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Anti protein A antibody-gold nanorods conjugate: a targeting agent for selective killing of methicillin resistant *Staphylococcus aureus* using photothermal therapy method

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The high prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) and developing resistance to antibiotics requires new approaches for treatment of infectious diseases due to this bacterium. In this study, we developed a targeting agent for selective killing of MRSA using photothermal therapy method based on anti protein A antibody and gold nanorods (GNRs). Polystyrene sulfonate (PSS) coated GNRs were conjugated with anti protein A antibody. The FT-IR and UV-vis analyses approved the formation of anti protein A antibody-gold nanorods conjugate. *In vitro* study of photothermal therapy showed 82% reduction in the MRSA cells viability which was significantly greater than the ablation effect of free GNRs and laser alone. Significant accumulation of anti protein A antibody-GNRs in the infected muscle in comparison with normal muscle approved the targeting ability of new agent. *In vivo* study of photothermal therapy resulted in a significant reduction (73%) in the bacterial cells viability in the infected mouse model. These results demonstrated the ability of anti protein A antibody-GNRs conjugate in combination with NIR laser energy for selective killing of MRSA in mouse model.

Keywords: Methicillin Resistant *Staphylococcus aureus*, photothermal therapy, gold nanorods, anti protein A antibody

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA), a Gram-positive spherical cells usually arranged in grape-like irregular clusters, is responsible for several difficult-to-treat infectious diseases in humans such as bacteremia, endocarditis, meningitis, impetigo, eczema, and folliculitis (Huang, 2006; Fernandes *et al*., 2012; Galanzha *et al*., 2012; Turos *et al*., 2012). Unfortunately, antiseptics such as hexachlorophane, triclosan, chlorhexidine, and povidoneiodine are not fully effective against MRSA and there are some evidences of resistance developing to these agents (Zharov *et al*., 2006; Zolfaghari *et al*., 2009). Given that MRSA has evolved mechanisms of resistance for most commercially produced antibiotics, it is necessary to develop novel strategies not rely on traditional therapeutic regimes (Turos *et al*., 2007; Huang *et al*., 2009). One such strategy involves photothermal therapy method using the rod shape of gold nanoparticles to destroy bacteria (El-Sayed *et al*., 2006; Chen *et al*., 2008; Pissuwan *et al*., 2008). Here we describe the approach involving physical damage to MRSA using the combination of NIR laser energy and absorbing nanoparticles selectively attached to the bacterial cells. When gold nanoparticles are irradiated, they absorb energy, which is quickly transferred through non radiative relaxation into the heat and accompanied effects, and eventually leads to irreparable damages. Gold nanorods are photostable and nontoxic material that manifest a low level of toxicity when introduced into the biological systems and are easily conjugated to the antibodies or proteins (Panyala *et al*., 2009). In addition, their maximum absorption can be shifted to near-infrared radiation (NIR) ranging from 700 to 900 nm, where the minimum absorption of light occurs in most human tissues, allowing deeper penetration of laser radiation into the infected tissues. (Grace and Pandian, 2007; Huff *et al*., 2007). Therefore, gold nanorods can be useful as a photothermal therapy agent since their use would minimize the risk of damages to healthy cells (Mohanpuria *et al*., 2008; Pissuwan *et al*., 2008).

To date, laser energy in combination with targeted GNRs has been successfully used in killing bacterial cells *in vitro*, but selective ablation of bacterial infection foci using photothermal therapy method has not been performed *in vivo*. Norman *et al*. (2008) used gold nanorods conjugated with primary antibodies for killing of pathogenic Gram-negative bacterium, *Pseudomonas aeruginosa*, *in vitro*. They found a significant reduction in bacterial cells viability following exposure to near-infrared laser. In this study, we performed the experiments in which GNRs were covalently conjugated with anti protein A antibody to produce a nanodrug for selective killing of MRSA using photothermal therapy method *in vitro* and *in vivo*. Anti protein A antibody is a monoclonal antibody against the specific product of *Staphylococcus aureus*, protein A, one of the major surface-clustered of proteins that is linked to the peptidoglycan portion of the bacterial cell walls. About 99% of *S. aureus* strains express protein A on their cell walls (Huang, 2006). So, it seems a good target for selective killg of MRSA.

