



The effect of photodynamic therapy by gold nanoparticles on *Streptococcus mutans* and biofilm formation: an in vitro study

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Abstract

In this experimental study, we aimed to evaluate the antibacterial and anti-biofilm effects of photodynamic therapy with a photosensitizer in conjunction with Gold nanoparticles against *Streptococcus mutans* as an important cariogenic bacterial agent. This experimental in vitro study evaluated the antibacterial and anti-biofilm effect of five groups as followed against *S. mutans*: methylene blue (MB), Gold nanoparticles (AuNPs), methylene blue conjugated with Gold nanoparticles (MB–AuNPs), MB mediated photodynamic therapy (MB mediated PDT) and methylene blue conjugated with Gold nanoparticles mediated photodynamic therapy (MB–AuNPs mediated PDT). InGaAlP laser (Azor-2 K) with 25 mW total output, 660 nm wavelength and laser probe cross-section of 0.78 cm² was used for methylene blue activation. Total dose of 19.23 J/cm² for 10 min was irradiated to each group. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and colony forming unit (CFU) were determined. Bacterial biofilm formation inhibition was assessed by crystal violet staining (The microtiter plate biofilm assay). The viability of *S. mutans* cells was assessed by MTT assay. MB mediated PDT and MB–AuNP mediated PDT were the most effective method for *S. mutans* biofilm inhibition ($P < 0.05$). MB alone, MB–AuNP alone and MB mediated PDT and MB–AuNP mediated PDT had the same effect against the planktonic phase of *S. mutans* ($P > 0.05$). Also they had similar pattern for bacterial growth inhibition and bactericidal effect ($P > 0.05$). Gold nano particle mediated photodynamic therapy represented antibacterial and antibiofilm activity against *S. mutans*; but this modality was not more effective than routine PDT.

Keywords Biofilm · Cell survival · Low level laser therapies

Introduction

Streptococcus mutans is a spherical gram-positive bacterium from the phylum firmicutes and the lactic acid bacteria group which plays an important role as a cariogenic bacterium [1–4]. Photodynamic therapy (PDT) is a safe treatment in which a photosensitizer is activated by a light with specific wave length. Singlet oxygen and free radicals are produced in the presence of oxygen and can damage cellular organelles [5]. PDT in dentistry is recommended for its antimicrobial effect, diagnosis and treatment of cancerous or dysplastic lesions. Antimicrobial effects of PDT have been demonstrated in previous studies [5]. PDT is safe and it is not mutagenic. PDT can inhibit biofilm formation and can cause decrease in microbial load [6, 7].

Different nanoparticles including gold nanoparticles, in previous studies have demonstrated antimicrobial

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properties [2, 8]. Colloidal gold nanoparticles (AuNPs) have proper bioconjugation surface for molecular probes and optical properties which make it a suitable choice for using it in nanomedicine. AuNP has been used as a carrier of drugs for selective killing of diseased cells and microbes [9, 10]. Cationic thiazine dyes such as methylene blue interact with AuNP strongly and increase ultraviolet (UV)-Visible absorption [11]. Nanoparticles in several studies have shown antibacterial properties [12–15]. Using both nano particles with photodynamic therapy may increase cell penetration and produce cytotoxic singlet oxygen strongly [16] and finally it seems to cause synergistic antibacterial effect.

To the best of our knowledge there is no study about the antibacterial effects of photodynamic therapy with gold nanoparticles or any other nanoparticles. There were some studies about the anticancer effect of photodynamic therapy with nanoparticles [16–18] In addition, some studies evaluated antibacterial properties of nanoparticles [13, 19]. Due to the properties of AuNPs and therapeutic effects of photodynamic therapy, we decided to evaluate the effect of photodynamic therapy with gold-nanoparticle on *S. mutans* as the most prevalent cariogenic bacteria.

Materials and methods

This experimental study has been approved by ethic committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1395.S916).

