1 Visible Light Plasmon Excitation of Silver

2 Nanoparticles Against Antibiotic-Resistant

3 *Pseudomonas aeruginosa*

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16

17 ABSTRACT

18 The interaction of metallic nanoparticles with light excites a local surface plasmon resonance

19 (LSPR). This phenomenon enables the transfer of hot electrons to substrates that release

20 Reactive Oxygen Species (ROS). In this context, the present study was aimed at enhancing

21 the antibacterial effect of citrate-covered silver nanoparticles (AgNPs), which already possess

22 excellent antimicrobial properties, via LSPR excitation with visible LED

23 against *Pseudomonas aeruginosa*, one of the most refractory organisms to antibiotic

24 treatment. The Minimum Inhibitory Concentration (MIC) of AgNPs was 10 µg/ml under dark
25 conditions and 5 µg/ml under light conditions. The combination of light and AgNPs led to
26 100% cell death after 60 minutes. Quantification of ROS via flow cytometry showed that
27 LSPR stimulated AgNPs increased intracellular ROS concentration by 4.8-fold, suggesting
28 that light-exposed AgNPs caused cell death via ROS production. Light exposition caused a
29 small release of silver ions (0.4%) reaching a maximum after 6 hours. This indicates that
30 silver ions play at most a secondary role in *P. aeruginosa* death. Overall, the results presented
31 here show that LSPR generation from AgNPs by visible light enhances the antimicrobial
32 activity of silver nanoparticles and can be an alternative for the treatment of topic infections
33 caused by antibiotic-resistant bacteria such as *P. aeruginosa*.

34

35 KEYWORDS: Silver nanoparticles, Surface Plasmon resonance, *Pseudomonas aeruginosa*,
36 Antibiotic resistance

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38 **Running title:** Light-enhanced Ag nanoparticles against *P. aeruginosa*

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40

41 Introduction

42 Metallic nanoparticle possess remarkable features, such as their manageable scale and the
43 ability of fine tuning their composition, structure and shape (1). The desired electronic,
44 optical and chemical properties of nanoparticles can be obtained through modern synthetic
45 techniques devised in the past decades (2). Even small variations in one of these parameters
46 may result in new properties that are absent in the analogous bulk material. Due to its high
47 range of controllable features, metallic nanoparticles are now used in many processes, such as

48 catalysis (3), SERS (4), electroanalytical sensors (5), plasmonics (6), photonics (7) and
49 others.

50 Silver nanoparticles (AgNPs)(2), exhibit a great potential of use in many areas, due to their
51 relative easy and controlled synthesis (8), ranging from small and simple spheroidal AgNPs
52 (9) to more complex geometries (10, 11) and even asymmetric shapes (12–14) with solid or
53 hollow interiors (15–17). Historically, silver has been extensively used as an antimicrobial
54 agent. Many different commercial products relying on silver biocidal properties have been
55 produced, most of them in the last 10 years (18, 19). The wide variety of AgNPs as well as
56 the intrinsic properties of metallic silver in relation to biological system and biomolecules
57 (20) fostered the extensive use of AgNPs in medical applications, such as cancer treatment
58 (21–23), drug delivery (24, 25), imaging (26, 27) and antimicrobial treatment (28, 29).

59 *P. aeruginosa* is a ubiquitous Gram-negative γ -proteobacterium and an opportunistic
60 pathogen associated with nosocomial infections. It also affects patients afflicted by chronic
61 respiratory diseases such as cystic fibrosis (30, 31). The relatively large genome and genetic
62 complexity of *P. aeruginosa* (32, 33) supports its versatility and ability to colonize the soil,
63 water bodies, as well as plants and animal tissues (33). *P. aeruginosa* infections are
64 frequently refractory to quinolone, aminoglycosides and β -lactam antibiotics (34). In fact,
65 owe to its outer membrane low permeability, expression of efflux pumps, and biofilm
66 formation, *P. aeruginosa* is one of the most difficult organisms to treat with antibiotics. Skin
67 infections by *P. aeruginosa* on burn victims are particularly problematic, in part due to non-
68 efficient treatments (35–37). The World Health Organization (WHO) included carbapenem
69 resistant *P. aeruginosa* in a list of critical priority bacteria to which the research and
70 development of new antibiotics and alternative treatments is of paramount importance (38).

