Laser thermal ablation of multidrug-resistant bacteria using functionalized gold nanoparticles

Abstract: The issue of multidrug resistance (MDR) has become an increasing threat to public health. One alternative strategy against MDR bacteria would be to construct therapeutic vectors capable of physically damaging these microorganisms. Gold nanoparticles hold great promise for the development of such therapeutic agents, since the nanoparticles exhibit impressive properties, of which the most important is the ability to convert light into heat. This property has scientific significance since is exploited to develop nano-photothermal vectors to destroy bacteria at a molecular level. The present paper summarizes the latest advancements in the field of nanotargeted laser hyperthermia of MDR bacteria mediated by gold nanoparticles.

Keywords: bacteria, photo-thermal ablation, gold nanoparticles, antibiotic resistance

Introduction
The problem of multidrug resistance (MDR) has become a growing threat to public health. A high number of infections and many deaths that occur each year in the USA are caused by bacteria that resist multiple antibiotics. Another issue is the rejection of certain drugs, a recent trend that has intensified the urgency of developing new therapeutic agents and approaches. Current studies are investigating the possible benefits of natural products, the changes that might be brought to certain classes of therapeutic agents and approaches. Current studies are investigating the possible benefits of natural products, the changes that might be brought to certain classes of therapeutic agents and approaches.
representing a novel and competent substitute for effective transportation and translocation of therapeutic molecules.\textsuperscript{15–22} Various types of GNP complexes (protein-coated GNP s, peptide-coated GNP s, GNP s coated with nucleic acids, drugs) are available for delivery to cells and organs.

One of the most remarkable properties of GNP s is the ability to transform light into heat under laser irradiation.\textsuperscript{2,6,18,23–26} This property is significant because it can be exploited to develop nano-photothermal vectors in order to destroy bacteria at a molecular level. The present paper summarizes the latest advancements in the field of nanotargeted laser hyperthermia of MDR bacteria mediated by GNP s (Table 1).

### Nano-photothermal therapy mediated by GNP s in MDR Staphylococcus aureus

**Gold nanoparticles**

Light-absorbing GNP s have been functionalized with specific antibodies to achieve targeted and selective laser bacterial killing.\textsuperscript{27} This therapeutic intervention was assessed in real time using photothermal microscopy. The antibacterial effect was caused by the combination of strong overheating in the laser impact zone and the generation of bubbles around gold clusters. These results were confirmed by photothermal imaging and time-resolved imaging. The technology was further employed to selectively kill \textit{S. aureus} by means of targeted delivery of 10, 20, and 40 nm GNP s functionalized with anti-protein A antibodies. Following exposure to laser pulses (420–570 nm, 12 ns, 0.1–5 J/cm\textsuperscript{2}, 100 pulses), several techniques such as electron microscopy, optical transmission, and viability testing assessed laser killing of bacteria for different laser fluences and nanoparticle size ranges.\textsuperscript{27}

In a study testing the antibacterial effect resulting from the association between antibody-targeted GNP s and pulsed laser irradiation,\textsuperscript{28} GNP s were functionalized with \textit{S. aureus}-specific antibodies, followed by methicillin-resistant \textit{S. aureus} (MRSA) and methicillin-sensitive \textit{S. aureus} incubation. Suspensions were further irradiated with a pulsed laser (8 ns, 532 nm, fluence range 1–5 J/cm\textsuperscript{2}). The change in effect caused by this type of exposure was assessed at fluences ranging from 0 to 5 J/cm\textsuperscript{2}. The results obtained in the group exposed to fluences of 0 J/cm\textsuperscript{2} showed a survival rate of 86%±20% (mean ± SD, n=6) compared to controls, with a decrease in survival from 75%±11% to 31%±8% in subjects exposed to fluences of 5 J/cm\textsuperscript{2}. After exposure to 2, 3, and 5 J/cm\textsuperscript{2}, there was a significant decrease in survival when compared to the first group, while differences were not as considerable for fluences of 1 J/cm\textsuperscript{2}. Estimations made using regression analysis indicated evidence of a linear relationship between the destruction of drug-resistant bacteria by photothermal therapy and laser beam fluence ($r^2=0.97$). The use of ligand-free nanoparticles or the absence of laser irradiation led to a considerably lower antibacterial effect (Figure 1).

