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Research Article

Antibacterial and Antibiofilm Activities of the Photothermal Therapy Using Gold Nanorods against Seven Different Bacterial Strains

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The objective of this work was to determine the bactericidal and antibiofilm activities of gold nanorods (AuNRs) using plasmonic photothermal therapy (PPTT) against oral microorganisms. AuNRs were synthesized by the seed and growth solution method and the gold nanoclusters were characterized with a size of 33.2 nm ± 2.23 length and 7.33 nm ± 1.60 width. The efficacy of PPTT related to its temperature was done reaching 67°C. Minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) of AuNRs and AuNRs PPTT were determined against *Enterococcus faecalis, Staphylococcus aureus, Streptococcus mutans, Streptococcus sobrinus, Streptococcus oralis, Streptococcus salivarius*, and *Escherichia coli* growth. The antibiofilm activity of AuNRs was explored by fluorescence microscopy. After experimental analyses, AuNRs PPTT shows better results in MICs and MBCs, when it was compared with AuNRs alone. The laser employed to activate the AuNRs had no antibacterial effect against oral microbes. The MICs and MBCs values were higher for *S. aureus* and *E. coli* and lower against *S. oralis*. Surprisingly, the AuNRs alone presented a high antibiofilm activity, inhibiting the biofilm formation of *S. mutans*. Altogether, these results strongly suggest that AuNRs could be an interesting option to control oral biofilms.

1. Introduction

Photodynamic therapy (PDT) is an effective treatment to kill both eukaryote and prokaryote cells [1, 2]. It uses a drug called photosensitizer and a particular type of light. When photosensitizer is exposed to a specific wavelength of light, it produces a form of oxygen that kills nearby cells. However, it is useless on infected areas without oxygen such as dentinal tubules and roots. The plasmonic photothermal therapy (PPTT) could be more efficient to eliminate microorganisms through its local hyperthermia mechanism [3]. PPTT has been important in the last years in the medicine field due to its capacity of absorbing visible light. At the same time, gold nanoparticles with nonspherical shape, such as gold nanorods, and polygonal particles have the capacity of absorbing near-infrared (NIR) light that is within an appropriate wavelength window for therapeutic applications. One of the potential applications of gold nanoparticles with NIR absorption capabilities is in the hyperthermia. Hyperthermia can be used as a possible mechanism to kill bacteria selectively by combining the use of laser and functional gold nanoparticles. When nanorods are in contact with the bacterial cells and this is followed by laser irradiation, the overheating effects can rapidly destroy the bacterial cells

[4, 5]. The position of the plasmon maximum is related to the particle size (larger diameter results in a longer wavelength) and the peak width to size dispersion [6]. Knowing this, we can increase the antimicrobial potential of nanoparticles to lyse entire bacterial communities.

The increasing development of multidrug-resistant strains among pathogen microbes has become one of most important problems in medicine worldwide [7]. Great advances in nanotechnology have provided a solid foundation for using nanoparticles (NPs) in the fight against pathogen microorganisms, including multidrug resistant bacteria [8–11]. Several studies have investigated the efficacy of PDT for disinfection of oral pathogens; however the use of PPTT against oral pathogens has not been enough explored. In this work we reported the bactericidal activity of gold nanorods against oral microorganisms. In this work the minimum inhibitory concentration (MIC) and minimun bactericide concentration (MBC) of the AuNRs combined with PPTT on seven different strains of oral bacteria were determined. Additionally the antibiofilm properties of AuNRs were analyzed by fluorescence microscopy.

2. Materials and Methods

2.1. Gold Nanorods Synthesis. For the preparation of AuNRs, seed and growth solutions were made using the method reported by Nikoobakht and El-Sayed (2003) as briefly described below [12]. Seed solution: 5 mL of 5×10^{-4} M $HAuCl_4$ solution was added to 5 mL of 0.20 M CTAB solution. To this solution, under magnetic stirring, 0.60 mL of ice-cold $NaBH_4$ was added. The stirring continued during 2 additional minutes. Growth solution: 0.35 mL of 0.0040 M $AgNO_3$ solution and 5 mL of 1×10^{-3} M of $HAuCl_4$ solution were mixed with 5 mL of 0.20 M CTAB solution. After gently mixing, 70 μL of 0.0788 M ascorbic acid solution was added. To form AuNRs the final step was the addition of 12 μL of the seed solution to the growth solution at 27–30°C. The color of the solution gradually changed within 10–20 min. After the synthesis, centrifugation at 13000 rpm at 25°C for 30 minutes was used to eliminate unreacted CTAB and other subproducts of the reaction [13].