71 Although anti-bacterial treatments with visible light, such as blue light therapy (BLT) have
72 been emerging as a non-invasive treatment for bacterial infections, the interaction of visible

light with AgNPs in the range of LSPR and its possible effect on microbes has not yet been studied (39). Inspired by these findings, in the present work we use the concept of LSPR in order to enhance the antimicrobial properties of AgNPs against *P. aeruginosa* by illuminating the system with a white LED lamp. This strategy revealed a quicker and more effective treatment against *P. aeruginosa*, being a promising improvement in the fight against this pathogen.

79

80 **Results and Discussion**

81 **AgNPs synthesis**

Citrate covered AgNPs have been widely used in biological applications. Citrate, a natural anti-oxidant is not harmful to cells and is used as a carbon source by some bacteria. We synthesized citrate-covered AgNPs with a diameter of 20 ± 3 nm (Figure 1a). The extinction spectrum displayed a full width at half maximum (FWHM) of 131 nm and a maximum at 436 nm, a wavelength that matches the maximum emission from the LED floodlight used here (Figure 1b). The extinction band is broadened due to the standard deviation in particle size distribution, which represents ca. 15% of the average diameter. This is a typical spectrum for AgNPs synthesized by the citrate method (40, 41). In order to optimally observe the effects of the LSPR phenomenon, the excitation of AgNPs with an external radiation source should be performed in the region of maximum absorption. Therefore, a floodlight with an emission spectrum that superposes the AgNPs extinction spectrum was utilized (Figure 1b). To avoid the mutagenic and carcinogenic effects of UV light, a light source that did not emit in the UV region was chosen.

95

96 **Bactericidal effect of AgNPs on *P. aeruginosa***

97 Preliminary assays showed that the synthesized AgNPs displayed antimicrobial activity
 98 against *P. aeruginosa*. The minimal inhibitory concentration (MIC) of AgNPs was 10 µg/mL,
 99 which is comparable to values reported by similar studies for the same bacterium(42–44).
 100 However, when the AgNPs were exposed to light the MIC dropped to 5 µg/mL. The minimal
 101 bactericidal concentrations (MBC) were 20 µg/mL and 10 µg/mL under dark and light
 102 conditions, respectively. In addition, a Kirby-Bauer assay using filters containing different
 103 concentrations of AgNPs was performed in the presence and absence of light (Fig. 2). In the
 104 absence of light (Fig. 2A), an inhibition halo became apparent with AgNPs concentrations
 105 higher than 15 µg/mL and larger halos were observed with AgNPs concentrations up to 30
 106 µg/mL. In contrast, when the AgNPs were exposed to light, 10 µg/mL sufficed to produce a
 107 halo similar to that observed when the bacteria were treated with 30 µg/mL AgNPs in the
 108 dark, suggesting that light enhances the bactericidal effect of silver nanoparticles (Figure 2B).
 109
 110 It is worth noticing that upon light exposition, the greenish color of *P. aeruginosa* colonies,
 111 caused by the synthesis of the pigments pyoverdine and pyocyanin visually decrease. These
 112 molecules are well-known virulence factors of *P. aeruginosa* are targets for the screening of
 113 antimicrobial compounds (45–47). Accordingly, some studies have shown that light reduces
 114 the concentration of pyocyanin in *P. aeruginosa* and that this effect was dependent on light
 115 intensity and wavelength (48). It has also been shown that high doses of blue light treatment
 116 inhibited the activity of pyocyanin, staphylolysin, pseudolysin and other proteases (37, 39).
 117 Furthermore, it has been demonstrated that pyocyanin confers on *P. aeruginosa* resistance to
 118 ionic silver (49). It is thus possible that pyocyanin inhibition by LED floodlight contributes to
 119 the bactericidal effect of AgNPs on *P. aeruginosa* and diminishes the virulence of the
 120 surviving bacteria.

