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# Plasmonic enhancement in the photoinactivation of *Escherichia Coli* using Rose Bengal and Gold Nanoparticles

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## ABSTRACT

In the present study we report on the ability of gold nanoparticles (AuNP) to enhance the antimicrobial activity of the photosensitizer Rose Bengal (RB), a very effective singlet oxygen generator. Our experiments were conducted using a suspension of *Escherichia Coli* in the presence of either RB or a combination of RB and AuNP. Nanoparticles were synthesized by laser ablation in water, which allows high purity, biologically friendly AuNP production, as compared to traditional chemical methods. Several relative concentrations of bacteria, photosensitizers and AuNP were studied. Bacterial survival rates were determined before and after LED light illumination. The phototoxicity of RB with and without AuNP was checked following illumination for 10 and 20 minutes. As a control, the dark toxicity of RB was verified. The results show that the survival rate of bacteria decreases significantly with the increase of RB concentration and illumination time, which is in accordance with previous works. Interestingly, our results also indicate a significant increase in the lethal photosensitization of RB in the presence of AuNP. We propose this effect is due to plasmonic light enhancement, considering the superposition of RB and AuNP absorption spectra, which favors electric field enhancement effects in the presence of AuNP. Similar experiments using the photosensitizer Methylene Blue (MB) allowed us to test our hypothesis for MB did not show any difference in its phototoxicity in the presence of AuNP. We propose this observed synergistic effect could be an effective way for improving photodynamic inactivation of microorganisms.

**Keywords:** photodynamic inactivation, photodynamic therapy, antimicrobial activity, *Escherichia Coli*, rose bengal, gold nanoparticles, plasmonic enhancement, laser ablation.

## 1. INTRODUCTION

Bio-nanotechnology offers promising opportunities to the development of novel nanodevices, which can be used in several physical, biological and biomedical applications<sup>1,2</sup>. Inorganic nanosized particles open up the possibility of creating a new generation of bactericidal materials, which is getting more and more important due to increasing resistance development of bacteria to bactericides and antibiotics in recent years<sup>3</sup>. In this given context, the development of an alternative treatment involving a light-activated agent, such as a photosensitizer dye, in combination with nanosized particles has been used to achieve lethal photoinactivation of microbial cells<sup>4,5</sup>. In special metal nanoparticles became very useful as agents for photoinactivation. Metal nanoparticles, on account of the phenomenon of surface plasmon resonance, possess strongly enhanced visible and near-infrared light absorption, several orders of magnitude more intense compared to conventional photosensitizer dyes<sup>6,7</sup>. In the present work we related the use of inorganic gold nanoparticles (AuNP) in conjunction with the photosensitizer Rose Bengal (RB) for the *in vitro* photoinactivation of gram-negative bacteria *Escherichia Coli*. Nanoparticles with an average size of 8 nm were produced by laser ablation, a physical technique that allows for high purity AuNP as compared to chemical methods.

