Assessment of photodynamic damage on *Escherichia coli* via atomic force microscopy

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ABSTRACT

Photodynamic antimicrobial therapy (PAT) may become a useful clinical tool to treat microbial infections, overcoming microbial resistance that is a major problem nowadays. The aim of our work was to verify the damage caused by photosensitization over a *Escherichia col*) via atomic force microscopy (AFM), looking for structural changes that might occur in cells after PAT. Cells culture were grown until a stationary phase to reach a concentration of approximately 10^8 cells/mL allowing the production of extracellular slime in a biofilm-like structure. The cells including the extracellular matrix were put in a slide and its structure was observed using AFM; subsequently a water solution of methylene blue at 60μM was applied over the cells and a pre-irradiation time of 3 minutes was waited and followed by illumination with a diode laser (λ=660nm, power 40mW, 3min, fluence 180J/cm^2, beam diameter 0.04cm^2). The same cells were observed and the images stored. A second set of experiments was performed with a smaller number of cells/area and without extracellular slime, using the parameters abovementioned. The results showed alterations on cellular scaffold markedly dependent on the number of cells and the presence of extracellular slime. The slime is targeted by the photosensitizer, and after irradiation a destruction of the matrix was observed; when fewer cells were evaluated the destruction is much more evident. The images suggested rupture of the cellular membrane and cellular fragments were observed. Our findings indicate that AFM seems is a useful tool to investigate parameters linked with photodestruction of microorganisms.

Keywords: Photodynamic therapy, antimicrobial, atomic force microscopy, photoactivated disinfection, bacterial topography.

1- INTRODUCTION

Photodynamic therapy (PDT) is a treatment modality that uses chemical compounds known as photosensitizers and a light source to destroy target cells, through the formation of reactive oxygen species (ROS) that leads to oxidative stress on cells. Photodynamic antimicrobial therapy (PAT) has as target microbial cells, including bacteria, fungus and virus[1].

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Microbial resistance to the commonly used antimicrobial approaches has been persistently reported and it is considered a major worldwide health problem[2]. Therefore new antimicrobial strategies are extremely necessary and PAT seems to be a suitable method, mostly for localized and superficial infection.

The susceptibility of different bacteria to PAT varies according to several factors, including type of microorganism, quantity of cells, stage of development, since cells in exponential growth phase are more susceptible than cells in stationary growth phase, environment, type of photosensitizer ant its charge, and irradiation conditions among others.[3]

The composition of the microorganism outer layer seems to be of great importance as Gram negative microorganism that possess a complex outer barrier with two lipid bilayers are more resistant to PAT than their Gram positive counterpart [3].

The ROS produced during PAT may target a lot of cell components including cell membrane via lipid peroxidation and it may cause the rupture of the cell membrane with consequent cell death. The balance between pro-oxidative and antioxidant components within the area where ROS are been formed seems to be crucial to the efficiency of this therapy.[4]

Another important variable is the quantity of possible targets to deactivate the ROS formed. In this context the extracellular slime produce by cells and the microbial organization into a biofilm, a well structure ecosystem, promotes an extra protection against any antimicrobial strategy including PAT, since planktonic cells are more susceptible to PAT than the ones present in a biofilm[5].

The morphological changes in cells caused by oxidative stress may be studied by different microscopic methods. Among them the atomic force microscopy (AFM) stands out as a technique that provides 2D and 3D images of the cell morphology, in addition to quantitative information with the same precision level in the three axis *x-y-z*.. AFM can be used to image cell surfaces on scales from micrometer to nanometer with the objective of characterize surface, texture and shape. Another important characteristic of this type of microscopy is that minimal sample damage occurs during its preparation, and there is no need of dehydration, coating, staining or submitting the sample to vacuum.

In this study we used Gram negative bacteria, in stationary growth phase, with evident production of extracellular matrix. AFM imaging were performed before and after treatment with methylene blue (MB) and red laser in order to observe the effect of PAT on cell morphology in different conditions as increased number of cells and different irradiation setup.

