

Photothermal killing of *Staphylococcus aureus* using antibody-targeted gold nanoparticles

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Purpose: The continued emergence of multidrug resistant bacterial infections and the decline in discovery of new antibiotics are major challenges for health care throughout the world. This situation has heightened the need for novel antimicrobial therapies as alternatives to traditional antibiotics. The combination of metallic nanoparticles and laser exposure has been proposed as a strategy to induce physical damage to bacteria, regardless of antibiotic sensitivity. The purpose of this study was to test the antibacterial effect of antibody-targeted gold nanoparticles combined with pulsed laser irradiation.

Methods: Gold nanoparticles conjugated to antibodies specific to *Staphylococcus aureus* peptidoglycan were incubated with suspensions of methicillin-resistant and methicillin-sensitive *S. aureus* (MRSA and MSSA). Bacterial suspensions were then exposed to 8 ns pulsed laser irradiation at a wavelength of 532 nm and fluences ranging from 1 to 5 J/cm². Viability of the bacteria following laser exposure was determined using colony forming unit assays. Scanning electron microscopy was used to confirm the binding of nanoparticles to bacteria and the presence of cellular damage.

Results: The laser-activated nanoparticle treatment reduced the surviving population to 31% of control in the MSSA population, while the survival in the MRSA population was reduced to 58% of control. Significant decreases in bacterial viability occurred when the laser fluence exceeded 1 J/cm², and this effect was linear from 0 to 5 J/cm² ($r^2=0.97$). Significantly less bactericidal effect was observed for nonfunctionalized nanoparticles or functionalized nanoparticles without laser activation.

Conclusion: Laser-activated nanoparticles targeted to *S. aureus* surface antigens significantly reduced the percentage of viable organisms and represents a promising new treatment modality that could be used either alone or as an adjunct to existing, conventional antibiotic therapy.

Keywords: MRSA, bacteria, pulsed laser, nanospheres, photoacoustic

Introduction

The development of multidrug resistance in pathogenic bacteria is a significant public health risk across the world. Methicillin-resistant *Staphylococcus aureus* (MRSA) has become one of the pathogens of greatest concern due to its ability to cause a wide range of infections ranging from localized skin conditions to life threatening pneumonia and sepsis and its high prevalence in hospital- and community-associated settings.¹ Skin and soft tissue infections (SSTIs) are the most common manifestation of MRSA infection in the community setting.² Recent studies found that MRSA now accounts for 59% of SSTIs presenting to emergency departments in the US,³ and the national cost associated with community-acquired MRSA SSTIs ranges from \$108 to \$343 million annually.⁴ The emergence of community-acquired MRSA infections over the last decade has been notable because those affected are typically young, healthy individuals without any apparent risk factors,² the infecting strains tend to be more

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virulent than nosocomial isolates,⁵ and it correlates with a coincident increase in the total number of hospitalizations in the affected patients.⁶ Current trends indicate the expanding reservoir of MRSA in the community is likely to become a source for recurrent transmission into hospitals where it would put many more patients at risk for developing highly virulent and multidrug resistant infections.⁷ Due to the steady decrease in the rate of new antibiotics reaching the market, an urgent need exists for the development of alternate therapeutic approaches.

One potential strategy for circumventing multidrug resistance mechanisms that has gained interest in recent years is the use of light-based therapies to induce chemical or physical damage to the bacteria.^{8–13} While photodynamic therapy involving use of photosensitizing dyes has been widely shown to be effective at killing bacteria via generation of reactive oxygen species,¹³ use of metallic nanomaterials as photoabsorbers offers many advantages over this approach. Gold nanoparticles (GNPs) in particular are viewed as a promising platform for light-based therapies because they are predicted to have 4–5 orders of magnitude higher energy absorption and greater photostability than conventional photosensitizing dyes.¹⁴ Additional advantages of GNPs include ease of synthesis, straightforward conjugation to a variety of targeting molecules, ability to tune the optical properties to absorb at specific wavelengths, and utility in multimodal applications such as simultaneous imaging and treatment.^{14,15} Importantly, gold nanomaterials are also considered to exhibit relatively good biocompatibility, and therapies involving use of GNPs are currently undergoing testing in clinical trials.¹⁶