2.2. Characterization of AuNRs. Vis-NIR absorption spectra were obtained using a CHEMUSB4-VIS-NIR (Ocean optics) spectrophotometer. Transmission electron microscopy (TEM) images were obtained before and after irradiation of AuNRs with the NIR laser to observe any structural changes using a JEOL JEM1230 microscope operating at an acceleration voltage of 100 kV. TEM images of the prepared NRs were used for the size distribution measurements; the size of 200 particles was measured to obtain the average size.

2.3. Light Source. A NIR laser (Quantum IR 810, Laser Systems) with a wavelength of 810 nm and a 200 mW broadcast power was used for all experiments.

2.4. Characterization of Photothermal Efficiency of AuNRs. Temperature test was done applying the 810 nm NIR laser for 20 minutes to 50 mL of deionized water and centrifuged AuNRs. Evaluation of the temperature was done every 30 seconds with a digital thermometer (FLUKE SI II) and the subsequent observation of the nanorods using the TEM [14].

2.5. Antibacterial Activity of AuNRs by Microdilution Assay. Strains of *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 29213), *Streptococcus mutans* (ATCC 25175), *Streptococcus sobrinus* (ATCC 27351), *Streptococcus oralis* (ATCC 10557), *Streptococcus salivarius* (ATCC 7073), and *Escherichia coli* (ATCC 25922) were used at 0.5 of the McFarland Scale as determined with a Spectronic 21D spectrophotometer (Milton Roy). To determine MIC and MBC we employed the broth microdilution method according to the NCCLS 2003 standard in three groups: group 1: AuNRs, group 2: AuNRs with PPTT, and group 3: NIR laser. Concentrations of AuNRs ranged from 196 to 0.37 $\mu g \cdot mL^{-1}$; 100 μL of bacterial suspension was added to each well (approximately 2×10^4 CFU·mL^{-1} in each well) [15]. The MIC was read after 24 h of incubation at 37°C. In some cases (groups 2 and 3), laser was applied in each well during 20 minutes. After determining the MIC, Each bacteria was extended on agar plates to determine the MBC. The agar plate was incubated at 37°C for 24 hours to determine the cutoff. All experiments were repeated three times to ensure the veracity of results.

2.6. SEM Observation of Enterococcus faecalis. After *E. faecalis* treatment with the AuNRs and AuNRs PPTT, *E. faecalis* dispersion was passed through a syringe filter (0.2 μm of pore size) and then they were fixed, dehydrated, and gold sputtered to observe them in a JEOL JSM-6510 scanning electron microscope.

2.7. Antibiofilm Activity of Gold Nanorods. With the intention to evaluate the ability of AuNRs for killing cells associated with oral biofilms, the antibiofilm activity of AuNRs was evaluated by fluorescence microscopy. The protocol for this study was as described above with a slight modification. In this study, the inoculums (*S. mutans, S. gordonii*, and *S. aureus*) maintained at 37°C were exposed to 150 μg/mL as final concentration of AuNRs at varying time intervals such as 0 and 18 hours after inoculation. As a positive inhibition control, 1.2 μg/mL of chlorhexidine was used. After incubation for 24 hours, the cells were stained with 20 μM SYTO 9 (green fluorescent nucleic acid stain for living cells (Invitrogen, Carlsbad, CA)) [16, 17] and allowed to stand for 30 minutes at room temperature in the dark. Subsequently, the cells were gently washed with sterile water and observed under fluorescence microscopy at 485 nm (Thornwood, NY). The images were analyzed by using AxioVision software (Thornwood, NY).