MB, a cationic phenothiazinium photosensitzer, has been widely studied for PAT and due to its charge it has the ability of connecting with cell membrane, but it also can penetrate cell and bond with DNA. Therefore it may has different mechanisms of action, and morphological evaluation of bacteria after treatment may provide further insight that may help the improvement on treatment parameters choice.

2- MATERIALS AND METHODS

2.1- Cell Culture

Escherichia coli (HB101) were cultured in Eppendorf vials containing brain and heart infusion broth (BHI) during 48h at 37 °C allowing the formation of a biofilm like extracellular slime on the bottom of the vial and providing a bacterial concentration of approximately 10^8 cells/mL. Afterward the cells were centrifuged at 4000rpm during 15min.

The cells were gently washed with PBS (phosphate buffered saline) and again centrifuged for another 15 min with the same rotation and the supernatant was removed. For the first assay ten μL aliquots of the cell mass was removed from the Eppendorf spread over a clean glass coverslip and air dried at room temperature. It has been reported that this method allows bacterial viability[6]. For the second set of experiments five μL aliquots of the cell mass was washed to remove the slime spread over another clean glass coverslip and also air dried at the same conditions as abovementioned.

2.2- Photodynamic Process

Methylene Blue (MB) was used as photosensitizer and it was purchased from Sigma-Aldrich (USA) and used without further purification. It has been demonstrated that commercial and purified MB does not have significant differences regarding the photodynamic efficiency[7]. The dye was prepared by dissolving appropriate quantity of the powder in sterile distilled water to a concentration of 60μM. The solution was used immediately after preparation.

For irradiation a λ=660nm diode laser (MMOptics, São Carlos, Brazil) was used with output power of 40mW, and beam area of 0.04cm^2. The laser output power was checked prior to irradiation with a powermeter (Lasercheck, Coherent, USA)

2.3-AFM imaging

Bacteria imaging was performed with the SPM 9500J3 scanning probe microscope (Shimadzu Corp, Hadano, Kanagawa. Japan) in dynamic mode at low scanning rate (0.5-1.0Hz). I-shaped cantilever tips (Nanosensors, Nanoworld AG, Switzerland) with radius of curvature of less than 10 nm, nominal resonance frequency of 330 kHz, and 42 N/m of spring constant were used.

On the first assay, surface topography of untreated bacteria at the higher concentration (10μL of the cellular mass), was imaged. Thereafter, ten-μL of MB solution was applied over the cells and a pre-irradiation time of 3 minutes was waited and followed by illumination (λ=660nm, output power 40mW, 3 min, fluence 180J/cm^2). The same cells were observed and the images stored.

On the second set of experiments surface topography of untreated cells in smaller concentration and without slime was analyzed. Afterward the MB solution was applied over the cells with the same dye concentration followed by irradiation as aforesaid, and the cells were imaged again. Subsequent periods of irradiation were performed until a total irradiation time of 10min corresponding to a total fluence of 600J/cm^2. Images were obtained at 3min, 6min and 10min.

The images were analyzed using the SPM-9500 Series Data Processing Software (Shimadzu, Corp. Hadano, Kanagawa, Japan). The mean cell width was measured before and after treatment.

3- RESULTS

Figure 1 shows a representative 2D AFM image obtained in the first experimental set with cells intact without treatment. The results displayed small cells that are rode-shaped. Some cells presented indistinct borders indicating the presence of the extracellular slime. The mean width observed was 830nn (±189nm).

In Fig 2, the same slide can be observed with cells after treatment. In a panoramic view we observed the removal of the extracellular slime after PAT with MB and red laser. The cells appeared flatter, losing their roundedness after treatment. The mean width calculated was 736nm (±226nm).