## 2. MATERIALS AND METHODS

### Preparation of AuNP

The synthesis of nanoparticles is usually done by chemical reduction methods that have to be specifically designed for each type of nanoparticle material. These chemical routes unavoidably lead to unreacted precursors and additives that tend to remain in the final colloidal product, therefore, requiring huge efforts for an efficient purification. Laser ablation in liquid (LASiS)<sup>8</sup> has emerged in the last decade as a valuable alternative approach for nanoparticle generation for it allows the production of nanoparticle colloids directly from nearly all classes of materials in pure solvents. In the present work, AuNP with an average size of 8nm were produced by LASiS directly in water. This technique is also known by other acronyms such as PLA<sup>9</sup> (pulsed laser ablation) and PLAL<sup>10</sup> (pulsed laser ablation in liquid). LASiS is a *top-down* physical technique that allows high purity AuNP production as compared to *bottom-up* chemical methods and it is considered a green alternative to nanoparticle production aiming at important applications in biology, medicine and biophotonics<sup>8-10</sup>. The production of AuNP in water through the laser ablation technique was performed in our labs using a Q-switched Nd:YAG laser delivering 1064nm, 200ns pulses at repetition rates of 1.5kHz. The maximum pulse energy output was of 3 mJ. The laser beam was focused on a high-purity (99,999%) gold target immersed in 3 ml to 10 ml of water in such a way that the water column was 1 mm thick above the target. A 5 cm focal length lens was used leading to a spot size of 40  $\mu\text{m}^2$ , and energy densities around 100 J/cm<sup>2</sup>. The target was kept in movement in a direction perpendicular to beam irradiation for periods of 3 to 10 minutes. In this process, once laser irradiation is present, a sound can be heard which relates to laser-light interaction, and the formation of a brilliant plasma plume is visible at all times. After only a few seconds, the characteristic reddish color of colloidal AuNP indicates the formation of nanoparticles. Typical production rates were measured to be 5 mg/h, and AuNP colloidal concentration was set to 0.1mg/ml. A schematic view of LASiS is presented in Figure 1. Following laser ablation, the AuNP colloids were characterized through diverse techniques, such as Dynamic Light Scattering (DLS), suitable for determining particle size and size distribution; UV-VIS spectroscopy, which allows for the determination of nanoparticle optical extinction and plasmon bands; and Transmission Electron Microscopy (TEM). In addition, zeta potential measurements were performed in order to access nanoparticle surface charge, and colloidal stability.

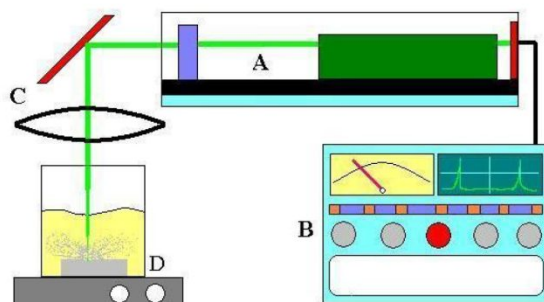


Figure 1. Schematic setup of nanoparticle generation by LASiS showing: laser (A) and laser control (B); mirror and focusing lens (C) for directing laser light onto target; and target immersed in liquid (D).

### Photoinactivation test and bacteria counting

In order to examine the phototoxicity effect of AuNP on gram-negative bacteria, colony-forming units (CFU) of *E. Coli* were cultured on Brain Heart Infusion (BHI) agar plates supplemented with the photosensitizer RB and nanosized AuNP with different relative concentrations. Control experiments were also carried out in the absence of AuNP. In addition, all experiments were performed with and without LED light illumination so photo- and dark- toxicity could be evaluated. The photoinactivation was performed using LED light illumination with LED emission centered at 505 nm (green), and power set to 3 mW, for two distinct exposition times: 10 minutes and 20 minutes. Similar experiments were carried out using the photosensitizer methylene blue (MB) excited by LED light illumination with its peak centered at 626 nm (red). LED wavelengths were chosen in order to match each photosensitizer absorption spectra (RB, absorbs light in the green region of the electromagnetic spectrum, and MB absorbs in the red region), and guarantee resonant light absorption

aiming at maximizing photosensitizers photo activity, i.e. reactive oxygen species (ROS) generation<sup>11</sup>. After the irradiation, the number of surviving bacteria was determined by viable counting. For the counting experiments, the six by six (6x6) method<sup>12</sup> was employed, and all experiments were carried out in triplicate. The experiments were conducted in a multiple-well plate also known as ELISA-plate (Figure 1), which is commonly used in immunoassays, totaling 96 wells in each plate. Since ELISA-plates are relatively compact, LEDs were soldered onto a circuit board in such a way that uniform illumination was achieved across the plate. Several “LED boxes” were built, some with green LEDs and others with red LEDs. These LED boxes would exactly attach to the ELISA-plate where bacterial cultures, AuNP and photosensitizers were placed. The boxes had a lid to guarantee no light other than that of the LEDs would influence and excite the photosensitizers. Therefore, several triplicates could be performed in each plate according to our experimental plans (see Figure 2).