Synthesis of gold nanoparticles

A solution of 0.1 g gold nanoparticles (AuNP) dissolved in 50 mL water was prepared by Iranian Nanomaterial Pioneers Company. This solution was dark brown with more than 99.97% purity. Size of AuNPs was 28 nm with spherical morphology. Crystallographic structure of nanoparticles was cubic. True density and bulk density of this solution were 19.32 g/cm³ and 0.85 g/cm³, respectively.

Characterization of AuNP

Characterization of AuNP particles was determined by the company which the solution was prepared from. Transmission electron microscopy (TEM) image of colloidal solution of AuNPs was obtained at 100 kV with 180,000 magnification (EM208, Philips). Image of scanning electron microscopy

(SEM) of AuNPs solution was obtained with 70,000 magnification (Leo 440i) The pattern of X-Ray powder diffraction (XRD) was recorded using EQUINOX (INEL, France) in the range of 30 to 120° in 2θ.

Bacterial strain

Standard strain of *S. mutans* ATCC25175 was used in this study. The culture of microorganism was prepared in Brain Heart Infusion (BHI) broth and incubated at 37 °C.

Photosensitizer and light source

The photosensitizer used in this study was Methylene Blue (MB). A stock solution of 1 mg/mL was prepared with distilled water. A InGaAlp laser (Azor-2 k) low level laser with 25mW output power and 660 nm wavelength was used for irradiation. Calculation of effective radiant exposure of light was as follow:

$$\text{dose} = \frac{\text{power} \times \text{time}}{\text{area}}$$

This irradiation area of laser beam was 0.78 cm² (laser probe cross section). Continuous irradiation with no distance from the plates, in 10 min can produce 19.23 J/cm² dose.

Determination of minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) and colony formation unit (CFU)

Micro-dilution method was used for determination of MIC and MBC of MB, MB–AuNP conjugates with and without irradiation and AuNP against *S. mutans* in fresh BHI supplemented with 1% sucrose and fresh BHI alone.

Evaluation groups with fresh BHI alone

- Group 1: MB.
- Group 2: MB–AuNP.
- Group 3: MB + Laser (MB mediated PDT).
- Group 4: MB–AuNP + Laser (MB–AuNP mediated PDT).
- Group 5: AuNP.

Evaluation groups with fresh BHI supplemented with 1% sucrose

- Group 6: MB.
- Group 7: MB–AuNP.
- Group 8: MB + Laser (MB mediated PDT).
- Group 9: MB–AuNP + Laser (MB–AuNP mediated PDT).

In two 96-wells microtiter plates, MB which was six fold serially diluted for four times (initial concentration 1 mg/mL) were added. In two series of MB serial dilution (one for *S. mutans* in BHI culture media alone and another for *S. mutans* in BHI supplemented culture media with sucrose), solution of AuNP with concentration of 0.2 mg/mL was added to each well for MB–AuNP conjugate. The standard count of bacterium (inoculum), equal to 0.5 McFarland turbidity, was prepared in PBS and added to all wells. Two wells in each plate were considered as positive and negative controls. One of the plates was considered for the experimental group without laser irradiation and the other one was considered for PDT study (activation of the methylene blue with laser).

In PDT study plate, after 10 min that MB was in contact with the inoculum, all wells were irradiated for 10 min, in dark with no distance between laser probe and plate. Dark environment can help to minimize the other confounding factors including environmental light which can affect the photosensitizer.

Both plates were incubated at 37 °C for 24 h under shake condition. Then the MIC, in which the lowest concentrations that inhibited visible bacterial growth was determined. MBC determination was assessed by culturing the test dilution on a blood agar plate incubated at 37 °C with 5% CO₂ for 24 h. MBC was taken from the highest dilution at which no bacterial growth was seen. The number of colony forming unit (CFU) was calculated as well.