3. Results

3.1. Synthesis and Characterization of AuNRs. Figure 1 shows TEM images of the AuNRs before and after irradiation with the NIR laser; the as synthesized AuNRs show a uniform size distribution with a size of 33.2 ± 2.23 nm length and 7.33 ± 1.60 nm width (Figure 1(a)). In the Vis-NIR absorption spectrum, transversal and longitudinal plasmon peaks were

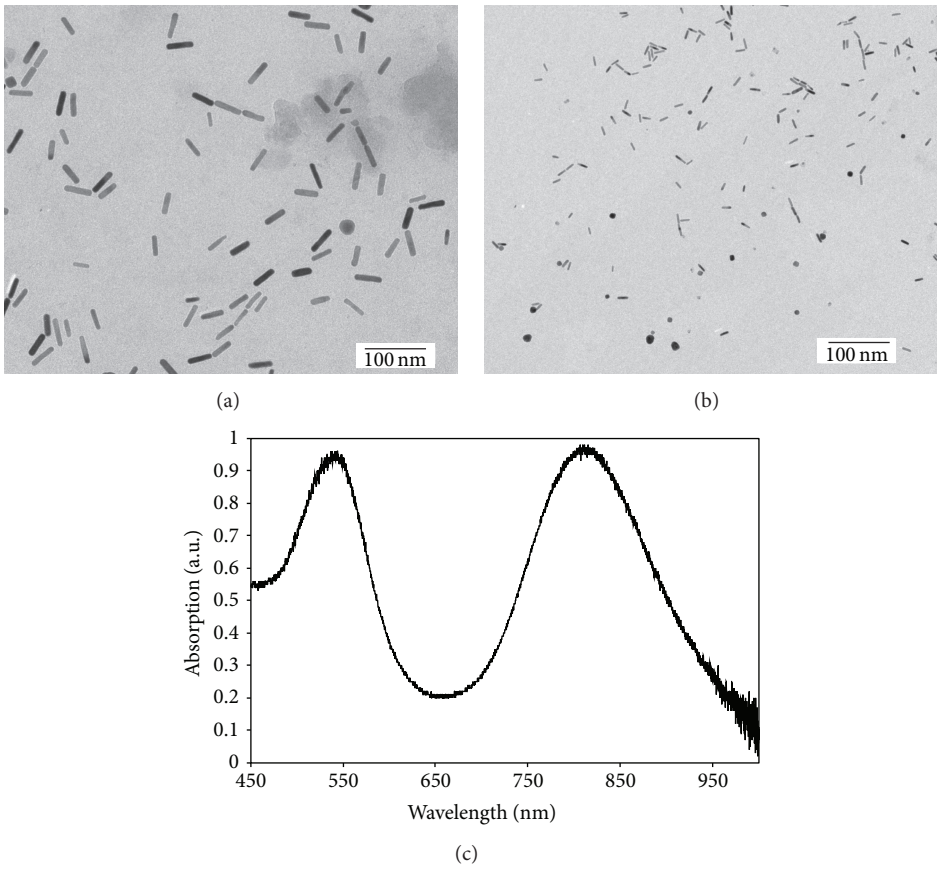

Figure 1: Characterization of gold nanorods. (a) TEM image of AuNRs: it clearly shows the rod shape of these nanostructures. (b) TEM image of AuNRs after laser exposition showing no changes in the morphology of gold nanorods. (c) Vis-NIR absorption spectrum: transversal and longitudinal plasmon peaks were observed at 540 nm and 810 nm, respectively.

observed at 540 nm and 810 nm, respectively (Figure 1(c)). No structural changes were found in AuNRs after 20 minutes of laser irradiation (Figure 1(b)).

The characterization of the photothermal efficiency of AuNRs is shown in Figure 2. A maximum temperature of 67°C was obtained within 15 minutes of laser application; after that time the temperature was constant until the experiment was completed (20 minutes).

3.2. Antibacterial Test. The results of the antibacterial test are presented as average values in Table 1 and Figure 3. MIC values ranged from 0.45 to 6.88 $\mu g \cdot mL^{-1}$; and MBC values ranged from 0.83 to 10.84 $\mu g \cdot mL^{-1}$. The MIC and MBC are lower for the AuNRs combined with PPTT if compared with AuNRs. The laser group has no antibacterial effect. For a given group, the MIC and MBC are higher for *S. aureus* and *E. coli* and lower for *S. oralis*.

In order to determine whether the gold nanorods are immediately effective for the bacterial growth inhibition an experiment with different analysis times at 0, 5, 15, 30, and 60 minutes was performed; results are shown in Figure 4, as we can see gold nanorods are able to Lower the bacteria population as low as 30% since the 5 minutes of contact.