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Materials and Methods

Polystyrene sulfonate (PSS, MW=18,000, Poly Sciences Inc), HEPES buffer, anti protein A monoclonal antibody, and gold nanorods (15 × 50 nm) were purchased from sigma-Aldrich, USA. BALB/c mice (7–8 weeks old and 25–35 g weight) were purchased from Razi Vaccine and Serum Research Institute, Karaj, Iran. Methicillin-resistant *Staphylococcus aureus* prototypic UK epidemic MRSA (EMRSA-16) was obtained from microbial bank of Institute Pasteur, Tehran, Iran.

Conjugation of anti protein A antibody with gold nanorods

Anti-protein A antibody was conjugated with gold nanorods according to the method described by Huang with some modifications (Huang, 2006). Briefly, GNRs were centrifuged twice at 10,000 rpm for 15 min to get rid of the extra free CTAB molecules. Then, GNRs surfaces were coated by adding 200 µl PSS (10 mg/ml) to 10 ml of GNRs solution with optical density of 1.0 at the longitudinal absorption maximum. The mixture was left to react for 15 min. Then, the extra PSS was separated by centrifuging the solution at 8,000 rpm and the pellet was redispersed in HEPES buffer (pH= 7.4). Then, GNRs were mixed with anti protein A antibody solution diluted in HEPES buffer and left to react for 20 min. The solution was centrifuged and the pellet was redispersed into PBS (pH=7.4) to form a stock solution with the optical density of 1.0 at 825 nm.

Anti protein A antibody-gold nanorods conjugate characterization

UV-vis spectrophotometer (CARY-100 BIO, Varian, USA) was preliminary used to investigate the interaction of anti-protein A antibody with GNRs in the wavelengths range of 400 to 900 nm at the resolution of 1 nm. The UV-vis spectra of free GNRs and PSS-GNRs were determined for comparison too. The shift of surface plasmon resonance band (SPR) from 810 nm (free GNRs) and 820 nm (PSS-GNRs) to a new peak at 825 nm shows the capping of GNRs surfaces with anti protein A antibody. The FT-IR spectra of GNRs alone, anti protein A antibody alone and anti protein A antibody-GNRs conjugate were recorded by Perkin-Elmer Fourier transform infrared spectroscopy (model FT/IR-Jasco 6300) to assure the formation of the conjugate (Grace and Pandian, 2007).

Stability in human blood

1 ml samples of human blood were prepared and heparinized at the final concentration of 10 units/ml to prevent coagulation. 100 µl of anti protein A antibody-GNRs conjugate was added to the blood samples. The samples were mixed vigorously and incubated at room temperature for 24 h. Then, the samples were transferred to 1.5 ml eppendorf tubes and centrifuged at 2,000 rpm for 15 min to precipitate the red blood cells. The supernatant was collected and transferred to a 1 cm cuvette. The light absorption of supernatant was determined using a UV-vis spectrophotometer (Grace and Pandian, 2007).

In vitro photothermal killing of MRSA

MRSA was cultured in tryptic soy broth and grown aerobically for 16 h at 37°C. The cells were harvested by centrifugation and resuspended in sterile PBS to the concentration of 2×10^8 CFU/ml (Pissuwan *et al*., 2007). 100 µ1 aliquots of saline suspension of the bacteria were transferred into the each well of a 24-well, round bottomed microtitration plate in 4 groups of 6. The study included 4 arms: 1) anti protein A antibody-GNRs conjugate and NIR laser, 2) free GNRs and NIR laser, 3) PBS and NIR laser and 4) PBS without laser (control). Anti protein A antibody-GNRs conjugate and free GNRs were added to the first and second groups of wells, respectively. The bacterial cells were washed with PBS for removing the unbound GNRs after 30 min incubation. Next, each well on the plate except the fourth group (control) were exposed to the laser light (825 nm) at power value of 80 mW and focus spot of 1 mm diameter for 3 min. Immediately, after laser irradiation, the content of each well was transferred to a plate containing tryptic soy agar medium, individually and incubated at 37°C for 36 h. Then, the colony counting was performed for bacterial viability (Huang *et al*., 2006; Turos *et al*., 2012). Additionally, in order to make an in-depth study of the antibacterial activity of the conjugate, the SEM analysis was performed.

