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# Photodynamic Antibacterial Chemotherapy (PACT) Against *Staphylococcus aureus* and *Escherichia coli* Using Gold Nanoparticles and LED's Irradiation

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Gold nanoparticles synthesized through sodium citrate (A-AuNPs) and sodium borohydrade (B-AuNPs) reduction of $Au^{3+}$ and a photochemically initiated method (C-AuNPs) were tested for possible application in Photodynamic Antibacterial Chemotherapy. None of them presented inhibitory activity against bacterial suspensions in dark conditions using the cylinders inhibition method; however, a total inhibition of *Staphylococcus aureus* ATCC 29213 and an extended-spectrum beta-lactamases-producing Escherichia coli ATCC 25922 growth were observed when incubated with 2 mM B-AuNPs and irradiated with LEDs at 525 nm for 12 h. Characterization of the interaction between *S.aureus* and B-AuNPs was demonstrated through an optimized procedure for TEM. These results probe that B-AuNPs have a good potential as photosensitizers for PACT.

**KEYWORDS:** *Gold Nanoparticles, Staphylococcus aureus, Escherichia coli, Photodynamic Antibacterial Chemotherapy (PACT).*

## INTRODUCTION

Resistant bacteria to antibiotics are becoming even more troubling. The emergence of *Escherichia coli* resistant to antibiotics is a problem that affects various countries. It has been reported that bacteria have developed $\beta$-lactamase that gives resistance against $\beta$-lactam antibiotics.[1] Organisms expressing extended-spectrum $\beta$-lactamases (ESBL) are widely distributed worldwide, although prevalence rates are significantly higher in certain geographical regions.[2]

*Staphylococcus aureus*, both in the healthcare setting and the community prompts great urgency in the development of and advocacy for prevention and treatment efforts. The precipitous spread of *S. aureus* methicillin-resistant strains (MRSA) has created new challenges for governments, healthcare systems, and drug development.[3]

Some antibiotics have been indiscriminatingly and unnecessary used, resulting in the emergence of bacteria able to resist many commonly used antibiotics. The research for new alternative antibacterial agents is not just necessary, but rather urgent. In the last decade, different approaches for the use of nanoparticles in PACT have been investigated. Stefano Perni[4,5] Copper, zinc, magnesium but especially silver and gold NPs display antibacterial activity and are used for various healthcare, hygiene and personal care purposes and also in water-treatment.[6]

Nanoparticles have already shown to have some potential activity against Gram negative and Gram positive bacteria;[7,8] Excitation of nanoparticles plasmon could, on the other hand, enhance its antimicrobial effect through a photothermal or photochemical mechanism.[9,10] The use of a light source and a chemical agent for treating microbial infections it is known as Photodynamic Antimicrobial Chemotherapy (PACT). The irradiation wavelength must be chosen such that it coincides with the maximum absorption of the photosensitizers. After the absorption of light, the excited photosensitizer can either transfer its energy to a substrate which then reacts with oxygen (Foote Type I reaction) or directly to oxygen in the surroundings (Foote Type II reaction), producing Reactive Oxygen Species (ROS).[11]

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Moreover, plasmon excitation represents a promising approach to efficiently destroy bacteria since after treatment these microorganisms do not recover their viability and after ten generations of partially photosensitized cells none of the bacteria develop resistance to the photodynamic process.[12]

This article describes the analysis of the potential of gold nanoparticles (AuNPs) as photosensitizers for PACT, and *ergo* as alternative antimicrobial agents.

## MATERIAL AND METHODS

### Synthesis of AuNPs

Three different AuNPs synthesis were performed in order to compare the results. A-AuNPs were synthesized according to Turkevich method by reducing the $HAuCl_4$ from Sigma Aldrich with sodium citrate as reducing agent,[13] B-AuNPs were made by reducing the $HAuCl_4$ with $NaBH_4$ as Low et al. described, with slight modifications.[14]

On the other hand, C-AuNPs were obtained through a photochemically initiated reaction, irradiating a solution of $Au^{3+}$ and Irgacure 259 with UV lamps for 30 min adapting the procedure proposed by McGilvray et al.[15]

### UV-Vis Characterization of AuNPs and Plastic Wellplates

Spectra of colloidal AuNPs (0,1 mM) were recorder employing an Agilent 8243 spectrophotometer with DAD detector, using a quartz cell with an optical path length of 1 cm.

### LEDs Setup

The electronic arrangement was specially built up to irradiate the well plate containing the samples. For this purpose, 24 LEDs (10 mm diameter) were placed in such a way that each LED was directly underneath each well. This LED's plate was controlled with an ad hoc power source set at 18 V, keeping constant 60 mA and yielding 10000 $mW/cm^2$ in the liquid culture surface.