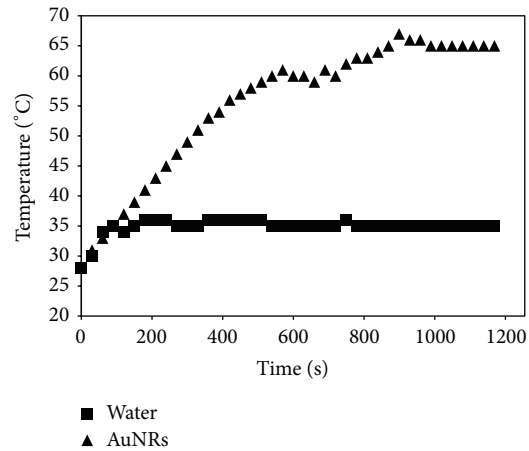

Figure 2: Characterization of the photothermal efficiency of AuNRs. A maximum temperature of 67°C was obtained within 15 minutes of laser application; after that time the temperature was constant until the experiment was completed (20 minutes).

Figures 5(b) and 5(c) show SEM images of *E. faecalis* after their treatment with AuNRs and AuNRs PPTT, respectively; compared with a control group (bacteria without any

Table 1: Minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC) of AuNRs and AuNRs PPTT.

Microorganisms	MIC	MIC range	MBC	MBC range
		AuNRs		
E. faecalis	**1.41 ± 0.69**	*1.50–0.37*	**4.7 ± 1.64**	*6.12–3.0*
S. aureus	**6.52 ± 4.78**	*12.50–1.5*	**10.84 ± 6.81**	*24.5–1.5*
S. mutans	**2.20 ± 1.19**	*3.00–0.37*	**5.08 ± 3.15**	*12.5–3*
S. sobrinus	**1.84 ± 1.75**	*6.12–0.37*	**2.86 ± 2.05**	*6.12–0.75*
S. oralis	**2.00 ± 0.75**	*3.00–1.5*	**2.5 ± 0.75**	*3.0–1.5*
S. salivarius	**4.38 ± 4.51**	*12.25–1.5*	**6.09 ± 3.77**	*12.25–3*
E. coli	**6.88 ± 7.97**	*24.5–0.75*	**7.44 ± 7.54**	*24.5–3*
		AuNRs PPTT		
E. faecalis	**0.45 ± 0.16**	*0.75–0.37*	**0.83 ± 0.25**	*1.5–0.75*
S. aureus	**3.9 ± 3.52**	*12.5–1.5*	**6.12 ± 2.74**	*12.5–3.0*
S. mutans	**0.99 ± 0.81**	*3.0–0.37*	**2.17 ± 1.68**	*6.1–0.75*
S. sobrinus	**0.58 ± 0.20**	*0.75–0.37*	**1.04 ± 0.45**	*1.5–0.75*
S. oralis	**0.62 ± 0.19**	*0.75–0.37*	**0.87 ± 0.37**	*1.5–0.37*
S. salivarius	**0.74 ± 0.46**	*1.5–0.37*	**1.16 ± 0.76**	*1.5–0.75*
E. coli	**1.25 ± 0.37**	*1.5–0.75*	**2.08 ± 0.90**	*3.0–0.75*

All values are expressed as μg/mL. The laser group shows no antibacterial effect.