Biodistribution study

Staphylococcus aureus was cultured in aerobic condition in tryptic soy agar medium for 16 h at 37°C. Then, a turbid suspension containing 2×10^8 CFU/ml of viable bacteria in 0.2 ml of normal saline was injected intramuscularly to the left lateral thigh muscles of 4 groups of 5 BALB/c mice for biodistribution assessment at 4 time intervals. The right thigh muscles were considered as the control. Twenty four hour later, when the swelling was appeared at the injection site, the infected mice were injected intravenously with 200 µl of anti protein A antibody-GNRs conjugate at the concentration of 2.7 mg/g GNRs via the tail vein. At 1, 4, 8, and 24 h post injection, the mice were sacrificed using CO_2 gas. The liver, heart, kidney, small and large intestines, stomach, blood, right and left thigh muscles (normal and infected) were dissected and weighted. Each tissue was lysed in a 15

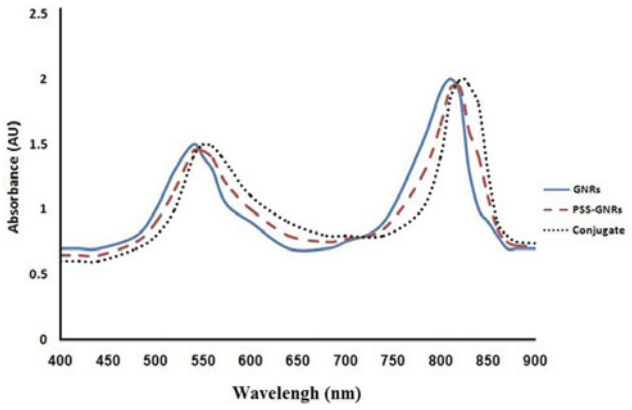

Fig. 1. UV–vis spectra of (A) free GNRs, (B) PSS coated GNRs and anti protein A antibody-GNRs conjugate.

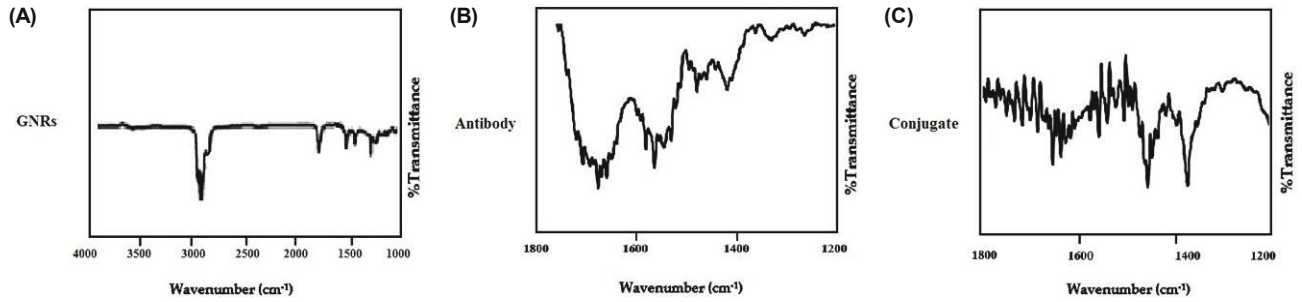

Fig. 2. FT-IR spectra of (A) free GNRs, (B) anti protein A antibody, and (C) anti protein A antibody-GNRs conjugate.

ml tube with aqua regia and subjected to the quantitative analysis. Atomic adsorption spectroscopy was used for quantitative measurement of the conjugate distribution in vital organs of the mouse model. The results were calculated as the percentage of injected doses per gram of each organ (% ID/g) (Ham *et al.*, 2007). All animal experiments were approved by animal care committee of Tarbiat Modares University, Tehran, Iran.