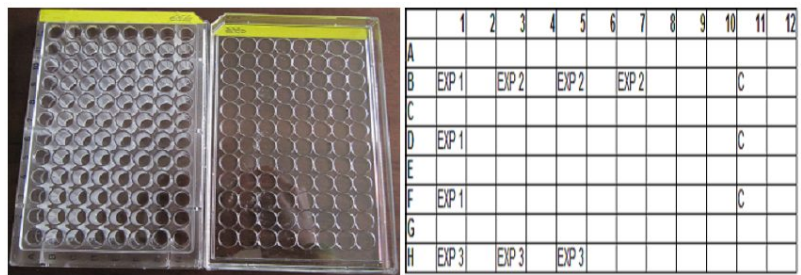


Figure 2. Left: ELISA-plate used in the experiments. Right: Experimental scheme, with three different experiments (EXP1, EXP2, and EXP3) performed in triplicate. C stands for the control experiments. Each pair of letters and numbers accounts for a specific well.

### 3. RESULTS AND DISCUSSION

In Figure 3 we present a TEM micrograph of the colloidal AuNP synthesized via LASiS. It can be seen that nanoparticles are mainly spherical in shape, and there is low size dispersion – an important feature for many applications in biophotonics. In fact, we here show the feasibility of synthesizing AuNP with small diameter and low size dispersion, which is still a major challenge in the field of laser ablation<sup>10</sup>. We believe that the combination of laser frequency and pulse duration together with the amount of liquid covering the sample used in our experiments plays a fundamental role for the synthesis of these particular AuNP. Figure 4 shows results related to DLS analysis of AuNP, indicating an average diameter of approximately 8 nm. We point out that this histogram is in good agreement with the TEM image presented in Figure 3.

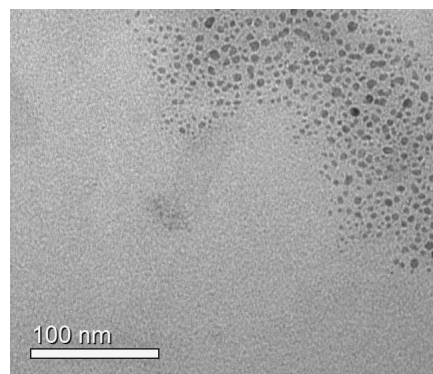


Figure 3. TEM image representative of the colloidal AuNP used in this work.

The extinction spectrum of AuNP is shown in Figure 5, and the peak at 520 nm corresponds to the surface plasmon resonance band which is a signature characteristic of spherical gold nanoparticles.

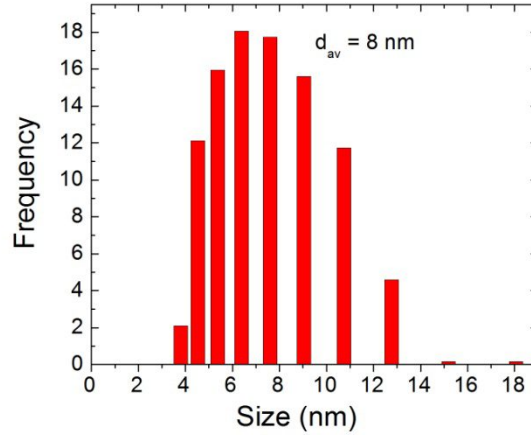


Figure 4. DLS histogram corresponding to the colloidal AuNP synthesized in water by LASiS.