121 To further explore the effect of light exposition on *P. aeruginosa*, bacteria were grown for 60
 122 minutes in MH medium without AgNPs in the presence or absence of light (Fig. 6). The
 123 calculated growth rates were 1.26 h^{-1} and 1.04 h^{-1} under dark and light conditions,
 124 respectively, a 17.5% reduction, suggesting that light mildly affects bacterial growth.
 125 Next, we measured the effect of AgNPs on cell viability at different time intervals. Bacteria
 126 treated for 15, 30 and 60 minutes with 1X MIC AgNPs showed a survival rate of 12.8%,
 127 4.4% and 1.6% in the dark and 4.5%, 0.7% and 0.2% under light conditions, respectively
 128 (Fig. 3a), while bacteria treated with 2X MIC showed, respectively, a survival rate of 20.6%,
 129 6.4% and 2.2% (dark conditons) and 3.6%, 0.4% and 0.0% (light conditions) at 15, 30 and 60
 130 minutes (Fig. 3b). An 1 h treatment with 10 or 20 $\mu\text{g/mL}$ AgNPs under light exposition
 131 resulted in 99.8% and 100% cell death, respectively. These results corroborate the
 132 bactericidal effect of AgNPs on *P. aeruginosa* and the enhancing effect of light exposure
 133 upon AgNPs treatment.

134

135 Effect of light and AgNPs on ROS generation

136 The antimicrobial mechanism of action of AgNPs is still not fully understood mainly due to
 137 the difficulty in evaluating the contributions of nanosized metallic silver and silver ions that
 138 are released by the former (50, 51). While some studies support the idea that silver ions play
 139 the main role in cellular damage (52), others claim that the strong antibacterial activity of
 140 AgNPs is associated with the introduction of nicks in the cytoplasmic membrane which
 141 ultimately lead to cell death (53). In addition, AgNPs (and silver ions) induce the formation
 142 of reactive oxygen species (ROS) (54), which damage the cell cytoskeleton, and oxidize
 143 proteins and nucleic acids, potentially leading to chromosomal aberrations and cell demise.
 144 Also, both ionic and nanosized silver can interact with sulphur-containing macromolecules,

145 such as proteins, resulting in their inactivation (55). Lately, a Trojan-horse like mechanism
146 has been proposed, in which AgNPs first enter the cell and only then release silver ions (56).
147 Previous studies showed that AgNPs promote the induction of reactive oxygen species (ROS)
148 (57, 58) and enhance the expression of superoxide dismutase, catalase and peroxidase (58).
149 These authors concluded that the main mechanism of antimicrobial action of AgNPs involves
150 the disequilibrium of oxidation and antioxidation processes and the inability to eliminate
151 ROS (58). High concentration of ROS in the bacterium lead to oxidative stress (59, 60). To
152 test whether AgNPs causes oxidative stress, the intracellular level of ROS in *P. aeruginosa*
153 was assessed. Bacteria were treated with 1X MIC AgNPs under light or dark conditions
154 followed by exposure to 2',7'-Dichlorofluorescein diacetate (DCFH-DA). This compound
155 diffuses into the cell where it is hydrolyzed by intracellular esterases (DCFH), resulting in the
156 production of 2',7'-dichlorofluorescein (DCF), which fluoresces upon oxidation. The
157 intracellular ROS level thus correlates with the level of fluorescence emitted by DCF in the
158 presence of ROS such as H_2O_2 , ROO^- and $ONOO^-$ (61). Fig. 4 shows that after 1 hour in the
159 presence of AgNPs, the level of ROS increased by 2.5-fold (dark) and 4.8-fold (light) when
160 compared to the respective controls.