### Bacterial Strains

The experiments were performed testing *S. aureus* ATCC 29213, *E. coli* ATCC 25922, and an extended-spectrum beta-lactamase-producing *E. coli* (ESBL) clinical strain. The screening and confirmatory methods to detect the presence of ESBLs was performed according to the Clinical and Laboratory Standard Institute (CLSI: M100-S20, 2010)[16] by the Bacteriology Service of the Sanatorio Aconcagua from Córdoba, Argentina.

Stock cultures were maintained in trypticase soy broth and stored frozen in 10% glycerol.

### Bacterial Suspensions Preparation

*S. aureus* ATCC 29213 and *E. coli* ATCC 25922 were chosen to conduct the experiments by the cylinders method. Suspensions of $10^6$ colony forming units (CFU) per mL in phosphate buffer saline (PBS) at pH 7 were prepared from overnight cultures and incubated at 37 °C.

Kinetic assays with *S. aureus* ATCC 29213 and an ESBL-producing *E. coli* clinical strain were done. Suspensions of $10^4$ CFU/mL in phosphate buffer saline (PBS) pH 7 from overnight cultures incubated at 37 °C were prepared.

### Assays for Antibacterial Activity of Au-NPs by the Cylinders Method

Antibacterial activity of the three different AuNPs against the reference strains, *S. aureus* and *E. coli* was tested applying the cylinders method. Bacterial suspensions (20 $\mu$L, $10^6$ CFU/mL) were placed and spread on the surface of Mueller Hinton agar (MH). Each cylinder was filled with 100 $\mu$L of

(a) 2 mM of A-AuNPs,
(b) 2 mM of B-AuNPs and
(c) 2 mM of C-AuNPs
(d) 0.2 mM of A-AuNPs,
(e) 0.2 mM of B-AuNPs and
(f) 0.2 mM of C-AuNPs.

PBS and 50 $\mu$g/ml ciprofloxacin were used as negative and positive control, respectively. Then, the plates were incubated at 37 °C for 24 h.

### Kinetic Study of Photodynamic Antibacterial Activity of B-AuNPs

In each well of a 24 wellplate, 250 $\mu$L of bacterial suspension ($\sim 10^4$ CFU/mL) of *S. aureus* ATCC 29213 or ESBL-producing *E. coli* and 250 $\mu$L of the tested solutions, (a) PBS or (b) 2 mM B-AuNPs were mixed softly in a shaker for 1 h, covered from any light. After this period, the wellplate was irradiated for 6, 9 and 12 h with 525 nm LEDs. The experiments were also run in parallel in dark conditions. Then, the content of the wells were diluted to the half with PBS and 20 $\mu$l were plated in MH agar. All plates were incubated at 37 °C for 24 h.

### Transmission Electron Microscopy (TEM)

Suspensions of *S. aureus* (10 ml, $10^6$ CFU/mL) were incubated with B-AuNPs (10 mL, 2 mM) for 1 h at room temperature in the dark, centrifuged for 15 min at 1000 rpm. Treated samples of *S. aureus*, were withdrawn and fixed at 1 h in 2% glutaraldehyde and 4% formaldehyde solutions in 0.1 M cacodylate buffer for 2 h at room temperature, and then post-fixed with osmium tetroxide at 1% in the same buffer, dehydrated and embedded in Araldite. Thin sections were cut with a diamond knife on a JEOL JUM-7 ultramicrotome, mounted on nickel grids and examined in a Zeiss LEO 906E electron microscope.

## RESULTS

### UV-Vis Characterization of AuNPs

All three types of AuNPs synthesized showed a strong absorption band in the green region of the visible spectra due to surface plasmon resonance (Fig. 1). The strong red colloidal color is caused when the frequency of the light wave became resonant with the electron motion. Surface plasmon resonance is produced by the motion of free electrons in the conduction band caused by interaction with an electromagnetic field. The absorption of the plasmon it is directly related to the size and shapes of the nanoparticles in colloidal solution.[17] It was observed that particles were spherical and quite stable as their sizes and shape remained constant after synthesis.

### LEDs Irradiation

Monochromatic LED irradiation at 525 nm was chosen in order to excite the AuNPs selectively at their wavelength of maximum absorption (Fig. 2). Therefore, we avoid bacterial damage produced directly by light energy.

### Assays for Antibacterial Activity of Au-NPs

The three types of AuNPs tested by the cylinders inhibition method did not show inhibitory activity against the reference strains of *S. aureus* or ESBL-producing *E. coli* at concentrations of 2 mM in dark conditions.

B-AuNPs were selected to perform the irradiation assays since it was easier to concentrate and was more stable compared to A-AuNPs or C-AuNPs.