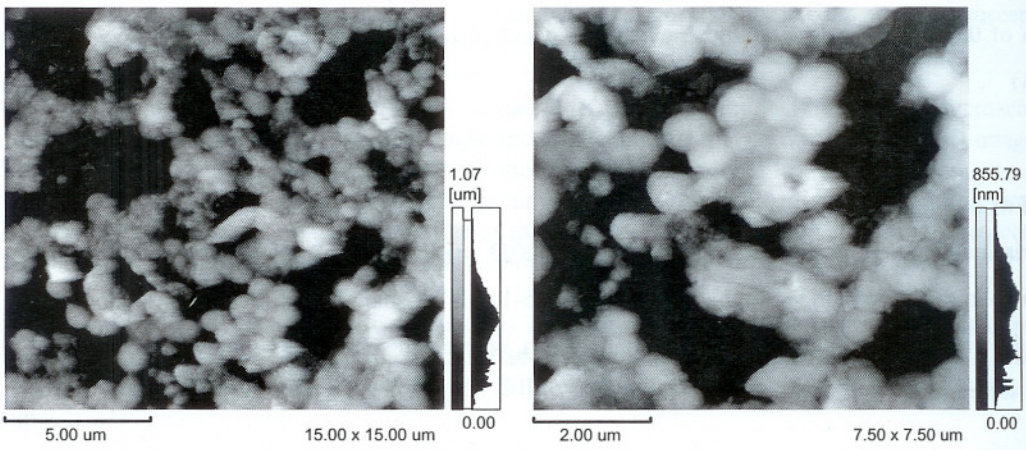

Figure 1 – AFM 2D images of *E. coli* cells before treatment.

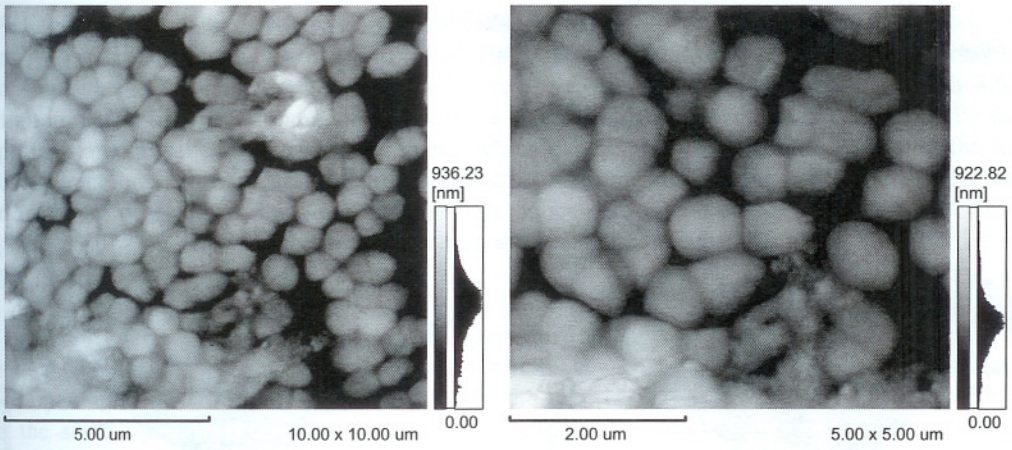

Figure 2 – AFM 2D images of *E. coli* cells after treatment.

Figure 3 represents the second set of experiments with a smaller number of cells, without extracellular matrix after PAT. Much more evident changes can be noted, the cells lost their original shape and a "blow up" aspect can be observed.

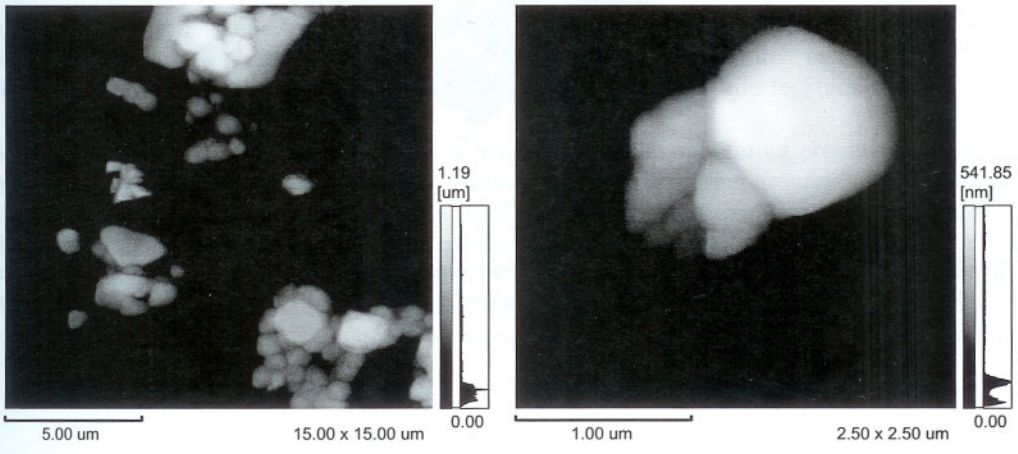

Figure 3 – AFM 2D images of *E. coli* cells from the second experimental set. A smaller number of cells were used and the slime was washed before PAT. The figures represent the images obtained after treatment with MB and 10 min of irradiation.