161 In a conducting medium, surface electrons of nanoparticles may oscillate in a resonant
162 condition with a coupled electromagnetic field, resulting in a phenomenon called Local
163 Surface Plasmon Resonance (LSPR), which confer on nanoparticles physical unique
164 properties. Among them, a local temperature increase, enhancement of optical near fields
165 surrounding the particle and the generation of hot carriers can be highlighted (62). The last
166 one received a special attention in the field of catalysis due to the possibility of electronic or
167 vibrational activation of substrates or reaction intermediates. Interestingly, recent studies
168 have demonstrated the formation of ROS at the surface of metallic nanoparticles (63–65). For

169 example, LSPR-based antibacterial treatment with gold nanoparticles has been reported (66–
170 68).

171 It has been suggested that the formation of ROS by AgNPs occurs either at the particle
172 surface or it is indirectly caused by Ag^+ ions released from aging nanoparticles (69, 70). In
173 addition, the light stimulus may contribute to the amount of ROS generated through a
174 mechanism of plasmon-produced hot electrons and their interaction with water and oxygen
175 dissolved in the medium (63–65). To assess whether exposure to light affects the production
176 of Ag^+ from AgNPs by an aging process, a closed dialysis membrane filled with an AgNPs
177 suspension was submerged in distilled water and the concentration of released Ag^+ to the
178 solution was followed during 24 h (Fig. 5). At 0 h the concentration of silver ions was below
179 the limit of detection of ICP-OES. The highest concentration of Ag^+ under dark conditions
180 was four times lower than that observed under light exposition. Although the concentration of
181 Ag^+ increased with light treatment, the highest observed concentration (at 6 h, under light
182 exposition) represented only 0.4% (w/w) of the total amount of silver in the sample.
183 Moreover, the release of Ag^+ occurred several hours after AgNPs have already killed the
184 bacteria (see Fig. 3) . At the time in which the bactericidal effect and ROS were evaluated (1
185 h), it is noticeable that the measured concentrations of Ag^+ under light and dark conditions
186 are statistically indistinguishable. Therefore, the release of Ag^+ is unlikely to be involved in
187 the mechanism through which AgNPs cause bacterial death.

188

189 Conclusion

190 AgNPs were shown to be effective against *P. aeruginosa*. The bactericidal effect of AgNPs
191 were intensified when the system was irradiated by light, indicating that the combination of
192 light and AgNPs ensures a quick and more effective treatment against this pathogen. A
193 treatment period of one hour was sufficient to cause complete cell death. These effects were

194 associated with an increase in intracellular ROS possibly generated by the hot carrier transfer
195 of LSPR on the AgNPs surface. AgNPs aging and the consequent release of silver ions
196 occurred only after long periods of exposition to light. Our data illustrate a viable treatment
197 that combines two pre-existing strategies: the use of both AgNPs and light treatment, that
198 synergistically enhance the treatment of *P. aeruginosa* topical infections.

199

200 **Materials and Methods**

201 **Materials**

202 Silver nitrate (AgNO₃, Sigma-Aldrich, ≥99%), sodium citrate (Sigma-Aldrich, ≥99%),
203 lysogeny broth medium (KASVI, Curitiba, Brazil), phosphate buffer saline (PBS, pH 7.4),
204 bacteriological agar (KASVI, Curitiba, Brazil), Mueller-Hinton broth (BD Difco) and 2',7'-
205 dichlorofluorescein diacetate (DCFH-DA, ≥97%, Sigma-Aldrich) were of analytical grade and
206 used as received. All solutions were prepared using deionized water (18.2 MΩ) unless stated
207 otherwise. Sterile filter paper discs of 6 mm, 96-well flat bottom plates (Corning) and 24-well
208 flat bottom plates (Corning) were used as received.

209

210 **Synthesis of Silver Nanoparticles (AgNPs)**

211 Preparation of AgNPs followed a slightly modified method of Lee and Meisel (71). AgNO₃
212 (90 mg) was dissolved in 500 mL of distilled water. The solution was heated until boiling
213 under magnetic stirring. Ten mL of a 34 mM sodium citrate solution were then added and the
214 mixing which proceeded for another 1 hour. The suspension was transferred to an amber
215 flask and stored at room temperature until further use.