Biofilms formation assay by crystal violet staining

Biofilm formation of *S. mutans* biofilm treated with MB, MB–AuNP conjugate with and without irradiation and AuNP were assessed via micro-titer plate biofilm assay, as

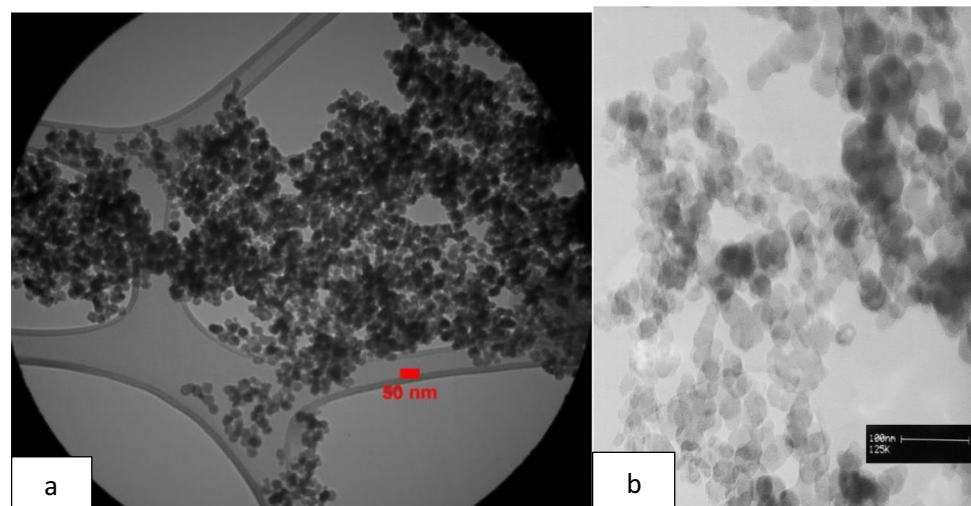
described previously (in 1% sucrose supplemented wells) and quantified by using crystal violet. For removing the not adhered bacteria, after incubation the medium was removed gently by washing three times with 200 µL PBS. Then 200 µL of 0.1% crystal violet was added to each well for biofilm staining. After incubating dye for 25 min, extra dye was removed by rinsing three times with 350 µL of sterile distilled water and immediately de-stained with 200 µL of 95% ethanol and remained for 15 min. Quantification of biofilm formation was then measured by optical density (O.D) of suspension at 490 nm, 570 nm and 630 nm [20] with an ELISA reader.

Interaction between MB and AuNPs causes a significant reduction in GNP absorption peak (534 nm) and change it to a new absorption peak (613–662 nm), measured by UV absorption spectroscopy. According to this reduction, a wide range of UV absorption for MB–GNP conjugation can be seen so in this study three different wave lengths have been considered for OD measurement [21].

MTT reduction assay

For the assessment of bacterial cell viability, the MTT assay was performed. A 5 mg/mL concentration of 3–4–5 diphenyl tetrazolium (MTT) dissolved in PBS was used for MTT assay. The solution was filter sterilized using 0.2 mm pore-size filter. As previously described in crystal violet assay, the adherent cells in treated and control *S. mutans* biofilm, were rinsed three times with 200 µL PBS to remove loosely adherent and planktonic cells. Afterward 100 µL of MTT solution was added to each well. Then the micro-titer plates were incubated at 37 °C for 2 h. Then 400 µL of acidic Isopropanol was added to each well. Colorimetric changes

Fig. 1 TEM(1-a) and SEM (1-b) images of gold nanoparticles



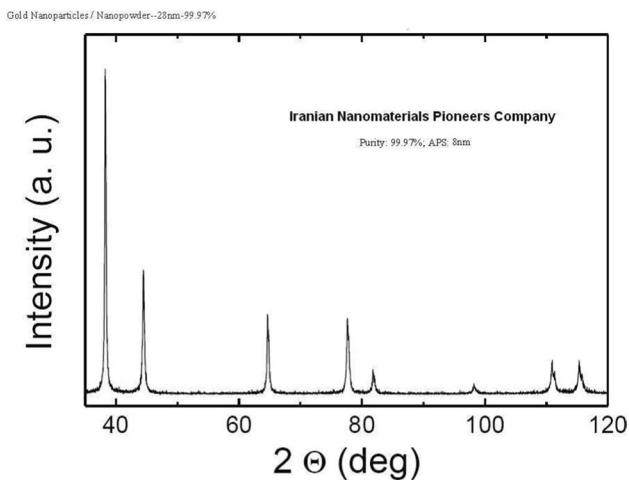


Fig. 2 XRD; X-Ray diffraction pattern of AuNP. *L* laser illumination, *O.D* optical density, concentration ($\mu\text{g/mL}$)

were measured using a microtiter plate ELISA reader at the wavelength of 530 nm.