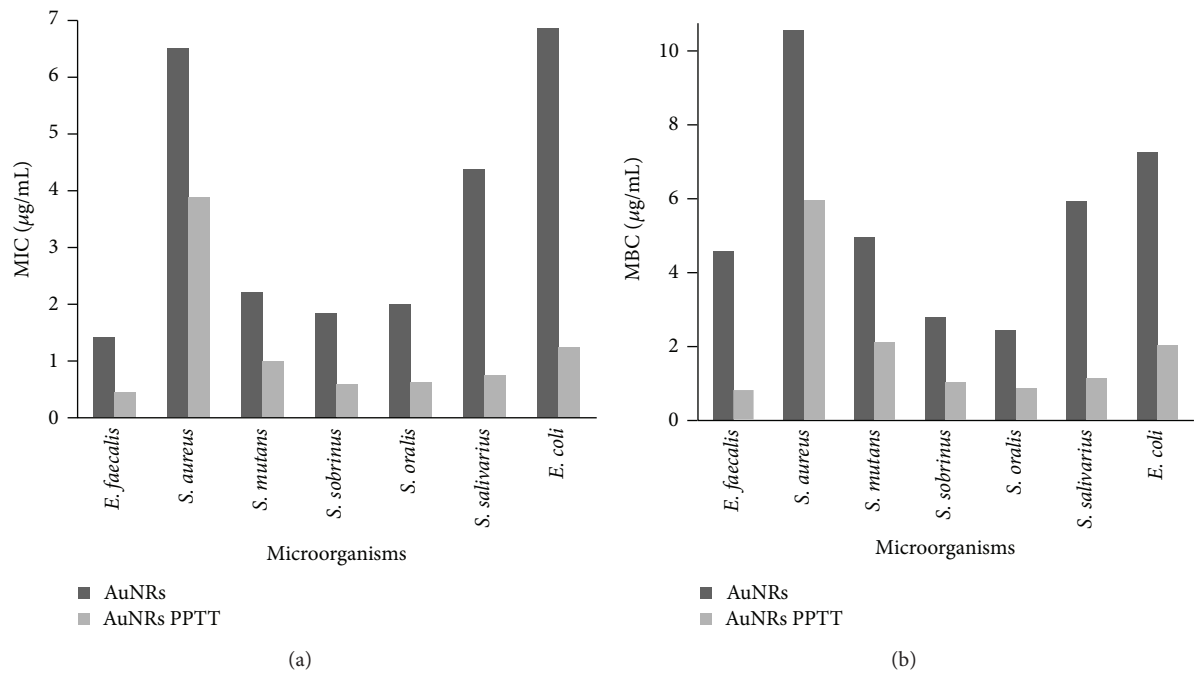

Figure 3: (a) Minimum inhibitory concentration and (b) minimum bactericide concentration of AuNRs and AuNRs PPTT.

treatment, Figure 5(a)) we can see that they have lost their morphology and volume, as a consequence of the damage in the bacteria surface.

3.3. Antibiofilm Activity of Gold Nanorods. In the previous experiments we measured the bactericidal activity of AuNRs. In order to analyze the possible biofilm inhibition of oral bacteria by gold nanorods, the antibiofilm activity of AuNRs was determined by fluorescence microscopy at different postinoculation times. The results showed a complete inhibition of biofilm formation by chlorhexidine and AuNRs, compared to control (Figure 6). The results did not change when AuNRs were added at different postinoculation times. We tested the biofilm inhibitory activity at 0, 4, 8, and 18 hours postinoculation times, obtaining similar results, a dark background indicating the albescence of living cells. These data indicate that AuNRs have an antibiofilm activity effective as chlorhexidine.

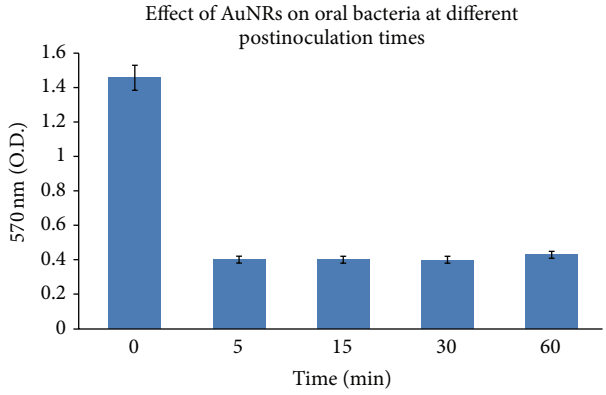

Figure 4: Bactericidal activity of AuNRs against oral bacteria growth. The Y -axis shows the optical density units of microbial growth. As growing control were used bacteria growing in culture media without inhibitor. AuNRs were used at final concentration of 150 μg/mL. The bactericidal activity of AuNRs was measured after 5, 15, 30, and 60 minutes after exposition. All experiments were done in triplicate to assess the veracity of results.