216

217 **Sample preparation for electron microscopy (SEM and TEM)**

218 One mL of the as synthesized AgNPs suspension was washed three times by successive
219 rounds of centrifugation at 17000 rpm. The pellet containing the AgNPs was resuspended in
220 20 μ L or 300 μ L of water. For the SEM analysis, 1 μ L of the concentrated suspension was
221 deposited onto a 1 cm^2 silicon wafer and let dry at room temperature. For the TEM analysis,
222 10 μ L of the suspension was transferred to a standard formvar coated copper TEM grid and
223 dried at room temperature. Particles size were analysed with the help of the ImageJ 2.0
224 software.

225

226 **Media and bacterial growth conditions**

227 *Pseudomonas aeruginosa* (strain PA14) was typically cultivated in LB medium at 37°C for
228 16 h. The culture was then diluted 1/100 in 5 mL of MH, and grown until the exponential
229 growth phase ($\text{OD}_{600} = 0.1$). Mueller-Hinton agar plates (MHA-DIFCO) were used in the
230 bacterial viability assays in solid medium. Bacterial growth rate was determined by growing
231 cells under dark or light conditions in MH medium and measuring the number of colony
232 forming units (CFU/mL) at different time points.

233

234 **Determination of the minimum inhibitory concentration (MIC)**

235 MIC determination was performed by the broth microdilution technique (Clinical and
236 Laboratory Standards Institute – CLSI, 2015). Briefly, 10^5 bacteria/ml were inoculated in 96-
237 well plates containing Mueller-Hinton medium and incubated in the presence of increasing
238 concentrations of AgNPs (from 0 to 80 $\mu\text{g/mL}$). One plate was covered with aluminium foil
239 (dark conditions) and the other was placed on the top of it (light conditions). The plate on the
240 top was positioned 10 cm away from a LED floodlight (50 W). Both plates were incubated
241 overnight at 30°C. On the next day the turbidity of the cultures was measured at 600 nm in an
242 EpochTM Microplate Spectrophotometer (BioTek).

243

244 **Antimicrobial activity of AgNPs (disc diffusion method)**

245 The antimicrobial activity of AgNPs in solid medium was evaluated through the Kirby-Bauer
246 disc diffusion method according to the guidelines provided (Clinical and Laboratory
247 Standards Institute – CLSI, 2015). Mueller-Hinton agar plates were evenly swabbed with 100
248 μL of a PA14 culture ($\text{OD}_{600} = 0.1$). Sterile filter paper discs (6 mm diameter, Oxoid) were
249 uniformly placed on the plate surface and 10 μL of different concentrations of AgNPs were
250 dropped onto each disc. The negative control was sodium citrate at the same concentration of
251 the AgNPs suspension. Plates were incubated overnight at 30 °C and the halos formed by
252 antibiotic activity against the bacteria were measured with the help of the ImageJ 2.0
253 software.

254

255 **Kinetics of AgNPs antibacterial effect**

256 Two 24-well plates were filled with 1.5 mL of a mixture of MH medium and AgNPs (0, 1X
257 and 2X MIC). A final concentration of 10^5 cells/mL at the exponential growth phase was
258 added to each well. One of the plates was covered with aluminium foil (dark conditions) and
259 the other one was placed on its top (light conditions). Both plates were statically incubated at
260 30°C, while the plate on the top was 10 cm away from a LED floodlight (50 W). Aliquots of
261 100 μL were withdrawn at 0, 15, 30 and 60 minutes from both plates and subsequently
262 diluted in 0.9% NaCl, plated on LB-Agar and incubated for 24 h at 37 °C. The number of
263 colony forming units (CFU/mL) was determined. This experiment was performed with
264 twelve biological replicates.