Statistical analyses

Data analysis was performed using SPSS version 18. The mean value of MIC, MBC, CFU of duplicate evaluations were reported. Kruskal Wallis test was used for comparing the mean of MIC and MBC.

Results

Characterization of AuNP

TEM and SEM images (Fig. 1) of AuNPs show spherical shape and the 28 nm size of the particles. In Fig. 2 a typical power XRD pattern of AuNP is observed.

MIC, MBC and CFU

The minimum inhibitory (MIC) and bactericidal (MBC) concentrations and CFU count of groups 1–9 are reported in Table 1.

Group 4 (MB–AuNPs mediated PDT) demonstrated the lowest MIC and MBC. The CFU count was at lowest level for group 3 (MB mediated PDT) and group 4 (AuNPs–MB mediated PDT) but there was no statistical difference ($P > 0.05$). AuNP alone with a relative high concentration had inhibitory effect (250 $\mu\text{g/mL}$) but MBC and CFU could not be detectable even at the highest concentration tested.

No significant difference was detected between different concentrations of MB ($P = 0.2$) and between different studied groups ($P = 0.5$) for MIC and MBC.

Assessment of biofilm formation by *S. mutans*

Biofilm inhibitory effect of groups 1, 2, 3, 4 (MB and MB–AuNP with and without laser illumination) are demonstrated in Figs. 3, 4, 5. AuNP alone did not have anti-biofilm effect.

Applied three wave lengths (490 nm, 570 nm and 630 nm) in most of evaluations showed no significant

Table 1 The MIC, MBC and CFU measured for MB, MB–AuNP, MB mediated PDT, MB –AuNP mediated PDT and AuNP on *S. mutans* in BHI broth and BHI supplemented with sucrose

Groups	Component	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)	CFU (Count)
1	MB	93.75	125	150
2	MB + AuNP	93.75	156.25	175
3	MB + L	93.75	187.5	100
4	MB + AuNP + L	78.125	93.75	120
5	AuNP	250	Not detectable	Overgrowth
6	MB + sucrose	140.625	187.5	115
7	MB + AuNP + sucrose	78.125	140.625	165
8	MB + L + sucrose	78.125	156.25	135
9	MB + AuNP + L + sucrose	78.125	93.75	90
10	AuNP + sucrose	Not detectable	Not detectable	Overgrowth

L laser illumination, *MIC* minimum inhibitory concentration, *MBC* minimum bactericidal concentration, *CFU* colony forming unit

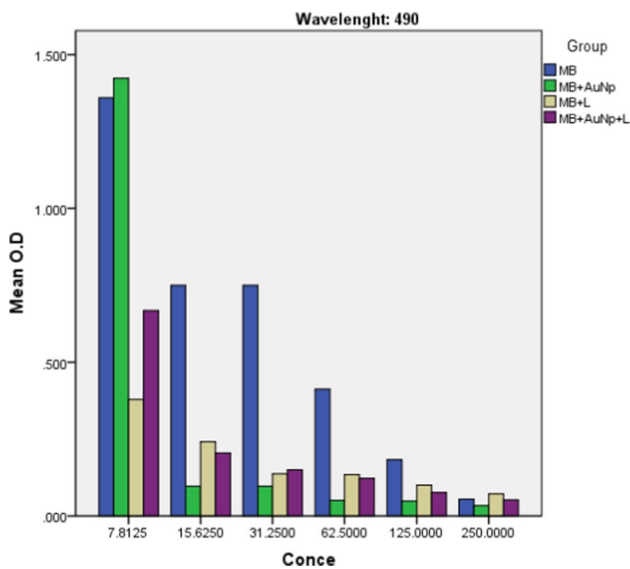


Fig. 3 Crystal violet assay; MB, MB–AuNP, MB mediated PDT and MB–AuNP mediated PDT inhibitory effects on biofilm formation with O.D measurement (490 nm). *L* laser illumination, *O.D* optical density, concentration ($\mu\text{g}/\text{mL}$)

difference between concentrations in evaluated groups was (P value > 0.05).