### Gold nanocages

\textit{S. aureus} was used as a proof-of-principle ESKAPE (\textit{Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species}) pathogen in a recent study\textsuperscript{29} to show that the loading of the correct antibiotic, such as daptomycin, into the polydopamine coating based on gold nanocages (AuNC@PDA) – where the strategy adopted for their synthesis was galvanic replacement – resulted in an outer edge length of 55±5 nm and an inner edge length of 38±5 nm. Another finding was that the conjugation of these complexes to antibodies targeting \textit{S. aureus} surface protein A can selectively and directly deliver the nanoscale constructs onto the bacterial cell surfaces, as seen in Figure 1. The fact that there was no binding in mammalian cells helped confirm target specificity. The effect of 808 nm diode laser irradiation on AuNC@PDA (200 μL, 0.04–0.4 nM) was represented by colony forming units (CFU) reduction below detection limits (20 CFU/well) at 0 hour. However, 24 hours after treatment, the cells were able to rebound above baseline. On the other hand, there was a decrease in bacterial cell viability below detection limits at 0 and 24 hours after laser irradiation of

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4 μg/mL AuNC@DapLo/PDA (polydopamine-coated gold nanocages). There was also evidence of a decrease in the antibacterial effects of antibiotics in the presence of unconjugated aSpa depending on the concentration of AuNC@Dap/PDA–aSpa nanoscale constructs. Therefore, the authors were able to confirm target specificity and greater synergistic antibacterial efficiency by means of bacterial cell surface localization of AuNC@Dap/PDA–aSpa instead of the more abstract effects of microtiter plate-based antibacterial assays.

**Gold nanorods**

An agent that can detect MRSA and selectively destroy it when used in combination with photothermal therapy was developed in another study using gold nanorods (GNRs) and an anti-protein A antibody. The authors synthesized GNRs functionalized with polystyrene sulfonate, binding anti-protein A antibody to their surface. The preparation of the final complex was confirmed by Fourier transform infrared spectroscopy and ultraviolet–visible spectra. After the colonies were counted, the results indicated greater cell death (82%) in groups undergoing treatment with the experimental nanoconjugate in association with photothermal therapy than in others ($P<0.05$). After treatment with free GNRs combined with near-infrared (NIR) laser therapy, bacterial cell death (16%) was not significantly greater than in controls ($P>0.05$). Moreover, no cell death occurred when using NIR laser therapy alone. The extent to which the GNRs conjugated to anti-protein A antibody were absorbed in the vital organs

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**Figure 1** Scanning electron microscope images of MSSA.

**Notes:** (A) Backscattered electron images of MSSA treated with PBG vehicle, nonfunctionalized GNPs, or fGNPs. (B) Secondary electron images showing MSSA treated with fGNPs and then sham exposed or exposed to pulsed laser irradiation at 532 nm. Red arrows indicate damaged bacterial cells. Copyright © 2015. Dove Medical Press. Reproduced from Millenbaugh NJ, Baskin JB, DeSilva MN, Elliot WR, Glickman RD. Photothermal killing of Staphylococcus aureus using antibody-targeted gold nanoparticles. *Int J Nanomedicine*. 2015;10:1953–1960.

**Abbreviations:** fGNPs, functionalized GNPs; GNPs, gold nanoparticles; MSSA, methicillin-sensitive Staphylococcus aureus; PBG, phosphate buffered saline containing 1% bovine serum albumin and 10% glycerol.
of infected BALB/c mice at time intervals of 1, 4, 8, and 24 hours following intravenous inoculation was expressed as the percentage of injected dose per gram of organ (% ID/g). Outcomes revealed that the nanocomplex accumulated at the site of infection to a greater extent than in the respective healthy tissues and organs at all time intervals and especially, at 8 hours after inoculation (P<0.05). A considerable decrease in bacterial viability (73%) was observed in the in vivo murine models following photothermal therapy.30

GNRs conjugated to toluidine blue photosensitizer (Au-anionic poly(acrylic acid)-toluidine blue) showed its duality in a study conducted by Kuo et al on MRSA, determining both antimicrobial photodynamic inactivation and hyperthermia.31 Following treatment with the above-mentioned conjugate and sequential exposure to helium–neon laser (633 nm, 1 minute) and continuous wave laser (808 nm, 25 minutes), there was a significant reduction in MRSA viability (to about 5%) and a 10-fold increase in antimicrobial activity, compared to photodynamic antimicrobial chemotherapy or hyperthermia alone. There was no bacterial damage in the absence of nanorods after both types of laser therapies, but there was a decrease in viability to 59% and 53% following treatment with nanorods.31