In vivo photothermal therapy of MRSA

The left lateral thigh muscles of twenty BALB/c mice were infected in the same way described above. Twenty four hour later, when the swelling was appeared at the injection site, the *in vivo* study was performed in 4 arms: 1) anti protein A antibody-GNRs conjugate and NIR laser, 2) PEGylated GNRs and NIR laser, 3) PBS and NIR laser and 4) PBS without laser (control). The infected mice were randomly divided into 4 groups of 5. Anti protein A antibody-GNRs conjugate and PEGylated GNRs were injected via the tail vein of the first and second groups of mice, respectively. Based on the results of biodistribution study, 8 h after the injection, all groups except the fourth group (control) were irradiated at the swelling site with laser light (825 nm) at 80 mW and spot size of 1 mm for 6 min. After laser irradiation, the mice were sacrificed using CO_2 gas and the left thigh muscles (infected muscles) were dissected. Infected muscles were cut off with a scissor and were separated into PBS buffer for 5 min. Next, 1 ml of each sample was placed on a plate containing tryptic soy agar medium. The plates were incubated at 37°C for 36 h. Then, the colony counting was performed for bacterial viability (El-Sayed *et al.*, 2006).

Statistical analysis

All the experiments were repeated 5 times for each test and the results were expressed as mean ± SD. The statistical comparisons were performed as analysis of variance (ANOVA), whenever applicable, using SPSS 18. The level of significations was taken at 0.05.

Results

Characterization of anti protein A antibody-GNRs conjugate

In UV-vis spectrum, appearance of a new peak emerging at 825 nm approved the conjugation of anti protein A antibody with GNRs (Fig. 1). The shift in the absorption peak is possibly due to the altered refractive index of local environment of gold nanoparticle caused by the presence of biological molecules. The FT-IR spectra of free GNRs alone, anti protein A antibody alone and anti protein A antibody-GNRs conjugate were shown in Fig. 2. The FT-IR spectra showed that the stretching frequency of carbonyl group in the free antibody (1,647 cm^{-1}) was shifted to the lower frequency in the conjugate molecule (1,615 cm^{-1}). This shift further approved the interaction between the antibody and

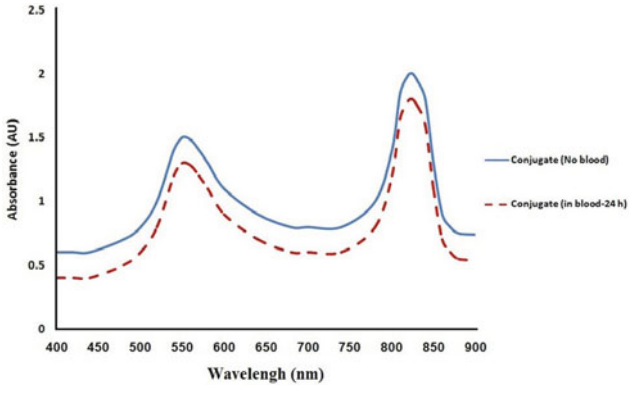

Fig. 3. Absorption spectra of the supernatant before and after incubating of anti protein A antibody-GNRs conjugate in human blood.

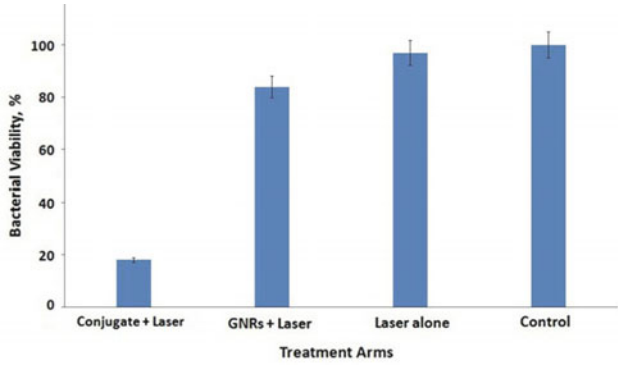

Fig. 4. The levels of MRSA viability *in vitro* in different trial groups. The results showed the significant destruction of the bacteria in the test group treated with anti protein A antibody-GNRs conjugate in combination with laser energy (82%) in comparison with other groups ($P < 0.05$).

