these two numbers is used as the bacterial height. Note, however, that in our measurements, this method is not applicable to all recorded bacteria since some of them cannot be well separated.

2.3. Bacteria samples

We used both gram-negative and gram-positive bacteria. A basic property that distinguishes these two types of bacteria, which differ in the structure of the cell wall, is that gram-positive bacteria possess a thicker and more robust cell wall. As a representative example of gram-negative bacteria we chose an \textit{E. coli} strain (DSM 1116), and for gram-positive bacteria we chose coagulase-negative \textit{Staphylococcus} (laboratory strain).

In the first step of the sample preparation, bacteria from a primary stock culture were inoculated on an agar plate (Mueller Hinton agar, Oxoid). After overnight incubation at 35$^\circ$C, a small sample of the colonies was smeared on an object slide using a medical swab. A suitable spot on the glass plate was chosen to record the untreated bacteria with the atomic force microscope in tapping mode. Afterwards, the selected region was plasma treated for a defined period of time. The distance from the aperture of the torch to the sample was always 2.5 cm where the gas temperature was below 30$^\circ$C. Reactive species can affect the bacteria since NO$_2$ was found between the torch and the sample (approximately a few ppm). Finally, the treated bacteria were imaged again.

It takes approximately 20 min to record the image with an atomic force microscope. In order to investigate the possibility that the bacteria die naturally during this time, we conducted qualitative tests: smears of \textit{E. coli} were prepared without further treatment as described above and subcultured both immediately and after incubation at room temperature for 40 min. Each time, a sample of the bacteria was taken from the object slide with a medical swab and suspended in a physiological salt solution. This solution was poured into an agar plate and incubated overnight at 35$^\circ$C after removal of excess fluid. The densities of the grown colonies were compared and no relevant difference was found. This shows that the bacteria survive the exposure on the glass plate for the time taken to conduct our experiments; this indicates that any morphological changes observed are due to the plasma treatment. Another question is whether the AFM measurements themselves might harm the bacteria. As will be shown later, as a result of the ‘short’ plasma exposure time of the measurements, this possibility can be ruled out.

3. Results

In the first experiment, we treated \textit{E. coli} bacteria for 30 s with our plasma. For that purpose, an area of approximately 10 $\times$ 10 $\mu$m$^2$ was chosen and recorded with the atomic force microscope. Figure 2(a) shows the scanning result containing about 15 bacteria. After plasma treatment this area was scanned. The corresponding image is shown in figure 2(b). A comparison of these two images does not reveal any relevant morphological changes in the bacteria. The height distributions of both images are similar (figure 2(c)). Additionally, the height of some bacteria
Figure 2. Topographic AFM images of *E. coli* before and after plasma treatment. (a) Area covered by untreated *E. coli* bacteria (edge length 10 µm). (b) The identical bacteria after 30 s of plasma treatment. (c) Corresponding height histograms of both images. The scale was adjusted to show the height distribution of the bacteria.

was determined based on the AFM measurements. They are marked by squares in figure 3. On an average, the height remained unchanged within the measurement accuracy. Hence, these height measurements do not indicate major alterations. As an aside, the comparison shows that repeated AFM scanning does not affect the bacterial morphology.

This experiment was repeated with another sample of *E. coli*. An area with an edge length of 15 µm was chosen. It contains 12 bacteria and is displayed in figure 4(a). After plasma treatment for 60 s this section was recorded again with the atomic force microscope. The result is shown in figure 4(b). As in the previous experiment, no relevant changes can be observed in the bacteria. This finding is also confirmed by the height measurements (triangles in figure 3). The average height change is again less than 2 nm.