But, in 490 and 570 nm wave lengths, group 3 (MB mediated PDT) and 4 (MB–AuNP mediated PDT) were significantly more effective against biofilm formation in comparison to group 1 (MB alone) ($P < 0.05$). In

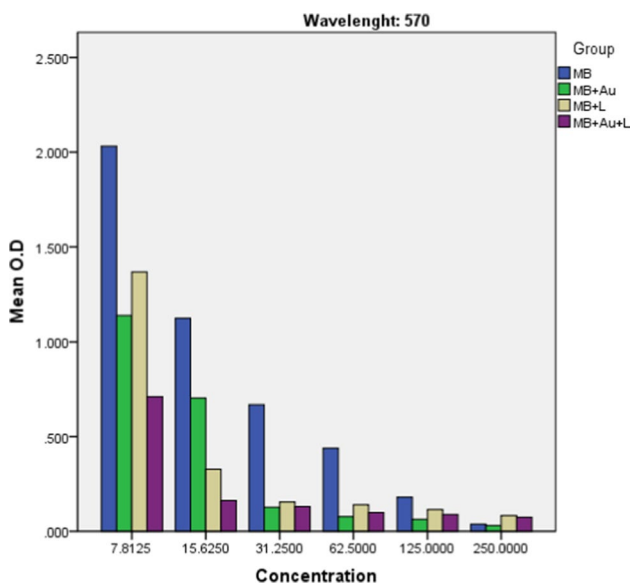


Fig. 4 Crystal violet assay; MB, MB–AuNP, MB mediated PDT and MB–AuNP mediated PDT inhibitory effects on biofilm formation with O.D measurement (570 nm). *L* laser illumination, *O.D* optical density, concentration ($\mu\text{g}/\text{mL}$)

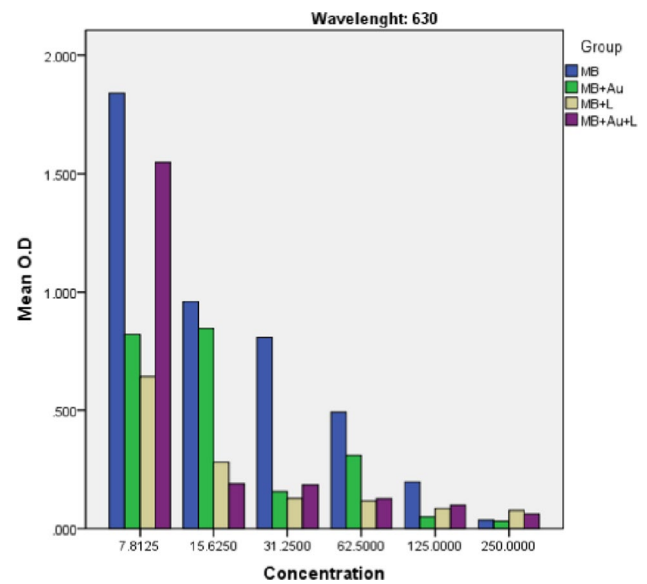


Fig. 5 Crystal violet assay; MB, MB–AuNP, MB mediated PDT and MB–AuNP mediated PDT inhibitory effects on biofilm formation with O.D measurement (630 nm). *L* laser illumination, *O.D*: optical density, concentration ($\mu\text{g}/\text{mL}$)

addition, in 570 nm, group 2 (MB–AuNP) and in 630 nm, group 3 (MB mediated PDT) showed more effective than group 1 in biofilm inhibition ($P < 0.05$).

In all three wave-lengths, the concentration of 7.81 $\mu\text{g}/\text{mL}$ of MB had the lowest biofilm inhibition than all other evaluated concentrations ($P < 0.05$).