Effective biofilm disinfection was obtained in another study32 using a GNR cluster that is easily excited by NIR laser irradiation, which generates heat rapidly through photothermal conversion. Wet chemistry was used for the synthesis of GNRs, and photolithography was used in the microfabrication of one GNR cluster. After being directly applied to the biofilm, the influence of the photothermal effect of the GNR cluster on the biofilm was assessed both prior to laser irradiation and after treatment, which showed a significant decrease in cell viability and biofilm thickness. As indicated by analysis of bacterial mortality rates and biofilm inactivation, greater efficacy was observed for longer exposures to laser irradiation. Clear morphologic damage was indicated by scanning electron microscopy images of bacteria following irradiation, including bacterial cell membrane rupture.

Core–shell gold–silver nanoparticles
Aspartame-stabilized gold–silver nanostructures (AuNP@Ag@Asm) with core–shell architecture were developed in an attempt to associate the antimicrobial activity of silver with the thermosensitive properties of gold (Figure 2).33 Broth microdilution antibacterial assay was used to determine minimum inhibitory concentrations. The experiment was performed on strains of *S. aureus* subsp. *aureus Rosenbach* (ATCC 25923), *Staphylococcus epidermidis* SE19, and *Escherichia coli* CF073. Experiments on 120 identical plates kept in the dark or exposed to LED irradiation (530 nm) and stored at a temperature of 37°C for 18 hours were carried out in parallel to determine the optical density of the bacterial cultures. Experiments were performed with and without irradiation therapy to assess the antibacterial effect of AuNP@Ag@Asm and the photothermal effect of gold. Results showed that bacterial killing was not only incomplete in the dark (even for below-detection values) but was also followed by regrowth. The opposite result was obtained following irradiation. Biocompatibility testing was performed using the MTS cell proliferation colorimetric assay, in which any significant effect on the proliferation of primary dermal fibroblasts following treatment with AuNP@Ag@Asm at different concentrations was not observed.33

**Gold nanostars**
In a recent study,34 monolayers of gold nanostars were synthesized by lauryl sulfobetaine-driven seeded growth method. The anti-biofilm activity was evaluated by preparing 32 round glass slides (1.0 cm in diameter) using localized surface plasmon resonance spectroscopy (802 nm, s =12) and setting the surface concentration of the adsorbate on the gold surface to 3.0 mg/cm² (s =0.4). Experiments were carried out using a strong biofilm producer, an MRSA LP strain. Following laser irradiation, the conjugate exhibited an excellent...
photothermal conversion property in the NIR region. After exposure to 808 nm laser, the monolayers of gold nanostars generated a rise in tumor temperature (hyperthermia), which resulted in the efficient killing of the *S. aureus* biofilm. A class of shape- and size-selective antimicrobial agents was developed in a study conducted by Borovička et al. The experiment consisted of the fabrication of imprinted colloid particles using cell templates by the fragmentation of silica shells. The increased contact area between the target cell and the colloidal imprint led to the mechanism of recognition, which can help develop “colloid antibodies” for efficient shape-selective binding (Figure 3). GNP’s were integrated into the silica shell of the colloid imprint to bind to matching yeast cells, which was followed by direct delivery onto the target cell surface. Laser irradiation of the cell dispersion prompted selective killing of the target cells. The impact of the photothermal colloid antibody on shape-selective recognition and killing of bacteria was also confirmed by a mixture of two differently shaped and differently sized types of bacterial cells.

**Nano-photothermal therapy mediated by GNP’s in MDR *P. aeruginosa***

**Gold nanorods**

Covalent binding of primary antibodies to GNR’s was used for the selective destruction of *P. aeruginosa* (Figure 4). The synthesis of nanorods for bacterial cell wall–targeted delivery was achieved by electrostatic or covalent binding of anti-PA3 primary antibodies to their surfaces. Bacteria were incubated with relevant antibody–nanorod complexes. Evidence of electron-dense GNR-covered bacteria was offered by transmission electron microscopy. Live and dead cell counts using the Live/Dead kit (Invitrogen, Carlsbad, CA, USA) revealed up to 80% cell viability in the case of PA3 cells without the attachment of nanorods or NIR irradiation, NIR-irradiated cells without the attachment of nanorods, and bacteria with attached nanorods and without NIR irradiation. A 75% decrease in cell viability was observed following NIR irradiation of nanorod-coated PA3 cells, along with a considerable increase in the number of dead or compromised cells (*P*<0.05). According to these findings, the functionalization of GNR’s with antibodies and their exposure to NIR light determined the selective targeting of PA3 cells. Therefore, these complexes are able to reduce cell viability to a great extent.