Previous studies have shown that use of GNPs with continuous wave or pulsed laser irradiation can significantly decrease the viability of several types of bacteria via photothermal cell lysis.^{9–12,17,18} Zharov et al¹⁰ proposed that the precision of microbial killing could be maximized and collateral host tissue damage minimized by combining nanomaterials functionalized with antibodies against specific bacterial cell wall components and nanosecond pulsed laser exposure. The antibody increases the specificity of nanoparticle binding, thereby targeting the thermally induced damage to the vicinity of the bacterial surface. Similarly, use of short laser pulses, compared to continuous wave irradiation, allows less time for heat diffusion to surrounding host tissue during exposure for more localized bacterial damage and reduced nonspecific damage to normal tissue; this may also allow integration of detection through photoacoustic or other mechanisms into the

treatment platform.^{8,10,19} Using this approach, one research group reported a $\geq 95\%$ killing of a methicillin-sensitive strain of *S. aureus*, in vitro, using laser pulses at 532 nm and a two-step nanoparticle targeting strategy involving binding of primary antibodies to the bacteria followed by treatment with secondary antibodies conjugated with gold nanospheres.¹⁰ The purpose of the current study was to test the antibacterial effect of this laser-induced photothermal technique against both methicillin-sensitive and methicillin-resistant *S. aureus* using a simplified, one-step method for targeting of the GNPs to the bacteria.

Materials and methods

Growth of bacterial cultures

Methicillin-sensitive *Staphylococcus aureus* (MSSA; ATCC[®] 29213[™]) and methicillin-resistant *S. aureus* (MRSA; ATCC[®] 33591[™]) strains of *S. aureus* were obtained from the American Type Culture Collection (Manassas, VA, USA). Most of the experiments were conducted using only the MSSA strain to simplify sample handling and for experimental convenience. MSSA and MRSA cultures were grown aerobically in tryptic soy broth or nutrient broth (BD, Franklin Lakes, NJ, USA), respectively, on a shaking incubator at 250 rpm and 37°C to an OD₆₀₀ of 0.5–0.7.

Analysis of antibody binding to MSSA and MRSA

Cultures were centrifuged at 5,000× *g* for 5 minutes at room temperature and the bacterial pellets were washed and resuspended in phosphate buffered saline (PBS; Gibco, Grand Island, NY, USA) with 0.1% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO, USA). Biotinylated monoclonal antibodies specific to *S. aureus* peptidoglycan (1.3 mg/mL, Clone 702, Catalog Number BM3066B; Acris Antibodies, San Diego, CA, USA) were added to the samples at a 1:100 volumetric ratio. Control samples were prepared for both bacterial strains as described above, except no antibody was added. All samples were incubated for 1 hour at room temperature on an orbital mixer, then pelleted via centrifugation. Bacteria were washed twice using PBS with 0.1% BSA and resuspended in the original volume in PBS containing 0.1% BSA, 1% goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA), and Alexa Fluor[®] 488-AffiniPure Goat Anti-Mouse IgG (1.5 mg/mL, Catalog Number 115-545-146; Jackson ImmunoResearch Laboratories, Inc.) at a 1:400 dilution. After incubation for 1 hour at room temperature on an orbital mixer, samples were centrifuged, washed three times with PBS, and resuspended

in PBS. Six aliquots (100 μL) of each sample were placed into individual wells of a 96-well microtiter plate. A Synergy HT Microplate Reader (BioTek, Winooski, VT, USA) was used to read OD_{630} for the amount of bacteria and fluorescence at excitation/emission settings of 485/528 nm for the amount of bound antibody. The fluorescent signal was divided by the OD_{630} reading to standardize for the amount of bacteria in each sample. Standardized values for MSSA and MRSA from three independent experiments were compared using a Student's two-tailed *t*-test (STATISTICA[®], v10; StatSoft, Inc., Tulsa, OK, USA), with $P < 0.05$ considered significant.