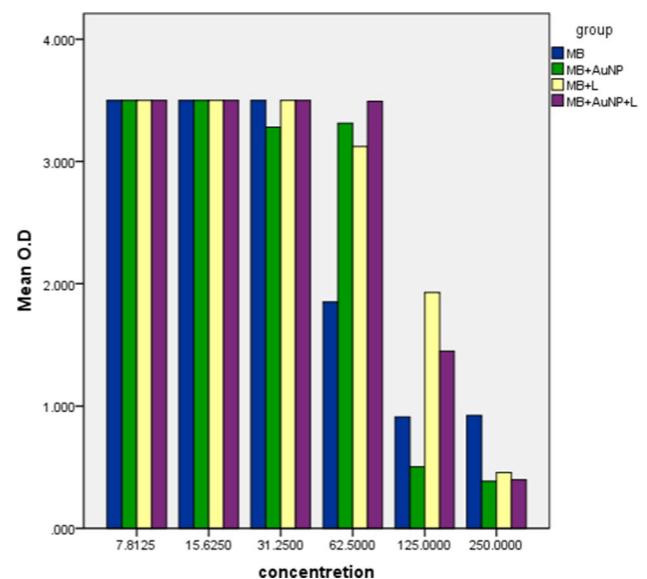


Fig. 6 MTT assay for bacterial viability; MB, MB–AuNP, MB-mediated PDT and MB–AuNP mediated PDT effect on bacterial viability with O.D measurement in 530 nm

Assessment of bacterial viability

The viability of *S. mutans* during different antibacterial assays in this study is revealed in Fig. 6.

No difference was detected between different concentrations in evaluated groups ($P > 0.05$).

Discussion

A photosensitizer is capable to absorb light of specific wavelength and transform it into useful energy [22]. A proper photosensitizer should be low toxic toward mammalian cells. In order to fulfill this property, they should be high binding affinity for microorganisms (positively charged photosensitizer for appropriate adherence to negatively charged bacterial cell wall) [23], low binding affinity for mammalian cells and low chemical toxicity and mutagenicity [24].

PDT cause the bacterial cell wall damage, increasing cytoplasmic membrane permeability and breakage of DNA [25]. PDT can destroy carbohydrates, amino acids or phosphate transport through carrier proteins of cell membrane [26]. Intracellular enzymes will be knocked out and disturb ATP production and cellular metabolism [27] cyclic dependent kinase inhibitor and cell growth inhibition causes over expression of P21 which irritates mitochondria [28]. Biological chlorophores in cells and organisms excites via proper dose and wavelength of laser illuminance, and produce singlet oxygen and superoxide. These toxic products destroy microorganisms by DNA and cell membrane destruction [29].

The different nanoparticles including gold nanoparticles, in previous reports have revealed antimicrobial properties [2, 8] besides, Cationic thiazine dyes such as methylene blue interact with AuNP strongly and increase ultraviolet (UV)-Visible absorption [11]. Evaluation of the antibacterial and anti-biofilm effect of photodynamic effect of methylene blue with gold nanoparticles against *S. mutans* as the significant producer of cariogenic biofilm was the idea of the present study.

According to the results of the present study, all testing groups except for AuNP onle, had antibacterial and anti-biofilm effect. AuNP alone with a very high concentration had inhibitory effect (more than twofold higher concentrations than the other groups) and MBC and CFU could not be measured.

The MIC, MBC and CFU counts of all evaluated groups (MB, MB–AuNPs, MB mediated PDT, MB–AuNP mediated PDT) for *S. mutans* cultured in BHI with and without sucrose supplementation, are in a similar pattern and there were no significant differences between them ($P < 0.05$). Biofilm assay confirmed the concentration dependency for better *S. mutans* biofilm inhibition. MB mediated PDT and MB–AuNP mediated PDT had the best inhibitory effect on

S. mutans biofilm formation among all groups in all three applied wave lengths. In MTT assessment no statistical difference between all evaluated groups was detected.

There are a few studies that evaluated the effectiveness of nanoparticles mediated PDT including an in vitro antimicrobial and anti-biofilm study of PDT with AgNP, TBO and AgNP-TBO, used 9.1 J/cm^2 irradiation dose with 630 nm wavelength for 70 s [19].