In the third experiment, the treatment time was increased to 5 min. Figure 5(a) shows two untreated *E. coli* bacteria. In this case the edge length of the recorded area is approximately 5 µm. The same two bacteria after 5 min of plasma treatment are shown in figure 5(b). After this prolonged treatment, major changes in the bacterial morphology could be observed. The general shape of the bacteria was still discernible, but alongside the original structure ‘additional’ matter was present. A possible interpretation of this finding is that the cell wall of the bacteria has been ruptured and the cell plasma was released to the outside. The change in morphology was accompanied by a considerable flattening of approximately 25%, as shown in figure 3 (circles).
The latter experiment was repeated with gram-positive *Staphylococci*. The untreated bacteria are shown in figure 6(a). This species typically forms clusters of spherical cells (cocci). Each ‘sphere’ in this structure is one bacterium. The same cluster after 3 min of plasma treatment is shown in figure 6(b). The edge length of the recording is 5 µm. A comparison of the two images shows that many of the single bacteria are not visible any more in the treated sample. The structure of these bacteria has dissolved. Again, rupture of the cell wall and release of cell plasma to the outside are a possible interpretation of this finding. Also, the height of the cluster decreased by approximately 10%, not as clear a change as in the previous experiment with gram-negative bacteria. This could be a consequence of the greater strength of the gram-positive cell walls.

One of the agents in the sterilizing process with many plasma sources is UV radiation. Especially wavelengths around 255 nm (UV-C) are known to cause dimerization of thymine molecules in the DNA, which inhibits cell replication. In order to investigate the effect of pure UV light on bacteria, we conducted experiments with a UV diode emitting light at 255 nm with 200 µW cm\(^{-2}\). The macroscopic result of UV treatment of bacteria with this diode is shown in figure 7. After 2 min of UV exposure, the affected region was completely sterilized.

The microscopic (AFM) features are displayed in figure 8. Figure 8(a) shows an area with untreated *E. coli* bacteria (edge length 10 µm). The same area after 2 min of UV irradiation is shown in figure 8(b). The UV treatment did not have a discernible morphological effect on

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**Figure 3.** Height variation caused by plasma and UV treatment. The height of the *E. coli* bacteria was measured before (x-axis) and after treatment (y-axis). The symbols represent the different treatment conditions. In most cases, the height of the bacteria is not affected; only after 3 min of plasma treatment was a considerable height reduction observed.
Figure 4. The AFM images show *E. coli* bacteria before (a) and after (b) 60 s of plasma treatment. The edge length of the displayed area is approximately 15 µm. Corresponding height histograms of both images are shown in (c).

Figure 5. AFM images of the identical *E. coli* bacteria before (a) and after (b) 5 min of plasma treatment. It seems that the cell wall is ruptured and cell plasma is released to the outside.

the bacteria, nor did it alter the height histogram (figure 8(c)). This finding is reflected in the height measurements of four selected bacteria (see figure 3, diamonds). A significant change in height was not observed. This observation suggests that the bactericidal effect of UV light is different from that due to plasmas: the UV radiation presumably penetrates into the inner part of the bacterial cytoplasm and damages the DNA, whereas the plasma (perhaps in addition) also appears to damage the cell wall.

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4. Discussion

Macroscopic measurements showed that our plasma torch can efficiently kill bacteria inoculated on an agar plate. After plasma treatment of 60 s (or longer) the affected area is decontaminated with only a few survivors.

To gain further insight into the sterilizing mechanism, we investigated bacteria before and after plasma treatment with an AFM. In the course of this microscopic investigation we compared the change in the bacteria’s morphology and height after 30, 60, 180 and 300 s of plasma treatment. In most of the cases no major changes in the morphology were observed. Also, the height measurements of the treated bacteria did not reveal significant changes. In a few cases after prolonged treatment, however, we observed clear indications of physical destruction. The AFM images suggest that the cell wall was partially ruptured and the cell plasma was

Figure 6. (a) An untreated sample of gram-positive coagulase-negative Staphylococcus (edge length 5 µm). (b) After 3 min of plasma treatment, morphological damages can be observed.
Figure 7. This image shows an agar plate covered with *E. coli* bacteria. Three spots of the freshly inoculated agar plate were exposed to a UV diode with increasing durations followed by overnight incubation of the agar plate at 35°C. The diode operates at 255 nm. After 2 min a defined area is sterilized.