Preparation of functionalized gold nanoparticles

Sterile-filtered 40 nm gold nanospheres coated with streptavidin were obtained commercially (15 OD; BioAssay Works, Ijamsville, MD, USA). Aliquots containing approximately 6.5×10^{10} nanoparticles were diluted with 1 mL of PBS containing 0.1% BSA and 10% glycerol (Sigma-Aldrich; vehicle containing PBS, BSA, and glycerol hereafter referred to as PBG) and centrifuged at $7,400 \times g$ for 10 minutes at room temperature to remove the original vehicle. The supernatant was removed, the pellet was suspended in 0.5 mL of PBG, and biotinylated monoclonal antibodies specific to *S. aureus* peptidoglycan (Acris Antibodies) were added at a 1:100 volumetric ratio. Pilot testing using different volumetric ratios from 1:50 to 1:50,000 indicated 1:100 was the lowest antibody concentration required to provide maximal bacterial killing (data not shown). Nonfunctionalized gold nanospheres were prepared as described above for functionalized particles except antibody was not added. All samples were incubated for 1 hour at room temperature on an orbital mixer.

Laser-induced photothermal killing of bacteria

Bacterial cultures were centrifuged at $5,000 \times g$ for 5 minutes at room temperature, the supernatant was removed, and the bacterial pellets were suspended in half the original culture volume in PBG. Tubes containing either the functionalized or nonfunctionalized GNPs were removed from the orbital shaker and 0.5 mL of the bacterial suspension was added to each tube. For vehicle control samples, 0.5 mL of the bacterial suspension in PBG was added to a tube containing 0.5 mL of PBG without any nanoparticles. All tubes were then returned to the orbital mixer for 90 minutes of incubation at room temperature. Pilot experiments indicated antibody alone

had no significant effect on MSSA colony forming ability (data not shown), and therefore this experimental group was not included in subsequent testing.

Laser exposures were performed using an Nd:YAG Q-switched laser (model CRF400; Big Sky/Quantel, Bozeman, MT, USA) with a wavelength of 532 nm, an 8 ns pulse duration, and a pulse repetition rate of 1 Hz. The optical system included a 250 mm focal length lens and a variable aperture arranged to provide a 2 mm diameter beam with a maximum energy density of approximately $5 \text{ J/cm}^2/\text{pulse}$.¹⁰ The approximate size and alignment of the beam was confirmed using ZAP-IT[®] paper (Zap-It Corporation, Salisbury, NH, USA). Neutral density filters were placed in the beam path to reduce the pulse energy in the experiments conducted to test the effect of laser fluence on bacterial killing. A Nova II Laser Energy Meter with a Pyroelectric Energy Sensor (model PE25BF-DIF-C) and StarLab 2.0 software (all from Newport, Irvine, CA, USA) were used to measure and record the pulse energies for each experiment. Laser dosimetry was performed immediately pre- and postexposure by recording the energy of ten successive pulses. These pre- and postexposure values were combined to calculate the mean exposure energy, which was used to calculate laser fluence (energy per unit area) assuming a 2 mm beam diameter.

Triplicate or quadruplicate 75 μL aliquots from each bacterial sample were exposed in quartz cuvettes with a 2 mm wide window and 10 mm light path (Precision Cells, Farmingdale, NY, USA). During exposures, cuvettes were placed in a holder with black side walls to minimize light scatter while allowing the laser beam to pass through the front and back windows. Vehicle controls and bacteria treated with GNPs alone were transferred to cuvettes and subjected to sham exposure for the approximate duration of laser treatment without laser activation. After irradiation with 100 pulses or sham exposure, the aliquots were gently mixed by pipetting, transferred to fresh tubes, serially diluted in PBS, and plated in triplicate on tryptic soy agar. Bacterial colonies were counted manually after overnight incubation at 37°C . Data in graphs are expressed as percent survival compared to the vehicle controls and represent the mean and standard deviation (SD) of three to six independent experiments. Statistical analysis of results was conducted using repeated measures analysis of variance (ANOVA) tests followed by post hoc Tukey or Dunnett tests (STATISTICA[®]; StatSoft, Inc.), with $P < 0.05$ considered significant.²⁰ Linear regression was performed using SigmaPlot[™] (v10.0; Systat Software, Inc., San Jose, CA, USA).