4. Discussion

Plasmon photothermal therapy has been extensively studied as an alternative technique in the biomedical area. Some studies have reported its effectiveness on the oral bacteria, but to our knowledge there are no reports presenting the minimum inhibitory concentration and minimum bactericide concentration of PPTT using gold nanorods. MacKey et al. described that plasmonic photothermal therapy using gold nanorods has a major effect related to the rod size; they determined that 28 × 8 nm AuNRs are the most effective in the plasmonic photothermal heat generation [18]. This size is similar to the one obtained in our experiments (33 × 7 nm) and this aspect ratio allowed us to overlap the 810 nm wavelength emission of the therapeutic laser with the transversal absorption plasmon of gold nanorods. When we irradiated 1 mL of gold nanorods with the 810 nm wavelength laser, this dispersion reached a temperature of 70°C and after this treatment nanorods were observed using TEM, keeping its morphology as can be shown in Figure 1(b), which is great, because it means they could be used several times in photothermal therapy [19].

The antibacterial effect of AuNRs and PPTT using AuNRs has already been studied. In this work, three groups were analyzed: AuNRs, PPTT with AuNRs, and laser group. Within our results, we found that in all cases AuNRs with PPTT present lower values in comparison with AuNRs alone (Table 1 and Figure 3). The MIC and MBC values were higher for *S. aureus* and *E. coli* and 3 times lower for *E. faecalis* and *S. oralis*. These differences could be due to divergence found in the cellular wall of each kind of bacteria. Mocan et al. reported a MIC for spherical gold nanoparticles against methicillin-resistant *S. aureus* with and without photoactivation of 6.25 and 25 μg·mL^{-1}, respectively. These values are higher than those reported in this investigation, probably due to the higher photothermal activity of AuNRs [20].

In endodontics, NaClO is used as the routine disinfectant due to its high effectiveness; Heling obtained a MIC of 1570 μg·mL^{-1} for NaClO against *S. sobrinus*, *E. faecalis,* and *S. salivarius*, and 3150 μg·mL^{-1} against *S. mutans*; the minimum bactericide concentrations reported in the Heling work reach the 25000 μg·mL^{-1}; our results using gold nanorods are at least 3 orders of magnitude lower [21]. Kim et al. claim that, when a dispersion of AuNRs is irradiated with a laser it reaches a temperature near 70°C and this temperature causes the *E. coli* lysis [6]; this temperature is very close to the temperature achieved in this study. Sean Norman et al. say that AuNRs PPTT produces a damage effect in the bacteria surface [5, 22] in our study; this could be the bactericide mechanism as confirmed by the SEM images.

In order to assess if, in parallel to its antimicrobial activity of AuNRs, gold nanorods had the potential to interfere with biofilm formation of oral bacteria, the antibiofilm activity of gold nanorods was studied. Surprisingly, the effect of AuNRs without PPTT on oral biofilm was their complete inhibition as can be observed in Figure 6. In the presence of chlorhexidine and AuNRs we just observed cellular debris on a dark background, mainly DNA of dead bacteria with accumulations of dye. Morphologically, these dye accumulations clearly differ from bacterial biofilm. This phenomenon was not changed if AuNRs were added at 0, 4, or 8 hours postinoculation times. These results are not strange; several early reports have described the antibiofilm properties of metal nanoparticles [8, 10, 11, 23]. Altogether, these results indicate that gold nanorods have an important antibiofilm activity to inhibit with biofilm formation of oral microorganisms.

5. Conclusions

The AuNRs PPTT presented a higher antibacterial activity on seven different ATCC strains used in this work, having a synergic effect if compared with the AuNRs by its own. To our knowledge this is the first study to report the CMI and CMB (based on the CLSI standards) of the AuNRs PPTT with any of the seven different strains.

Conflict of Interests

The authors of this paper declare that there are no potential competing interests among authors of this work.