A complete inhibition of bacterial growth was observed in the irradiated samples treated with B-AuNPs, there was a CFU reduction of more than $3 \log_{10}$ compared to the control without AuNPs, showing a bactericidal effect after 12 hours of irradiation.

Since we were interested in evaluate the effect of the B-AuNPs in a clinical strain resistant to antibiotics, we have chosen an ESBL-producing strain for the kinetic assays.

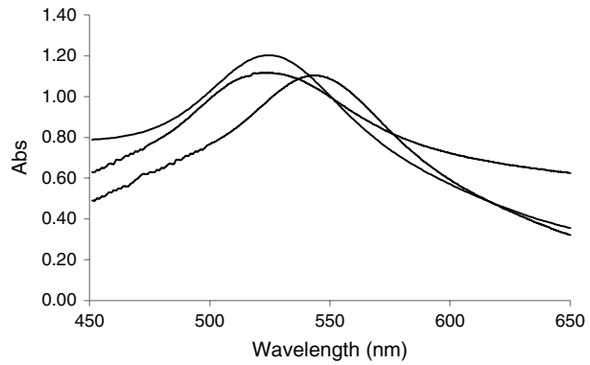


**Figure 1. Absorption spectra of different AuNPs synthesized (0, 1 mM).**

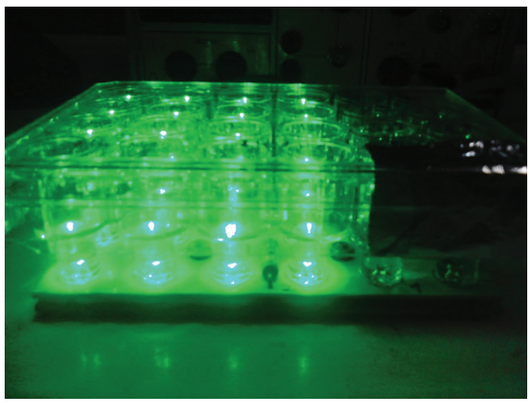

**Figure 2. Experimental set up using 525 nm LEDs panel built *ad hoc*.**

Antibacterial kinetics was analyzed by plotting CFU versus incubation time, which provides evidence for the effectiveness of the AuNPs. As the irradiation time increases, CFU of the bacterial population (*S. aureus* and ESBL-producing *E. coli*) decreased. There was a growth reduction in both strains after 9 h of irradiation, and there was complete inhibition when treated with B-AuNPs and irradiated for 12 h; the CFU reduced more than $3 \log_{10}$ compared to the untreated control, showing a bactericidal effect in a Gram positive (Fig. 3(A)) and a Gram negative strain (Fig. 3(B)).

### Transmission Electron Microscopic Analysis

B-AuNPs treated *S. aureus* was thinly sectioned for TEM imaging in order to study the mechanism of the antibacterial interactions. The concentration of 2 mM was selected because it displayed strong antibacterial effect. TEM demonstrated that nanoparticles were mainly concentrated on bacterial cell walls. Most of the AuNPs were surrounding each bacterium after 1 h of incubation, indicating some kind of specific interaction with the bacterial wall and showing strong affinity for the peptidoglycan layer (Fig. 4-TEM). There are no aggregated Au-NPs complexes inside of cells.

### Statistical Analysis

The student's *t*-test was used to carry out the statistical analysis. A $P$-value $< 0.05$ was considered significant.

## DISCUSSION

The application of AuNPs as a tool to deliver other antimicrobials or as a factor enhancing photodynamic destruction of bacteria was described. They are also very suitable as stabilizers for various antimicrobial photosensitizers.[18]

On the other hand, it has been reported that biophysical interactions occur between NPs and bacteria including biosorption, NPs breakdown or aggregation, and cellular uptake, with effects including membrane damage and