Scanning electron microscopy

Selected samples of MSSA were collected on 0.22 μm filters immediately after sham or laser exposure. The bacteria on the filters were washed once in PBS and twice in double-distilled H_2O , fixed in 2.5% glutaraldehyde in PBS, and then dehydrated through a series of increasing alcohol concentrations. After mounting the filters on specimen stubs, samples were coated using a Hummer 6.2 Sputter Coater (Anatech USA, Union City, CA, USA) with a gold–palladium (50%–50%) target and imaged using a Sigma-VP40 field emission scanning electron microscope (Carl Zeiss Inc., Jena, Germany) in high vacuum mode.

Results and discussion

Targeted photothermal destruction of MSSA

Figure 1 shows the results of testing the antibacterial effect of nonfunctionalized or antibody-functionalized GNPs either alone or in combination with 532 nm pulsed laser irradiation against MSSA. Exposure of bacteria to nonfunctionalized or functionalized GNPs without laser treatment resulted in $88\% \pm 8\%$ and $79\% \pm 5\%$ (mean \pm SD, $n=4$) survival, respectively, relative to the vehicle control group. When laser irradiation was combined with nonfunctionalized GNPs, viability remained at $75\% \pm 7\%$ of the controls and was not significantly different from survival observed in the two experimental groups treated with nanoparticles alone. In contrast, use of GNPs coated with anti-*S. aureus* antibodies with pulsed laser

exposure decreased viability to $36\% \pm 7\%$ of controls, and this was significantly less than survival in the controls and other three treatment groups ($P=0.0002$). Though the combination of nonfunctionalized GNPs and laser exposure caused a slight but significant reduction in viability compared to the control group, the results illustrate the positive influence of the targeting antibody on bacterial killing and are in agreement with prior reports showing use of a targeting moiety was necessary to induce pulsed laser-induced photothermal killing in bacteria and mammalian cells.^{10,15}

Selected MSSA samples were collected on filters for scanning electron microscopy (SEM) imaging to visually confirm attachment of antibody-functionalized GNPs to the bacteria and determine the presence of morphological changes indicative of laser-induced photothermal damage to the cells. Backscattered electron images of MSSA incubated with vehicle alone as the control, nonfunctionalized GNPs, or antibody-functionalized GNPs are shown in Figure 2A. In MSSA samples treated with nonfunctionalized GNPs, almost all of the visible particles, which show up as bright white dots, appear on the collection filter rather than in close contact with the bacteria. In contrast, a much greater number of GNPs appear attached to the bacteria when functionalized with the anti-*S. aureus* antibody. These findings are in line with the results reported in Figure 1 and further support the hypothesis of a highly localized photothermal effect generated by the pulsed laser around the nanoparticles, necessitating close contact between the GNPs and bacteria for optimal killing.

Secondary electron images of MSSA incubated with functionalized GNPs followed by sham or pulsed laser exposure are shown in Figure 2B. Sham exposed samples appear as typical clumps of cocci with GNPs attached individually or as clusters, whereas laser exposed samples indicate the presence of damaged bacteria that appear as shrunken or flattened cells. Mechanisms of pulsed laser-induced photothermal damage to bacteria may involve generation of acoustic and shock waves, bubble formation, thermal injury, and melting or fragmentation of the nanoparticles.^{10,18,19} Not all of the bacteria showed overt signs of damage after laser exposure, including some that had nanoparticles attached to the surface. It is possible that the typical growth pattern of *S. aureus* as clumps may shield some of the bacteria from the incident laser energy or binding of a sufficient number of GNPs to induce complete killing.