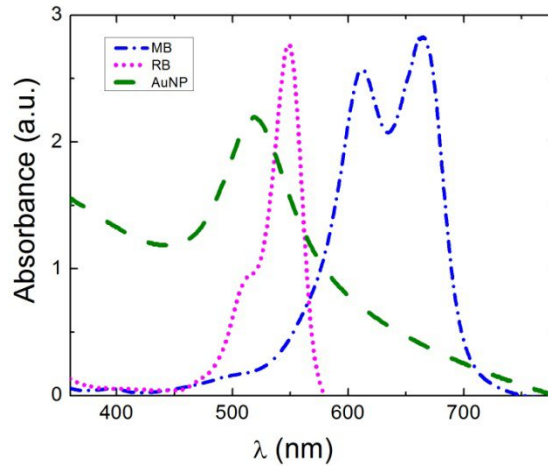


Figure 5. Extinction spectrum of AuNP, and absorption spectra of the photosensitizers Rose Bengal (RB) and Methylene Blue (MB) used in this work.

In Figure 5, we also present the absorption spectra of both photosensitizers, RB and MB. In this figure, we can see the superposition between RB spectrum and AuNP plasmon band. This plasmon resonance may lead to an enormous enhancement of optical cross section; therefore, metal nanoparticles act as “small antennas” for capturing radiation<sup>13</sup>. This plasmonic light enhancement effect is more intense in the presence of small nanoparticles and; in addition, should be more pronounced when AuNP were in the presence of RB. Thus, we predict a synergic effect when AuNP and RB are combined in photodynamic inactivation processes and, in fact, our experimental results confirm this hypothesis. Figure 6 shows the survival rate of *E. Coli* incubated with RB in the presence and in the absence of AuNP, using two different irradiation times. The phototoxicity of RB with and without AuNP was checked following illumination for 10 and 20 minutes. As a control, the dark toxicity of RB with and without AuNP was also verified under different concentrations.

The results show that the survival rate of bacteria decreases significantly with the increase of RB concentration and illumination time, which is in accordance with previous works. Interestingly, our results also indicate a significant increase in the lethal photosensitization of RB in the presence of AuNP for irradiation times of 10 minutes. Note that increasing the irradiation time did not cause any significant effect in the survival rate of *E. Coli* for both concentrations of RB, probably because, after 10 minutes, survival rate reached its minimum value under the circumstances studied. Similar experiments using the photosensitizer Methylene Blue allowed us to further test our hypothesis. Because MB absorbs light in the red region of the electromagnetic spectrum, we found no significant difference in its phototoxicity in the presence of AuNP with light illumination. Since there is no significant correspondence between MB absorption spectrum and AuNP plasmon band (Figure 5) no plasmonic enhancement effects should be expected. Further experiments are under way in order to better understand the relationship between plasmonic enhancement and reactive oxygen species (ROS) generated in the process.

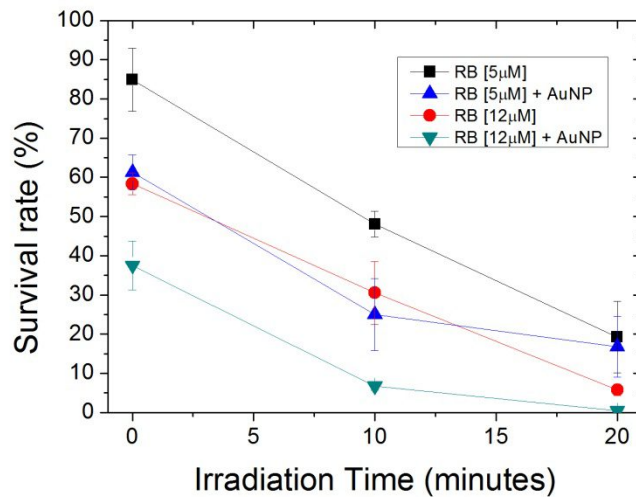


Figure 6. Photoinactivation of *E. Coli* by RB (5µM and 12µM) in the absence and presence of AuNP (10<sup>12</sup> NP/ml), after green LED light illumination for 10 and 20 minutes. The dark toxicity of RB in the absence and presence of AuNP is represented as 0 minutes. Note the increase in RB phototoxicity at 10 minutes due to the presence of AuNP.