**Gold nanocrosses**

The characterization of high NIR absorbing multibranched gold nanocrosses (~35 nm) was presented by other authors. Antibody-functionalized gold nanocrosses were used for targeting PcrV, a protein involved in the translocation of type III secreted toxins of *P. aeruginosa*. The bacterial suspension of *P. aeruginosa* PAO1 was tested in the presence of the conjugates using confocal fluorescence microscopy to assess the effect produced by the photoexcitation of gold nanocrosses with strong NIR absorption. The experiment consisted of the following steps: the preparation of 500 μL PAO1 bacterial suspension (1×10⁸ cells/mL), followed by incubation with antibody-functionalized gold nanocrosses for 1 hour (final concentration 0.1 mg/mL). Following the washing step, the effect produced by photoexcitation at 800 nm (power density of ≈3.0 W/cm²) for 5 minutes was assessed using confocal microscopy. Total destruction of *P. aeruginosa* was accomplished by the nanocomplex following NIR irradiation therapy, with
the absence of bacterial activity at 48 hours after treatment. This confirms the effective photothermal killing of bacteria and the prevention of bacterial regrowth. These gold nanocross conjugates proved to be successful for effectively targeted and localized photothermal ablation of MDR bacteria, reducing the risk or severity of damage to normal tissue.37

Nano-photothermal therapy mediated by GNPs in MDR Salmonella bacteria

In their study, Lin and Hamme developed an assay for the quick and accurate detection of bacteria by measuring the affinity binding of antibodies, the two-step, seed-mediated synthesis of popcorn-shaped GNPs (GNPop), the use of surface-enhanced Raman scattering, and the introduction of the inductively coupled plasma mass spectrometry analytical technique.38 A qualitative assessment indicated the presence of Salmonella in 10 minutes using Raman spectroscopy, while 100 isolates of Salmonella typhimurium definitive type $10^4$ were detected in 40 minutes in 1 mL (100 CFU/mL) during a quantitative assessment. Concentration-dependent (CFU/mL) laser therapy ($1 \text{ W/cm}^2, 670 \text{ nm}$) indicated the strong relationship between this type of treatment and the killing of Salmonella. Photothermal damage achieved by the conjugation of monoclonal M3038 antibody to GNPop resulted in the killing of nearly 100% of the bacteria ($Salmonella, 10^5 \text{ CFU/mL}$) in 19 minutes. Another finding revealed an almost complete concentration-dependent ($10^2$–$10^7 \text{ CFU/mL}$) bacterial destruction following laser irradiation for 6–30 minutes.38

Monoclonal antibody-conjugated popcorn-shaped gold nanotechnology was employed for selective killing of S. typhimurium DT104 resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline. Following exposure to 670 nm laser (200 mW/cm$^2$), tryptic soy agar was used to assess complete killing of the bacteria.39

Nano-photothermal therapy mediated by GNPs in Enterococcus faecalis MDR bacteria

Another conjugate based on a catheter coating containing gold nanoshells (AuNSs) functionalized with carboxylate-terminated organosulfur compounds was developed to assess the antimicrobial effect against E. faecalis. The gold standards for nanoparticle characterization, transmission electron microscopy, and scanning electron microscopy, were used for imaging the morphology of AuNSs. Energy-dispersive X-ray spectroscopy and X-ray photoelectron spectroscopy were used to detect and map their components and analyze their surface chemistry. Also, ultraviolet–vis spectroscopy and infrared thermography helped quantify the optical and thermal properties of the conjugate. The time–exposure ratio that would cause killing of bacteria subjected to heat
was assessed following treatment with an infrared (810 nm) diode laser using different exposure times. Conversely, the clonogenic assay was used to assess the survival of the bacteria after the inoculation of AuNPs conjugates, in either the presence or absence of NIR radiation. When compared to controls, killing of *E. faecalis* occurred to a greater extent after 5 minutes of treatment with NIR radiation, and this increased even more significantly after 10 minutes. In conclusion, efficient killing of *E. faecalis* adherent to silicone can be induced by irradiated AuNPs conjugates.