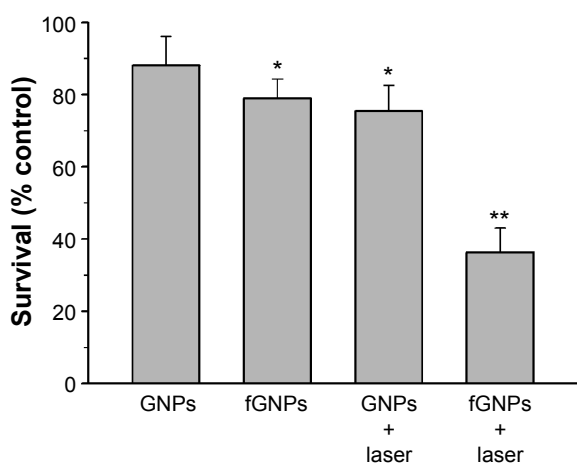


Figure 1 Targeted killing of MSSA using antibody-functionalized GNPs combined with pulsed laser irradiation at 532 nm.

Notes: Laser irradiation consisted of 100 pulses at 5 J/cm^2 . Bacterial survival was determined by colony forming unit assays. Survival in the vehicle control group, which did not receive nanoparticles or laser treatment, was set to 100%. Values represent the mean \pm standard deviation of four independent experiments. * $P \leq 0.005$ compared to the control group and ** $P=0.0002$ compared to controls and the other three treatment groups as determined by repeated measures ANOVA followed by post hoc Tukey test.

Abbreviations: ANOVA, analysis of variance; MSSA, methicillin-sensitive *Staphylococcus aureus*; GNPs, gold nanoparticles; fGNPs, functionalized gold nanoparticles.

Effect of laser fluence on photothermal destruction of MSSA

The antibacterial effect of targeted GNPs and pulsed laser irradiation was tested at varying laser fluences from 0 to 5 J/cm^2

