Exploring glutathione as an adjuvant of anti-biofilm strategies against *Pseudomonas aeruginosa*

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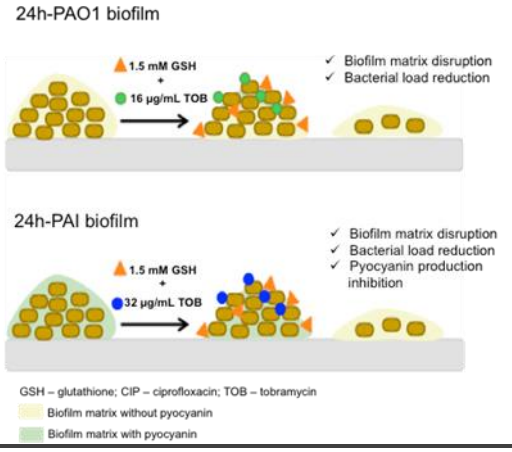

Pseudomonas aeruginosa biofilms are involved in several infectious diseases and their eradication remains a challenge. Disrupting the biofilm matrix is the most attractive way to facilitate biofilm-cells eradication and glutathione has been exhibiting great potential as a biofilm disrupter agent. Therefore, this study aimed to explore anti-biofilm strategies based on the combination of glutathione with two in-use antibiotics. Biofilms were formed and subjected to the action of combinatorial arrangements of glutathione with ciprofloxacin or tobramycin. Preliminary results showed that PAO1 biofilms were eradicated using glutathione+ciprofloxacin, in contrast to biofilms formed by a clinical isolate (PAI). Better outcomes were obtained using glutathione+tobramycin since biomass reduction occurred with lower dosages. Also, a different action than the usually described for glutathione is suggested. In conclusion, glutathione-tobramycin could be a potential anti-biofilm strategy.

P. aeruginosa is a gram-negative, ubiquitous environmental bacterium frequently found in diverse environments such as water, soil and plants in the form of biofilms. Biofilms are defined as highly structured, surface-attached communities of cells encased within a self-produced extracellular polymeric matrix forming a shell around a microbial community conferring to the microorganisms a protective environment [1]. *P. aeruginosa* biofilms have been linked to several human infectious diseases, such as nosocomial infections and cystic fibrosis, and to medical equipment [2–4]. The eradication of these biofilms poses several challenges because the antimicrobial resistance of biofilms is multifactorial resulting from the combination of different mechanisms, including restricted penetration of antimicrobials through the exopolysaccharide matrix, slow growth of bacteria within biofilms and cell-to-cell communication systems [5]. Attempting to eradicate *P. aeruginosa* biofilms, several treatments have been used but, unfortunately, *P. aeruginosa* infections still persist. Therefore, new strategies to eradicate *P. aeruginosa* biofilms are required. Biofilm matrix disruption has been considered an attractive approach because it can expose biofilm-cells to the action of antimicrobial agents. Typically, *P. aeruginosa* biofilm matrix contains a lot of compounds including significant amounts of pyocyanin, a blue redox-active phenazine that confers structural integrity to the biofilm, and extracellular DNA (eDNA) [6,7]. Glutathione is an human antioxidant that reacts with pyocyanin and eDNA intercalation being thus proposed as a potential matrix disruptor agent [7–9]. We hypothesized that combined with antibiotics, glutathione could weaken biofilms affecting the biofilm integrity and so augmenting the efficacy of the antimicrobial agents using reduced concentrations. Therefore, this study aimed to determine the anti-biofilm potential of glutathione in the disruption of biofilm matrix and to develop anti-biofilm strategies based on a possible synergistic effect resulting from combining glutathione with two different antibiotics, ciprofloxacin and tobramycin.

In this study, two *P. aeruginosa* strains were used, a laboratory strain PAO1, antibiotic sensitive and non-pyocyanin producer, and a clinical isolate PAI, antibiotic resistant and pyocyanin producer in order to reflect some of the *P. aeruginosa* biofilms diversity found in the hospital and industrial settings.

Biofilms were formed on a 96-well microtitre plate for 24, 48 and 72 h to obtain different biofilm ages (immature and mature biofilms). Briefly, 200 µL *per well* of $1x10^7$ CFU/mL overnight bacterial suspensions prepared in TSB were transferred into a 96-well microtitre plate and incubated aerobically at 37 °C under agitation (120 rpm). After biofilm formation, the content of the wells was discarded and biofilms washed to remove weakly attached cells and further treated with the combinatorial strategies. Checkerboard arrangements of glutathione, ranging from 1 to 8 mM, with ciprofloxacin, ranging from 1 to 32 µg/mL, or tobramycin ranging from 1 to 64 µg/mL were performed to determine the most effective anti-biofilm strategy. All these dual-arrangements were in contact with biofilms for 24 h. Biofilm biomass (determined by crystal violet assay [10]) and viable cells counting were determined before and after the application of the antimicrobial strategies to evaluate their anti-biofilm efficacy.

The preliminary results showed that the efficacy of the anti-biofilm approach of glutathione+ciprofloxacin reduced as the biofilm became more mature, since 24 h-old-biofilms were more susceptible than 48 h- and 72 h-old-biofilms. To augment anti-biofilm efficacy, increased concentrations of ciprofloxacin were needed and, even so, eradication did not occur for the two *P. aeruginosa* biofilms. The antibiotic susceptibility of *P. aeruginosa* strains seemed to have a great impact on the efficacy of this anti-biofilm strategy since all PAO1 biofilms suffered biomass reduction or were eradicated using glutathione+ciprofloxacin, in contrast to PAI biofilms in which no effect was observed. This result may be explained by the ciprofloxacin tolerance of PAI.

Interestingly, it was verified that glutathione+ciprofloxacin combination seemed to have action over PAO1 biofilms where there is no pyocyanin within the matrix. This finding led us to speculate that glutathione may not only act over pyocyanin and eDNA intercalation.

The combination glutathione+tobramycin exhibited improved preliminary results; although the same loss of efficacy was observed as biofilms became more mature. PAO1 biofilms were eradicated using low concentrations of glutathione and tobramycin, 1.5 mM and 16 µg/mL, respectively. The increased susceptibility of PAO1 biofilms to this last combination, reinforced our assumption that glutathione may play another role in biofilm matrix disruption. Young 24 h-old PAI biofilms were load reduced using low dosages of glutathione and tobramycin,1.5 mM and 32 µg/mL respectively. However, mature PAI biofilms with 48 h and 72 h of growth were again more tolerant to glutathione and tobramycin combination and increased concentrations of glutathione and tobramycin, 2 mM

and 64 µg/mL, respectively were needed to obtain a significant load reduction of biofilm cells and biomass.
Moreover, it was also observed that glutathione, ciprofloxacin and tobramycin did not exhibit increased or equal efficacy against *P. aeruginosa* biofilms when applied alone highlighting the beneficial result of co-applying different kind of agents to eradicate biofilms.

In conclusion, the combination of glutathione with tobramycin could be a potential anti-biofilm strategy to be applied in clinical biofilms. This finding was quite relevant to continue exploring other biotechnological solutions based on the synergistic effect between antimicrobials and glutathione to eradicate *P. aeruginosa* biofilms.

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