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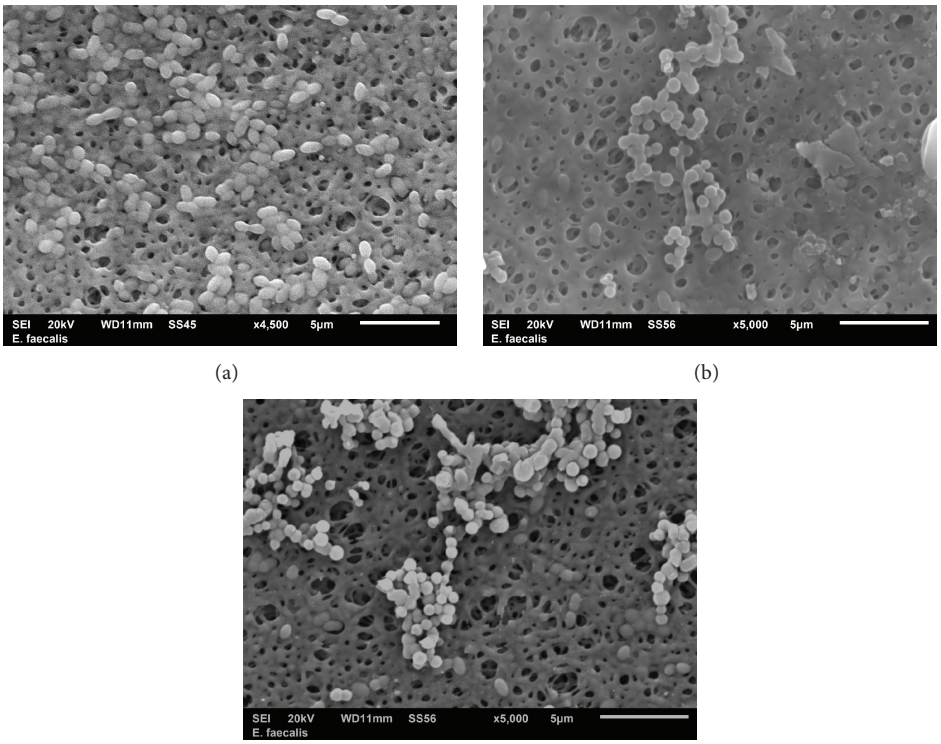

(a) (b)

(c)

Figure 5: SEM images of *E. faecalis*. (a) Control group (bacteria without any treatment); (b) after their treatment with AuNRs; and (c) after their treatment with AuNRs PPTT.

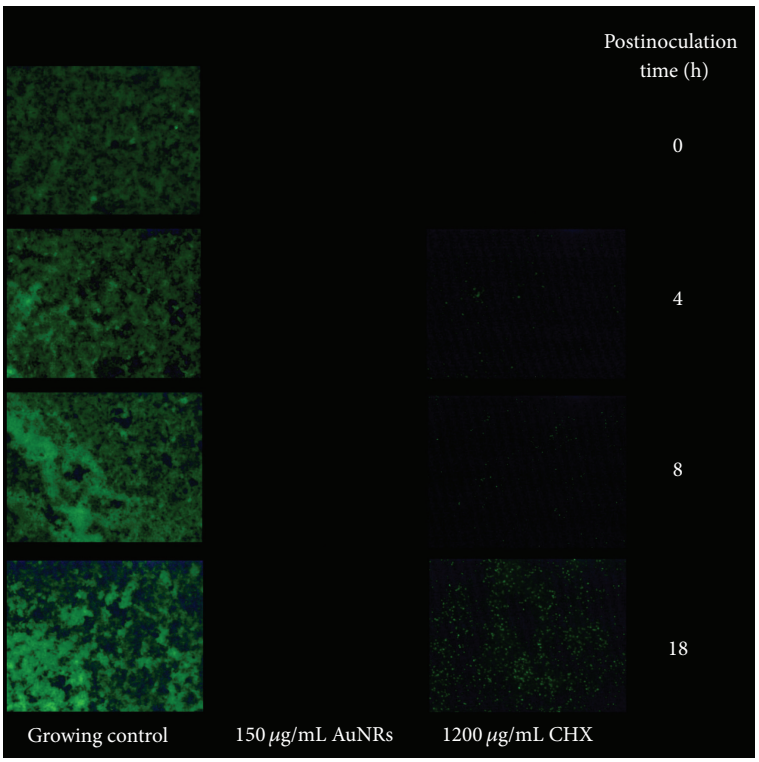

Figure 6: Inhibition of oral biofilm formation by gold nanorods using fluorescence microscopy. As growing control were used microorganisms growing in culture media without inhibitor and 1200 g/mL of chlorhexidine was employed as positive inhibition control. AuNRs were used at a final concentration of 150 μg/mL. All experiments were done in triplicate to assess the veracity of results.

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