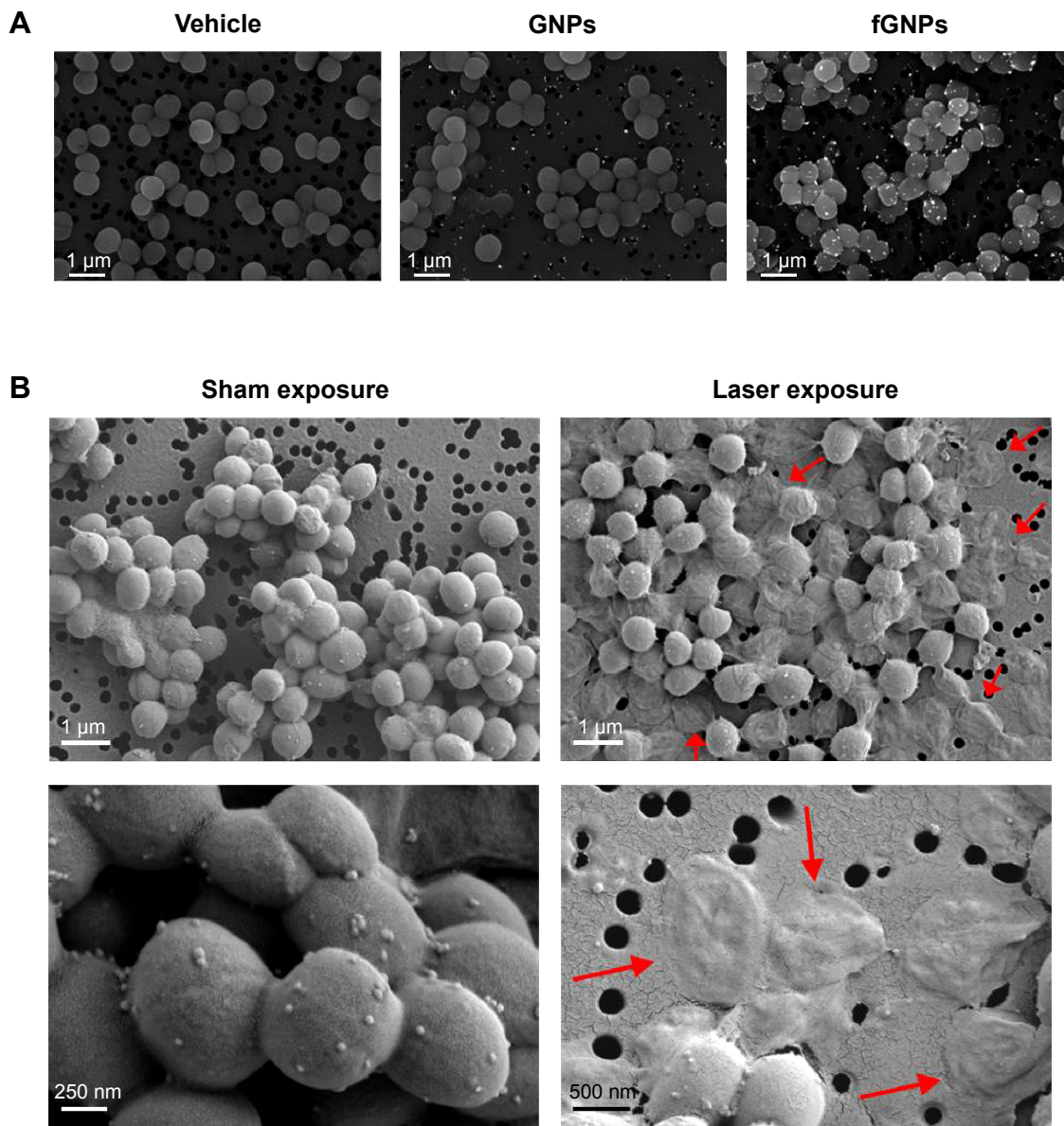


Figure 2 SEM images of MSSA.

Notes: (A) Backscattered electron images of MSSA treated with PBG vehicle, nonfunctionalized GNPs, or fGNPs. All images are at 25,000× magnification. Scale bars = 1 μm. (B) Secondary electron images showing MSSA treated with fGNPs then sham exposed (left column) or exposed to pulsed laser irradiation at 532 nm (right column). Red arrows indicate damaged bacterial cells. Top two images at 25,000×, bottom left image at 100,000×, bottom right image at 65,000× magnification.

Abbreviations: SEM, scanning electron microscopy; MSSA, methicillin-sensitive *Staphylococcus aureus*; PBG, phosphate buffered saline with bovine serum albumin and glycerol; GNPs, gold nanoparticles; fGNPs, functionalized gold nanoparticles.

to characterize the dose–response relationship (Figure 3). Survival in the 0 J/cm² (sham exposed) group was 86%±20% (mean ± SD, n=6) compared to the vehicle controls, and increasing the laser dose from 1 to 5 J/cm² resulted in decreasing levels of viability from 75%±11% to 31%±8%. Bacteria exposed to 2, 3, or 5 J/cm² had significantly lower survival rates compared to the 0 J/cm² group, whereas viability in the bacteria exposed to 1 J/cm² did not differ significantly from the sham exposed group. Regression analysis of the data revealed a linear relationship

between photothermal killing and laser fluence with an r^2 value of 0.97.

Overall, the data show a similar pattern of decreasing viability as that previously published for MSSA exposed to targeted GNPs and nanosecond pulsed laser irradiation at 532 nm from 0 to 5 J/cm².¹⁰ The prior report showed ≤10% bacterial survival for laser fluences at or above 3 J/cm², which is somewhat lower than that achieved here. This disparity may be due to use of different strains of *S. aureus* in the two investigations or use of a one-step approach