#### 4. CONCLUSIONS

In this work we focus on the *in vitro* synergic effect of RB and AuNP to inactivate the gram-negative bacteria *Escherichia Coli*. The AuNP colloids were synthesized through laser ablation (LASiS) in pure water. It is interesting to mention that LASiS constitutes a low-cost alternative method which is specially promising for nanoparticle production aiming at biophotonic applications. Our experimental results indicate a significant increase in the lethal photosensitization of RB in the presence of AuNP. We propose this effect is due to plasmonic light enhancement, since there is a superposition of RB and AuNP absorption spectra, which favor electric field enhancement effects in the presence of small AuNP, the observed synergic effect between RB and AuNP could be exploited as an effective way of improving photodynamic inactivation of microorganisms.

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## REFERENCES

- [1] Prasad, P. N., [Nanophotonics], Wiley-Interscience, Hoboken, N.J., (2004).
- [2] Andrews, D., Scholes, G. and Wiederrecht, G., [Comprehensive Nanoscience and Technology], Academic Press, London, (2011).
- [3] Vicente, M., Hodgson, J., Massidda, O., Tonjum T., Henriques-Normark B., Ron, E. Z., “The fallacies of hope: will we discover new antibiotics to combat pathogenic bacteria in time?,” FEMS Microbiol Rev 30, 841-852 (2006).
- [4] Sondi, I, Salopek-Sondi, B., “Silver nanoparticles as antimicrobial agent: a case study on E. coli as a model for Gram-negative bacteria,” J Colloid Interface Sci 275, 177-182 (2004).
- [5] Narband, N., Tubby, S., Parkin I. P., Gil-Tomás, J., Ready, D., Nair, S. P., Wilson, M., “Gold Nanoparticles Enhance the Toluidine Blue-Induced Lethal Photosensitisation of Staphylococcus aureus,” Curr Nanoscience 4, 409-414 (2008).
- [6] Huang, X., Jain, P. K., El-Sayed, I. H., El-Sayed, M. A., “Plasmonic photothermal therapy (PPTT) using gold nanoparticles,” Lasers Med Sci 23, 217-228 (2008).
- [7] Lohse, S. E., Murphy, C. J. “Applications of Colloidal Inorganic Nanoparticles: From Medicine to Energy,” J. Am. Chem. Soc. 134, 15607–15620 (2012).
- [8] Amendola, V., Meneghetti, M., "Laser ablation synthesis in solution and size manipulation of noble metal nanoparticles," Phys. Chem. Chem. Phys. 11, 3805-3821 (2009).
- [9] Zeng, H., Du, X., Singh, S. C., Kulinich, S. A., Yang, S., He, J., Cai, W. “Nanomaterials via Laser Ablation/Irradiation in Liquid: A Review,” Adv. Funct. Mater. 22, 1333–1353 (2012).
- [10] Barcikowski, S., Compagnini, G., “Advanced nanoparticle generation and excitation by lasers in liquids,” Phys.Chem. Chem. Phys. 15, 3022-3026 (2013).
- [11] Vatansever, F., de Melo, W. C. M. A., Avci, P., Vecchio, D., Sadasivam, M., Gupta, A., Chandran, R., Karimi, M., Parizotto, N. A., Yin, R., Tegos, G. P., Hamblin, M. R. “Antimicrobial strategies centered around reactive oxygen species – bactericidal antibiotics, photodynamic therapy, and beyond,” FEMS Microbiol Rev 37, 955-989 (2013).
- [12] Chen, C. Y., Nace, G. W., Irwin, P. L., “A 6x6 drop plate method for simultaneous colony counting and mpn enumeration of *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli*,” Microbiological Methods 55, 475-479 (2003).
- [13] Lindsay, S. M., [Introduction to Nanoscience], Oxford University Press, Oxford & New York, 329-331(2010).