265

266 **Intracellular ROS assay**

267 Three independent overnight cultures of *P. aeruginosa* were diluted 1:100 in fresh MH
268 medium, incubated under agitation until reaching the concentration of 10^8 cells/mL. Different
269 concentrations of AgNPs and 20 μ M DCFH-DA were then added. The cultures were
270 incubated at 30 °C for 1h in the presence and absence of light exposition. Then, 1 mL of each
271 culture was withdrawn and centrifuged at 8000 rpm for 5 minutes. The pellets were washed
272 with 1 mL of PBS and suspended again in 100 μ L of PBS. The fluorescence emitted by the
273 intracellular oxidation of the dye was determined using the BD Accuri™ C6 flow cytometer
274 at the excitation wavelength of 488 nm and at an emission wavelength of 535 nm.

275

276 **Quantification of released Ag⁺**

277 Six dialysis membranes each filled with 20 mL AgNPs were placed in 800 mL beakers filled
278 with 500 mL distilled water under magnetic stirring. Three of them were placed 10 cm away
279 from the floodlight, while the other three were covered in aluminium foil. After 1, 2, 4, 6 and
280 24 h, 5 mL aliquots of water from each beaker were withdrawn and stored for further
281 analysis. Silver ions were detected by iCAP™ 7000 Plus Series ICP-OES (Thermo
282 Scientific™).

283

284 **Statistical**

285 Statistical significance was calculated using the analysis of variance (ANOVA) followed by
286 Tukey pairwise comparison. Values of $p \leq 0.05$ were considered statistically significant.

287

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292

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498 Legends to Figures

499 **Figure 1.** Characterization of AgNPs. (a) Representative TEM and HRTEM (inset)
500 micrographs of citrate-coated AgNPs with an average diameter of 20 ± 3 nm. (b) Extinction
501 spectrum of AgNPs showing the SPR peak at 436 nm superposed with the LED floodlight
502 emission spectrum. The floodlight emission has a maximum intensity at 448 nm, with no
503 measurable emission from 190 to 406 nm.

504 **Figure 2.** Effect of AgNPs on *P. aeruginosa* survival. (a) Disc diffusion assay: Paper discs
505 containing each 10 μ L of 0, 10, 15, 20, 25 or 30 μ g/mL AgNPs were placed on Mueller-
506 Hinton plates spread with bacteria and incubated overnight under dark conditions. (b) Effect
507 of light on the bactericidal effect of AgNPs. Discs containing 10 μ g/mL AgNPs were placed
508 over *P. aeruginosa* lawns in the absence (left side) or presence (right side) of light.

509 **Figure 3.** Combined effect of light and AgNPs on bacterial viability. Bacteria were grown in
510 MH medium containing 1X MIC AgNPs (a), or 2X MIC AgNPs (b), in the absence (black) or
511 presence (white) of light. Aliquots of 100 μ L were withdrawn at 0, 15, 30 and 60 minutes to

512 determine bacterial survival. The initial number of cells in all experiments was 1.64×10^5
 513 CFU/mL. Each bar represents the mean $\pm$ SD of 12 independent cultures. * $p < 0.05$

514 **Figure 4.** Light increases intracellular ROS generation in *P. aeruginosa* treated with AgNPs.
 515 Bacteria were exposed for 1 hour to 10 $\mu\text{g/mL}$ AgNPs in the absence or presence of light.
 516 Fluorescence intensities of DCFH-DA were measured by flow cytometry. Each point
 517 represents the mean $\pm$ SD of 3 independent cultures. * denotes statistical significance with $p <$
 518 0.05.

519 **Figure 5.** Concentration of silver ions released by AgNPs through a dialysis membrane over
 520 time in the presence (blue color) or absence of light (white color), respectively. A known
 521 amount of AgNPs was added to a tightly closed dialysis membrane and the system was
 522 submerged in distilled water under magnetic stirring. (a) Analysis of Ag^+ content by ICP-
 523 OES. (b) Schematic representation of the dialysis method used to quantify silver ions.

524 **Figure 6.** Effect of light on bacteria growing in Mueller-Hinton medium. Bacterial growth
 525 rate was determined by growing cells under dark or light conditions in MH medium and
 526 measuring the number of colony forming units (CFU/mL) at different time points.

a)