The MIC and MBC of TBO-AgNP mediated PDT (with an initial concentration of 1 mg/mL TBO and a constant concentration of $25 \mu\text{g/mL}$ AgNP) showed a twofold higher activity against *S. mutans* than TBO alone. In comparison, our results showed less MIC and MBC for MB–AuNP-mediated PDT than MB mediated PDT alone against *S. mutans*, however, these differences were not significant.

In Misba et al. study [19], crystal violet assay showed anti-biofilm effect in a concentration dependent manner. A more significant reduction of biofilm formation with TBO-AgNP mediated PDT was detected in comparison with TBO mediated PDT. Better effect of photodynamic therapy generally in comparison to use of methylene blue alone is in line with our results in the presenting study. In addition to the concentration dependent pattern, MB–AuNP-mediated PDT and MB-mediated PDT had a higher effectiveness in biofilm inhibition than MB and MB–AuNP alone. Misba et al. [19], observed a better effect when adding AgNP to TBO mediated PDT than TBO mediated PDT alone. This is not in accordance with the present study, which could not detect significant differences between the PDT groups, that is, no extra effect was obtained with the addition of AuNP to MB mediated PDT. Moreover, MB itself has antibacterial effect [30] also higher concentrations of AuNPs for preparing the MB and nanoparticle conjugation might have been needed for better a antibacterial effect. This may justify this controversy.

In addition, Misba evaluated the bactericidal activity by converting the value of XTT, which was found to be concentration dependent as well. TBO-AgNP mediated PDT had a noticeable bactericidal effect on *S. mutans* in comparison with TBO at the same concentrations. We used MTT assay for bacterial viability assessment and MB–AuNP mediated PDT had no superiority for bactericidal properties than MB mediated PDT and other groups at the same concentrations.

Other study declared that polymeric nanoparticles are potent to load MB and act as a carrier of MB for PDT system [31]. Khan et al. study showed that AuNPs (GNP) conjugates with photosensitizer (PS) in PDT exhibited synergistic antimicrobial activity against *Candida albicans*. The GNP-PS mediated PDT against *Candida* biofilm formation showed significant biofilm reduction and adverse effect against *Candida albicans* [32].

In Sherwani et al. study, the effect of GNP-PS based PDT on *C. albicans* was also evaluated, which confirmed

the synergistic activity of GNP-PS conjugation against *C. albicans* [8].

Chemical structure of photosensitizer, incubation period of photosensitizer and bacterial cell, bacterial species, time of irradiation, photosensitizer concentration and laser energy dose would affect the PDT effectiveness [22, 25].

MB is a phenothiazinium dye, which has a very low toxicity in dark, penetrates the bacterial cell membrane, affect the bacterium genome and kill them [30]. MB is hydrophilic with low molecular weight and positive charge. These properties allow MB to pass across porin-protein channel in the outer membrane of gram-negative bacteria [33, 34].

Bacterial colonies are more resistant when they accumulate as a biofilm, due to better communication between cells [35]. PDT has a direct effect on extracellular polysaccharides matrix of biofilm are susceptible to photo damage [36].

As explained previously, energy of light exposure is an important factor in PDT efficacy. Thus it has been indicated that light exposure with a high power density over a short period of time compared with low power density over a longer time even with the same energy density in both, may cause different antimicrobial effects [25].

Nowadays, metallic nanoparticles are mostly used for bio-conjugation, drug delivery and also diagnostic application [37, 38]. Metallic nanoparticles are large in surface area which affects its antimicrobial effect [39]. The antimicrobial activity of nanoparticles has an inverse relationship with nanoparticles' size. A range size between 1 and 10 nm have the best antibacterial activity. In comparison with conventional and narrow target antibiotics, bacteria are less likely to acquire resistance against metallic nanoparticles [40, 41].

Several studies indicated that positive charge on the metal ion is essential for antimicrobial activity. Bacterial cell membrane has negative charge. Thus an electrostatic attraction happens between the positive charge of metal ion and negative charge of cell membrane [42].

Gold nanoparticles (GNP) is known to have special chemical and physical and optical properties due to its remarkable surface plasmon resonance for imaging and fluorescence enhancing [43, 44]. Molecular cytotoxicity mechanism of GNP is only disruption of protein conformation [6].