Figs 4 are 3D visualization of all the conditions tested. In a clockwise direction on top left side the cells without treatment, followed by cells after treatment, where we can observe the formation of grooves on cell surface that were not present before therapy The bottom image represents the final stage of bacteria after 10 min of irradiation. It is quite evident that cells shape and morphology are completely destroyed after 10 min of irradiation. Small structures with a

mean size of about 300nm appeared on the images, which may represent membrane fragments or even organelles inside cytoplasm that were forced out of the cell due to membrane rupture.

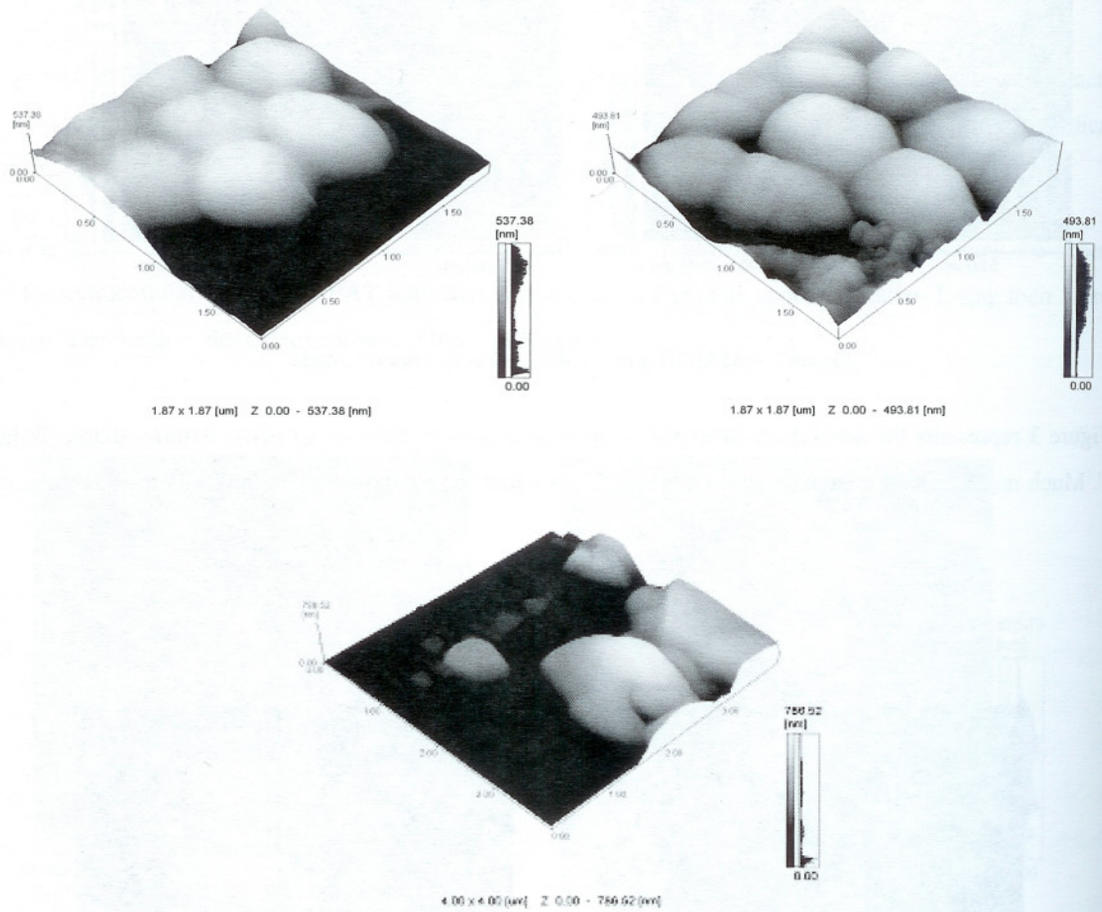

Figure 4 – 3D visualization of all tested conditions. In a clockwise direction on top left side the cells without treatment, followed by cells after treatment with PAT for 3min and on the bottom the image obtained after 10min of irradiation.