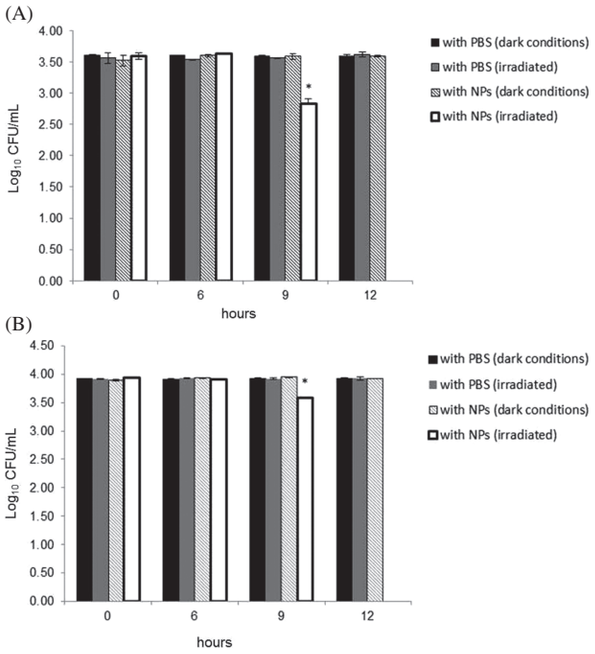


**Figure 3. The antibacterial effects of AuNPs on *S. aureus* ATCC 29213 (A) and ESBL-producing *E. coli* (B). Bacterial cell cultures were set up at $10^4$ CFU/mL from an overnight culture. Suspensions were exposed to 2 mM B-AuNPs and incubated for 6, 9 and 12 hours in dark conditions or under 525 nm LED's irradiation. Experiment was repeated three times and the results are shown in mean ± SD. Significant differences were observed on treated samples and irradiated with LEDs' 525 nm and the control without AuNPs. *$p < 0.05$.**

toxicity. However, the mechanisms of NPs inhibiting bacterial growth remain less well understood.[7]

Interestingly AuNPs are particularly and extensively exploited in organisms because of their biocompatibility. Due to the optimal transmission to tissue in the near infrared (NIR) region, upon NIR irradiation the Au-based nanomaterials with characteristic NIR absorption have been used to destroy cancer cells and bacteria via photothermal processes.[19]

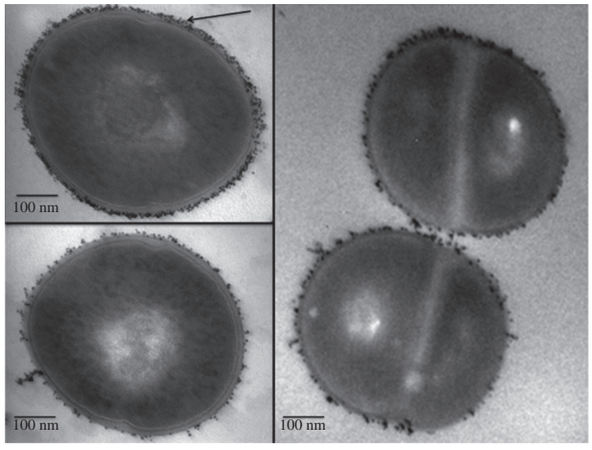


**Figure 4. TEM images of *S. aureus* ATCC 29213 shows the interaction between B-AuNPs and the bacterial wall after 1 h of incubation at 37 °C. Scale bar: 100 nm.**

To the best of our knowledge, in this study we reported the first information regarding to the antibacterial activity of AuNPs made by reducing the $HAuCl_4$ with $NaBH_4$ against *S. aureus* and an ESBL-producing *E. coli* strain.

Differences were observed in the bacterial inhibition mechanism in presence of B-AuNPs with or without irradiation. Those results clearly show that the effect observed could be attributed to the effect of the plasmon. It could be inferred from this result that the mechanism involved is via photothermal heating. When a AuNPs is excited, it became a "hot" particle. A "hot" nanoparticle could melt the lipids in a cell membrane, therefore the cell will be killed, but only that cell. The heat decreases so rapidly that at just a typical cell length away from the particle the heat will have decreased so much that it is harmless. As TEM shows, B-AuNPs are surrounding each bacteria, so they are completely capable of produce this photothermal damage. But this effect it is not evidence on macroscopic scale, so no increase in the liquid sample media could be measure.[20]

Other authors have reported that the level of aggregation of citrate AuNPs was higher in Luria Bertain medium as compared to in deionized water, since the medium is full of nutrients as well as free ions. Both components could exchange with citrate on the nanoparticles surface and cause aggregation.[17] Our AuNPs also aggregated in rich broth, so we preferred to conduct the experiments in PBS.

Chwalibog et al.[21] have demonstrated the interaction between hydrocolloids of silver, gold, and platinum nanoparticles with *S. aureus*. They have found that the interaction with AuNPs is not the same as with AgNPs. They described that their Au-NPs did not surround *S. aureus* but were trapped within the biofilm produced by the cell. However in our results, we noted that AuNPs surrounded all the cell wall. Bacteria cell wall tends to attract positive charged AuNPs due to the total charge of cell wall being negative.[22]

In conclusion, we have demonstrated that AuNPs display excellent antibacterial potential for the Gram negative bacteria *E. coli* and the Gram positive bacteria *S. aureus* with activity against multiresistant strains. More experiments will be needed to further investigate the mechanism of action of these AuNPs.

## Competing Interests

The authors declare no competing interests.

## Authors' Contributions

MJS designed the experiments, prepared NPs and characterized them, performed the bacterial culture, counted

CFU, performed TEM observation and drafted manuscript. MCB conceived research, participated in experimental design, and in manuscript writing. GAA participated in revising the manuscript. All authors read and approved the final manuscript.

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