Figure 8. AFM images before (a) and after (b) 2 min of UV treatment. Again, no morphological changes are observed. Corresponding height histograms of both images are shown in (c).

released to the outside. Although our observations are based on the quantitative analysis of only a few individual bacteria, we are convinced that the direct comparison of the morphology before and after treatment provides a well-studied measure of the effect of plasma treatment.

The details of the bactericidal effect presumably depend strongly on the plasma density, temperature and composition of reactive species, i.e. the ‘plasma design’. Several candidates
for sterilizing mechanisms have been suggested. One is electrostatic disruption of the cell wall [12, 13]. Thus it might be expected that electrostatic disruption is easier for gram-negative bacteria than for the more robust gram-positive bacteria. In our experiments, however, we did not observe a clear differentiation, since we also found an indication of cell wall rupture in gram-positive bacteria, depending on the treatment duration.

Reactive oxygen species (ROS) are also suggested to play an important role and to cause physical destruction of bacteria after plasma exposure [14]. Montie et al [14] suggest different chemical pathways of ROS for gram-negative and gram-positive bacteria, but both lead to irreversible cytoplasmic membrane damage. A diffusion of reactive species through the outer membrane of gram-positive bacteria is also discussed by Laroussi and Leipold [15], who found that, in addition to ROS, reactive nitrogen species (RNS) lead to damage of the cell integrity, which eventually results in destruction of the bacteria [15]. With their device (DBD), they claim that reactive species play the most important role in the sterilizing process.

In our experiments we did not observe physical damage to E. coli within 60 s of plasma treatment. Therefore, RNS and ROS cannot completely explain our results, since we know from our macroscopic measurements that bacteria are killed. The germs were inactivated before the occurrence of morphological alteration.

Several authors state that UV radiation does play a role in the sterilization process with their respective devices [16]–[18], which is also the case with our plasma torch. We could, however, show that UV radiation alone does not lead to physical destruction of the bacteria even if they are exposed to high dosage at 255 nm.

5. Conclusion

We investigated the role of isolated sterilizing agents in a low-temperature atmospheric plasma. Gram-positive and gram-negative bacteria were exposed to Ar plasma. To identify a sterilization mechanism, we investigated changes of the morphology of individual bacteria by imaging the identical bacterium before and after treatment.

In summary, we cannot assign one single agent to the sterilization of bacteria. We suggest that several sterilizing mechanisms that include reactive species, charged particles and UV must exist in plasmas. Together, they lead, possibly in combination, to inactivation of bacteria. This is in agreement with published results (e.g. [16]–[18]), where other possible agents like e.g. reactive species and metastable particles are discussed.

Physical destruction of the bacteria was not observed at a treatment time lower than 3 min in our experiments. Based on this observation, one may conclude that the different sterilizing mechanisms proceed at different timescales. This assumption is supported by various published survival curves, as they are summarized in [9]. These curves describe the dependence of the number of surviving bacteria (measured, e.g., as colony-forming units) on the treatment time. Many of these curves exhibit a double- or multi-slope shape. Such a behaviour is indicative of the relevance of several distinct sterilization mechanisms. Both, these investigations and our observations, can be understood, if the hypothesis of different mechanisms and different reaction times is correct.

However, we are aware of the fact that our conclusions are based on only a few measurements. More experiments have to be conducted to evaluate the hypothesis described above, for example investigations with different plasma exposition times and with different
bacteria strains. In this study, we started to investigate possible isolated sterilizing agents and we plan to continue this with other candidates.

From the methodological point of view, the AFM has proved to be an attractive tool for the investigation of plasma effects in terms of the morphological change of bacteria. The major advantage is the possibility of observing the same bacterium before and after treatment at high resolution without special preparation. Thus, the AFM can contribute to understanding the open question of the sterilizing mechanism(s) of plasma.

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