In another study, antibody-conjugated oval-shaped gold nanotechnology was employed for selective targeting and killing of pathogenic bacteria. Synthesis of nanoparticles using bacteria followed by NIR radiation induced the photothermal lysis of pathogenic bacteria, which subsequently reduced cell viability.

**Nano-photothermal therapy mediated by GNPs in E. coli MDR bacteria**

GNR-covered kanamycin-loaded hollow SiO$_2$ (HSKAu rod) nanocapsules were developed for drug delivery and photothermal killing of bacteria, demonstrating that the association between a chemical drug, physical sterilization, and the physical damage of biological systems can make the sterilization more effective and deliver the expected results. SiO$_2$ nanocapsules loaded with kanamycin and covered with GNRs were incubated with *E. coli* BL21 and exposed to NIR laser irradiation for 20 minutes. The treatment resulted in an overall rate of sterilization of 53.47%, and 34.49% for the individual components of the hybrid bactericidal material (carrier, chemical sterilization of kanamycin, and physical sterilization induced by NIR laser-excited GNRs). As they proved to accumulate on bacterial surfaces and possess two-photon-induced photoluminescence properties, positively charged gold nanospheres were used against bacteria by laser pulses with a duration recorded on the femtosecond time scale. Whether incubated or not with gold nanospheres, bacterial cells were subjected to femtosecond laser pulse irradiation with a power of 15 mW, at 820 nm, using the raster scan method and 8 ms dwell time. The Live/Dead cell viability assay was used to assess the killing effect triggered by these conjugates. SYTO 9 green fluorescent nucleic acid stain labeled all bacterial cells, while propidium iodide red fluorescent counterstain labeled compromised bacterial cells. When *S. aureus* or *E. coli* was not treated with gold nanospheres, no significant numbers of bacteria exhibited red fluorescence and were considered as dead cells, as the intact bacterial cell wall kept the red fluorescent stain from bursting in. The extent of bacterial killing was not considerable in these bacteria following irradiation. Treatment with gold nanospheres (7.5×10$^{-12}$ M) and radiation therapy enhanced bacterial killing. One treatment was enough to cause 88.8% antibacterial effects against *S. aureus* and 82.6% against *E. coli*.

In another study, the development of a photon-to-thermal conversion nanosystem and exposure of GNRs to laser irradiation produced a quick increase in temperature in the media. Laser-excited GNRs were expected to cause photothermal lysis of the bacteria, which was examined by synthesizing GNRs (aspect ratio 3:4) and determining their optical and thermal properties. Short GNRs proved to absorb laser pulses (808 nm, 450 mW) to a greater extent than long GNRs, and temperature was significantly higher in glass vials. When induced by continuous wave laser irradiation, these nanorods were able to increase the temperature to above 100°C in bulk media; thus, *E. coli* cell lysis was obtained by exposure of the bacteria-containing culture to NIR laser irradiation. Huang et al reported the synthesis of polygonal GNPs with affinity for absorbing NIR light, using a photochemical reaction. Vancomycin, an antibiotic that binds the D-alanine (D-Ala) residues, was attached to the GNPs and the resulting construct was used for growth inhibition of pathogenic bacteria under NIR irradiation at 808 nm. The conjugate was also able to recognize gram-positive and gram-negative cell walls, as indicated by transmission electron microscopy (Figure 5). Nonetheless, the formation of bonds between unmodified GNPs and these bacteria was absent. GNPs were able to destroy >99% of bacteria (*S. aureus, Streptococcus pyogenes, E. coli, MRSA*) in 5 minutes under NIR irradiation as a result of the photothermal effect of this type of therapy.

**Conclusion**

Although proof-of-principle experiments in which laser thermal ablation of MDR bacteria using bio-functionalized nanoparticles are reported in literature, yet, there is a need for further in vitro and in vivo, investigations. The potential for translation to human therapies is high, especially because GNPs exhibit low toxicity and chemical inertia.

This review shows that gold nanomaterials can be successfully used for photothermal treatment, paving the way for future materials with strong and selective biocide effects determined by combining colloid antibodies and strategies that induce cell killing, with possible applications in novel
therapies against MDR bacteria. The new materials based on a chemical drug associated with physical sterilization trigger a synergistic effect and increase the effectiveness of sterilization, and are thus promising candidates for adjuvant antibacterial treatment and disease control.

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Author contributions

All authors contributed equally to this paper; made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

References

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