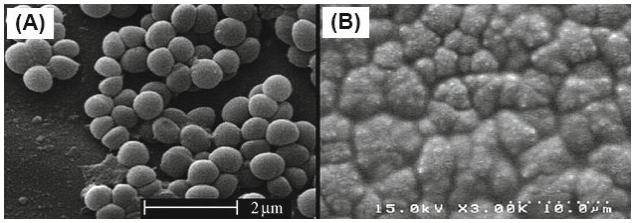

Fig. 5. SEM images of MRSA bacteria before (A) and after (B) photothermal therapy treatment.

the GNRs (Fig. 2).

Stability in human blood

Figure 3 depicts the light absorption of Anti protein A antibody-GNRs conjugate as a function of wavelength after 24 h being in human blood. The absorption peak remained intact around 825 nm up to 24 h that showed high stability of the conjugate and the lack of aggregation in human blood.

In vitro photothermal killing of MRSA

Figure 4 shows the results of photothermal killing of MRSA *in vitro*. Counting of colonies showed a significant cell death (82%), when the bacteria were treated with anti protein A antibody-GNRs conjugate plus NIR laser irradiation in comparison with other groups ($P < 0.05$). The bacteria treated with free GNRs plus NIR laser irradiation showed just small fraction of cell death (16%) in comparison with control group ($P<0.05$). The cells irradiated only with NIR laser did not show any cell death.

SEM

Figure 5 shows SEM micrographs of MRSA bacteria before (A) and after (B) photothermal therapy treatment (Phillips XL 3000 scanning electron microscope). The untreated *S. aureus* cells display a round and undamaged appearance (Fig. 5A). The cell deformation and indentation can be clearly observed in photothermal treated MRSA despite the relatively high thickness of the bacterial cell wall (Fig. 5B). These results further approved the antibacterial activity of the conjugate against MRSA using photothermal therapy method.

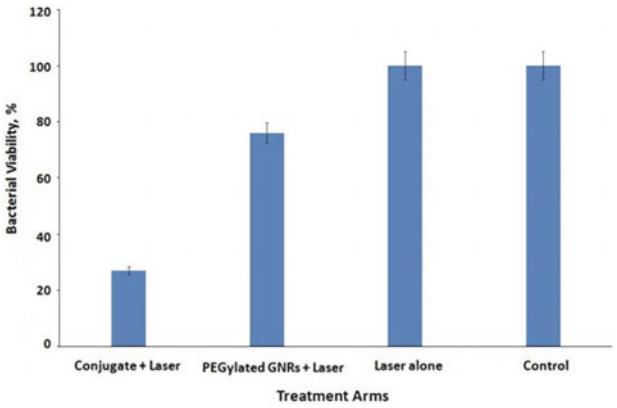

Fig. 7. **The results of NIR photothermal therapy *in vivo*.** It can be seen a significant difference in cell death in the test group treated with anti protein A antibody-GNRs conjugate in combination with laser energy (73%) compared to other groups ($P < 0.05$).

Biodistribution study

Figure 6 shows the biodistribution data of anti protein A antibody-GNRs conjugate in the vital organs of infected BALB/c mice at 1, 4, 8, and 24 h after IV administration expressed as the percentage of injected dose/g of each organ (%ID/g). The results showed significant accumulation of the conjugate in the infected muscle in comparison with normal muscle in all the measured times specially at 8 h post injection ($P < 0.05$).

In vivo photothermal therapy of MRSA

Figure 7 shows the results of *in vivo* photothermal treatment of infection foci due to MRSA in the mouse model. A significant bacterial cell death (73%) was observed, when the infection foci was treated with anti protein A antibody-GNRs conjugate plus NIR laser irradiation in comparison with other groups ($P < 0.05$). The infection foci treated with PEGylated GNRs plus NIR laser irradiation showed small fraction of bacterial death (24%) in comparison with control group ($P < 0.05$). The infection foci irradiated only with NIR laser did not show any cell death.