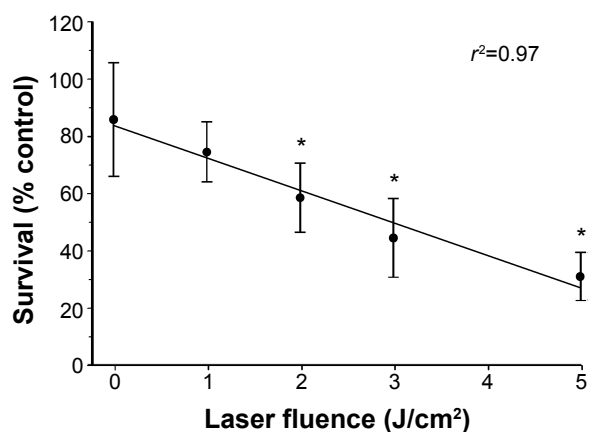


Figure 3 Linear relationship between laser-induced photothermal killing and laser fluence in MSSA.

Notes: MSSA samples were incubated with 40 nm gold nanospheres coated with anti-*S. aureus* antibodies then exposed to 100 laser pulses at varying laser fluences. Bacterial survival was determined by colony forming unit assays. The vehicle control group, which did not receive nanoparticles or laser treatment, was set to 100% survival. Values are expressed as mean \pm standard deviation of six independent experiments, and the fit line and associated r^2 value were derived from linear regression analysis. * $P \leq 0.00003$ compared to the 0 J/cm² group as determined by repeated measures ANOVA followed by post hoc Dunnett test.

Abbreviation: ANOVA, analysis of variance; MSSA, methicillin-sensitive *Staphylococcus aureus*.

for targeting the GNPs to the bacteria in the present study instead of the two-step method utilized previously. In addition, antibodies specific to peptidoglycan were employed in the current investigation, rather than anti-protein A antibodies as used in the earlier study, because growth phase- and *S. aureus* strain-dependent variations in protein A expression may affect efficacy of antibody-targeted antimicrobial therapies.²¹

Efficacy of photothermal killing against MSSA and MRSA

The efficacy of the pulsed laser-induced photothermal treatment was tested against an MRSA strain in addition to the MSSA strain used in the above experiments. As shown in Figure 4, laser exposure alone did not induce a significant reduction in viability in either strain compared to the vehicle control group. Incubation with functionalized GNPs alone did not significantly affect viability of the MRSA strain (Figure 4B) but decreased survival to 59% \pm 11% (mean \pm SD, n=3) in the MSSA strain, though this was of borderline statistical significance with $P=0.04$. The combination of functionalized GNPs and pulsed laser exposure, however, caused a significant reduction in survival to 31% \pm 8% and 58% \pm 10%, respectively, in the MSSA and MRSA strains. Though comparison of results from the two strains indicate a potential difference in sensitivity, the data from MSSA and MRSA were not compared statistically because testing for MSSA and MRSA was conducted in separate experimental runs.

Differential sensitivity of these two bacterial strains could be due to multiple factors including variations in the epitope to which the antibody binds and other structural characteristics affecting the inherent sensitivity of the cells to photothermal effects. Increased cell wall thickness, for example, has been associated with antibiotic resistance in *S. aureus*^{22,23} and could decrease susceptibility of MRSA to photoacoustic damage. In addition, variations in surface antigenicity of MSSA and MRSA^{24,25} could influence the affinity of the