4 - DISCUSSION

This study evaluated the morphological changes occurring in bacteria after PAT via AFM. Three different variables were analyzed, first, the extracelular slime effect, second the number of cells, and third, the irradiation time.

Any antimicrobial therapy works better in planktonic cells than on a biofilm. Different pathways explain the reason for such effect, depending upon the antimicrobial mechanism of action. In PAT the slime may difficult the interaction of the photosensitizer with microbial cells.

Our results demonstrated that the slime is actually the first target affected after PAT. The bacteria images obtained after PAT presented a much clearer environment, so PAT had a "wash out" effect. This data may have a clinical implication since maybe PAT should be performed with two different targets when dealing with an infected site that present a biofilm in it. The first PAT treatment may have as target the disorganization of the biofilm by removing some of the slime presents around cells. The second treatment, which can be performed immediately after the first one, might actually target the microorganisms. Even thought a single treatment with different parameters such as longer exposure time, or even changing other conditions as photosensitizer concentration for instance, may present the same results, variables as photosensitizer bleaching, consume of oxygen and even the presence of the physical barrier represented by the slime may represent different challenges.

The number of cells is another variable tested. Demidova and Hamblin[8] showed lower photo-activity of TBO, rose Bengal and chlorine upon increasing bacterial concentration. The ratio between reactive oxygen species and targets was considered one important factor in such case. Although the ratio is clearly an important issue, other phenomena can take place at the same time worsening the photodynamic effect. The work of Maisch et al[9] showed that oxygen consumption by bacteria and also the molecular oxygen availability on the solution are directly linked with singlet oxygen production and also with porphyryn antimicrobial activity against gram positive and also gram negative microorganisms. According to the author the molecular oxygen availability is extremely important for the photodynamic effect; therefore *in vitro* as well as *in vivo* tests with low oxygen concentrations will render lower antimicrobial effect, and aerobic microorganisms will consume the oxygen and more microorganism represent an increased consumption of the available O_2. In fact our results did not demonstrate an expressive alteration on cell morphology when an increased number of cells were targeted. The mean width of the cells was not changed after PAT as demonstrated by our results.

On the other hand, increasing the irradiation time has been demonstrated as a very efficient manner of increasing the PAT effect, mostly with photosensitizers that do not rendered bleached after longer illumination periods. Our results showed a marked difference on cell shape after a 10min irradiation period compared to 3 min. Although it might be a good solution for increasing PAT efficacy, the compliance of the therapy can be compromised by to long periods of illumination.

AFM has been recognized as an attractive and powerful method to study biological samples bcause of the high resolution images obtained combined with the fact that samples can be preserved in their native state[10], [11]. Our results indicated that it is possible to monitor cell alterations due to different variables using this technique.

The leakage of cellular material observed in our study with particles measuring around 300nm are consistent with the results presented by Sahu et al[12], whose work also found particles measuring around 260nm leaking from cells after PAT with orto-toluidine blue. The AFM allows a rapid acquisition of both qualitative and quantitative data and therefore it may be a useful tool to provide new information about alterations on cell morphology after PAT and also to discover the best treatment parameters that might improve the therapeutic results. Another important factor that may be studied by this technique is the different mechanisms of action of several photosensitizers, mostly understanding the differences between positive and negatively charged photosensitizers that may or may not connect with cellular membrane.

5- CONCLUSION

Our results using AFM to monitor alterations on *E* coli cellular morphology after PAT with methylene blue and red laser showed marked differences in cell topography as formation of grooves, leakage of cytoplasmic material and cell flattening. AFM seems to be a useful tool to monitor events occurring in cells after PAT.

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