Interaction between MB and AuNPs causes a significant reduction in GNP absorption peak (534 nm) and change it to a new absorption peak (613, 662 nm), measured by UV absorption spectroscopy. According to this reduction, a wide range of UV absorption for MB–GNP conjugation was seen [20]. The interaction between the negative charge of GNP and positive charge of MB has been confirmed by TEM image [31]. A noticeable quenching of MB fluorescence in addition with GNP was reported. AuNPs interaction with MB in the form of a 2 ± 0.5 nm layer of MB around AuNPs causes the new peak of UV absorbance [31].

The mechanism of quenching the fluorophore by AuNPs are a radioactive energy transfers from fluorophore to GNP and collision dynamics between fluorophore and AuNPs [45, 46].

Pagonis et al., revealed that the permeability of cell walls for MB carrier increases by the concentration of nanoparticles on bacterial cell wall [47].

Photodynamic therapy can be used as a method for decreasing the oral cariogenic bacterial count especially in patients with rampant caries or in patients with greater susceptibility to dental caries including patients with history of xerostomia, chemo and radio therapy.

Although there is a good concept about nanoparticles efficacy, side effects must also be considered. For example, actively excreted form of copper and silver accumulation within normal body [48]. To understand the toxic kinetic properties of nanoparticles, information about their absorption, distribution, metabolism and excretion is necessary [49].

Performing the evaluation in laminar flow, which produce a sterile, isolated environment for such lab research, could assure the researcher about no external contamination. In this study the anti *S. mutans* effect of PDT mediated by different compositions of MB and MB–AuNPs, in several media (BHI and sucrose supplemented BHI) were assessed. To determine the effective component of these approaches, different groups were designed. The antibacterial effects of MB, MB–AuNP and AuNPs alone without any light activation were also analyzed. Higher concentrations of AuNPs for preparing the MB and nanoparticle conjugation should be assessed in further evaluations.

In our study, the cover of plates was not removed during laser application since multiple plate contamination had been occurred before in pilot studies. The presence of this translucent barrier could reduce the irradiation dose. According to some studies, Vahabi et al. [50], using shorter time and higher dose of irradiation can induce more prominent effect. Our laser device could not produce enough dose of irradiation in a short period. In six series of repeating the evaluation, there were somehow different and contradictory results; while we did our best effort to homogenize and statistically analyze the situation on each effort.

It can be suggested to evaluate different AuNP concentrations and different irradiation dose. To have more complete evaluation, it can be proposed to study the developed biofilm inhibition of *S. mutans*.

While there is a global interest to introduce nanotechnology as an effective modality for different medical interventions, treatments or preventive procedures, according to our results there was no significant superiority for nanoparticle association with PDT. Choosing a proper photosensitizer and light activation wavelength and irradiation dose can induce enough antibacterial effects.

Conclusion

Our study revealed MB-mediated PDT and MB–AuNP-mediated PDT were the most effective method for *S. mutans* biofilm inhibition. Increasing the concentrations of photosensitizer along with gold nanoparticles reduces the bacterial biofilm formation more effectively, however, side effects must also be an important concern. Different groups in this study had the same effect against the planktonic phase of *S. mutans*. MB alone, MB–AuNP alone and MB-mediated PDT and MB–AuNP-mediated PDT had similar pattern for bacterial inhibition and bactericidal effect. Based on this study, both MB-mediated PDT and MB–AuNP-mediated PDT had proper antibacterial biofilm formation activity.

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Authors contributions FL conceived of or designed study, performed research, analyzed data, contributed new methods or models, wrote the paper. FM conceived of or designed study, performed research, analyzed data, contributed new methods or models, wrote the draft paper. SA conceived of or designed study, performed research, analyzed data, wrote the draft paper.

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Data availability Not applicable.

Declarations

Conflict of interest There is no conflict of interest to declare.

Ethical approval The study has been approved by ethic committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1396.S727).

Code availability Not applicable.

Consent to participate Written consent form was taken before initiation of the study.

Consent for publication Not applicable.

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