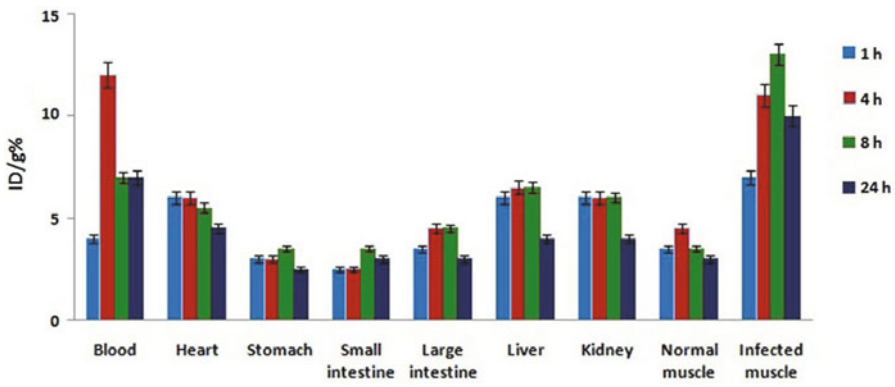

Fig. 6. **Biodistribution data in the vital organs of mouse model at time intervals of 1, 4, 8 and 24 h post injection of anti protein A antibody-GNRs conjugate (n=5).**

Discussion

In this study, we developed a targeting photothermal agent based on anti protein A antibody and gold nanorods capable of inducing physical damage to MRSA in combination with NIR laser energy. PSS was used for conjugation of anti protein A antibody with GNRs because of possible electrostatic interaction between its negative charge and the positively charged segment of the antibody (Gole and Murphy, 2005). PSS is a poly electrolytes biochemical agent that is used for preventing particles from aggregation in the presence of salts and provides a strong electrostatic repulsive force. On the other hand, PSS-coated GNRs have biocompatibility and remain stable even when they are mixed with plasma proteins and ions. Then, the various analytical techniques like UV-vis and FT-IR analyses were performed to approve the interaction between anti protein A antibody and GNRs.

The stability of conjugate within blood microenvironment is a key issue for successful targeting of GNRs to infection foci *in vivo*. The result of stability study demonstrated the high stability of the conjugate in blood microenvironment. Free sulfhydryl group reacts with gold surface to make a dative bond, also known as a coordinate covalent bond; thus the attachment of antibody to gold nanoparticles through their sulfhydryl side chain is very stable. The stability of conjugate in human blood up to 24 h is quite enough for using it as a photothermal therapy agent.

The killing of bacteria was examined *in vitro* using anti protein A antibody-GNRs conjugate as under illumination with NIR laser. Counting of live colonies showed the significant difference between the test group and other groups ($P < 0.05$). These results revealed the ability of the prepared agent to destroy MRSA cells selectively. Zharov *et al.* (2006) also used phothothermal therapy method for selective killing of MRSA by targeting the bacterial cells based on gold nanoparticles conjugated with anti protein A antibody *in vitro*. They reported the bacterial damages at different laser fluencies. The similar results were achieved in our study too. It has been suggested that numerous factors, including nanoparticle explosion, shock waves, thermal disintegration and, especially, bubble-formation phenomenon around GNRs may potentially cause physical damages to the bacteria (Zharov *et al*., 2006; Norman *et al*., 2008).

The results of biodistribution study demonstrated the accumulation of the conjugate in the infected muscle in comparison with normal muscle showing the specific targeting of the new nanodrug. The biodistribution study revealed that the best time for photothermal therapy *in vivo* was 8 h after the injection of the conjugate. Consequently, the *in vivo* photothermal ablation of the bacteria was studied 8 h following the conjugate injection and illumination with NIR laser focused at the infected site in mouse model. Counting of live colonies confirmed the results of *in vitro* laser treatment ($P < 0.05$). Overall, exposing the targeted MRSA to NIR radiation resulted in a significant decrease in cell viability compared to unexposed bacteria. Quantification of these results was represented graphically as percent cell viability in Fig. 7. Counting of live versus dead cells showed that MRSA cells treated with PEGylate GNRs alone and laser alone have approximately 76 and 100% cell viability, respectively. However, following NIR exposure of GNRs selectively attached to the bacteria, there was seen a 73% decrease in cell viability. This approach may also be suitable for killing of gram-positive and gram negative bacteria especially antibiotic-resistant bacterial strains and may be applicable as a therapeutic strategy for infections caused by other types of bacteria, or potentially viral infections (Zharov *et al*., 2006).

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