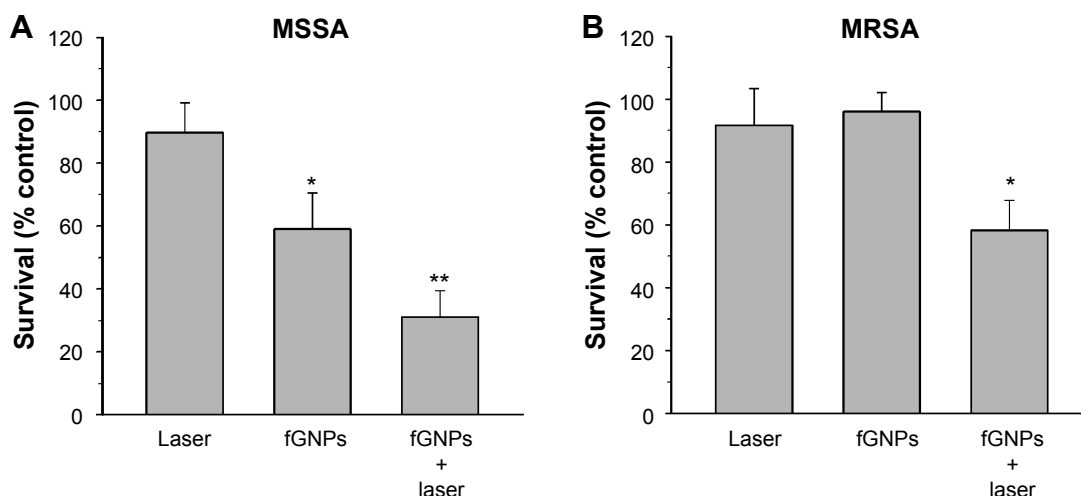


Figure 4 Antibacterial effect of targeted gold nanoparticles and pulsed laser irradiation at 532 nm against (A) MSSA and (B) MRSA.

Notes: Bacterial samples were incubated with 40 nm gold nanospheres coated with anti-*S. aureus* antibodies then exposed to 100 laser pulses at 5 J/cm². Bacterial survival was determined by colony forming unit assays. The vehicle control group, which did not receive gold nanoparticles or laser treatment, was set to 100% survival. Values are expressed as mean \pm standard deviation of three independent experiments. Statistical significance was determined using repeated measures ANOVA tests and post hoc Tukey tests. (A) * $P=0.04$ compared to the controls and ** $P \leq 0.005$ compared to the controls and laser alone group. (B) * $P \leq 0.01$ compared to the controls and the other two treatment groups.

Abbreviations: ANOVA, analysis of variance; MSSA, methicillin-sensitive *Staphylococcus aureus*; MRSA, methicillin-resistant *Staphylococcus aureus*; fGNPs, functionalized gold nanoparticles.

targeting antibody to the individual strains leading to differences in sensitivity. With this possibility in mind, binding of the biotinylated anti-*S. aureus* primary antibody to the MSSA and MRSA strains was tested using a fluorescently labeled anti-mouse secondary antibody in a microtiter plate assay. Values for fluorescence intensity standardized to OD₆₃₀ were 249,366±37,294 and 276,922±16,189 (mean ± SD, n=3) for MRSA and MSSA, respectively, and were not significantly different ($P=0.31$). These results indicate that differences in the level of binding of the antibody to the bacteria do not account for the variation in killing between strains observed in this study. However, further testing using a larger panel of bacterial strains and antibodies against other surface antigens is required to determine if true variations exist in the efficacy of the laser-induced photothermal treatment against different strains of *S. aureus*.

Conclusion

In conclusion, our findings support the use of laser-activated, functionalized GNPs as an adjunct therapy for *S. aureus* infections. The treatment can be targeted to specific organisms, and the bactericidal effect is linearly related to the laser fluence. While not completely eliminating all viable bacteria, at least in the treatment's present form, this novel therapy has a potential application as an adjunct to conventional therapy for reducing the infectious load in a lesion, thus facilitating the complete eradication with the concomitant administration of a suitable antibiotic. Because the systemic elimination of nanoparticles is incompletely understood at present,²⁶ this therapy is most suited for treatment of cutaneous infections, in which the subsequent removal of the particles following the treatment is not a serious concern. Finally, the efficacy of the nanoparticle therapy may likely be optimized in future studies by adjusting the size and shape of the nanoparticles and the laser parameters to regulate the nature of the induced shock waves, the extent of thermal diffusion from the metal particle, and laser-tissue interactions. It may also be possible to improve the therapy by modifying the particular bacterial antigen targeted by the functionalizing ligand on the nanoparticle.

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Disclosure

The authors report no conflicts of interest in this work.

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