Targeted laser therapy synergistically enhances efficacy of antibiotics against multi-drug resistant \textit{Staphylococcus aureus} and \textit{Pseudomonas aeruginosa} biofilms

Dickson K. Kirui, PhD, Gregor Weber, PhD, Jennifer Talackine, BS, Nancy J. Millenbaugh, PhD*
Maxillofacial Injury and Disease Department, Naval, Medical Research Unit San Antonio, JBSA-Fort Sam Houston, TX
Revised 7 May 2019

Abstract

The growing prevalence of biofilm-associated multi-drug resistant (MDR) bacteria necessitates the innovation of non-traditional approaches to improve the effectiveness of mainstay antibiotics. Here, we evaluated the use of gold nanoparticle (GNP)-targeted pulsed laser therapy to enhance antibiotic efficacy against in vitro methicillin-resistant \textit{Staphylococcus aureus} (MRSA) and MDR \textit{Pseudomonas aeruginosa} biofilms. Treatment with antibody-conjugated GNPs followed by nanosecond-pulsed laser irradiation at 532 nm (~1.0 J/cm²) dispersed 96–99% of the biofilms relative to controls. GNP-targeted laser therapy combined with gentamicin or amikacin caused a synergistic 4- and 5-log reduction in the viability of MRSA and \textit{P. aeruginosa} biofilms, respectively, whereas GNP-targeted laser therapy or antibiotics alone decreased biofilm viability by only ~1 log. Notably, GNP-targeted laser therapy was able to increase the antibiotic susceptibility of the biofilms to the level of drug sensitivity observed in planktonic MRSA and \textit{P. aeruginosa} cultures, further indicating effective biofilm dispersal via this novel approach.

Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Keywords: Biofilms; Multi-drug resistance; Laser therapy; Targeted gold nanoparticles; Photothermal

The continued emergence of multi-drug resistant (MDR) bacteria is a growing public health concern because it is quickly outpacing the development of new antibiotics and is widely associated with increases in morbidity, mortality, and cost of care.\textsuperscript{1,2} This problem is exacerbated by the propensity of many bacterial strains to form biofilms, which are present in 65%–80% of human infections\textsuperscript{3} and commonly exhibit 100–1000 times lower susceptibilities to antibiotics than planktonic (free-floating) bacteria.\textsuperscript{4} The heightened drug tolerance of biofilms is attributed to lower susceptibilities to antibiotics than planktonic (free-floating) bacteria.\textsuperscript{5} Often, the most effective means of treating biofilm-related infections is by elimination of the biofilm's growth surfaces from wound sites using debridement\textsuperscript{6,9} or removal of medical implants such as catheters and joint prostheses.\textsuperscript{10}

Recent advancements in nanotechnology research has led to the development of cutting-edge nanoparticle-targeted laser therapies that may overcome these challenges via photothermal destruction of the biofilm matrix and resident bacteria. These potential therapies exploit the unique surface plasmon resonance properties of noble metals, particularly gold nanoparticles (GNPs), which strongly absorb energy in the visible light spectrum (10\textsuperscript{4}–10\textsuperscript{5} times greater absorption than traditional photoabsorbing dyes) and release it as thermal energy to the surrounding medium.\textsuperscript{11,12} A notable advantage of this strategy is that it may be effective at eradicating pathogens regardless of their level of antibiotic resistance or metabolic status within biofilms.\textsuperscript{13} In addition, GNPs can be utilized for site-specific delivery of laser therapeutics by attachment of targeting agents (e.g., antibodies and aptamers) to the particle surface, thus minimizing collateral damage to healthy tissue.\textsuperscript{14,15}

Previous investigations have demonstrated that GNP-targeted laser therapies are capable of destroying drug-resistant bacteria in planktonic and biofilm cultures\textsuperscript{16–19} and enhancing the efficacy of antibiotics against biofilms.\textsuperscript{20} However, most of these studies employed continuous wave (CW) irradiation with exposure durations and power densities that may result in bulk
sample temperatures of up to ~50–60 °C. A significant limitation of using CW lasers for therapy, therefore, is the build-up of heat in host tissue to levels that exceed the temperature threshold of 47–50 °C for thermal injury. This risk can possibly be mitigated by using nanosecond (ns)-pulsed laser irradiation, which causes cellular damage via opto-acoustic wave generation and short-duration heating localized to the area near the targeted GNPs (tGNPs). Zharov et al. demonstrated bacterial killing using this technique against in vitro and in vivo planktonic *Staphylococcus aureus* and proposed that the antimicrobial effect was due to generation of intense heating, vapor nanobubbles, and shock waves around the nanoparticles resulting in disruption of bacterial cell walls. However, the effectiveness of GNP-targeted pulsed laser therapy against established biofilms, as would likely be present in topical wound infections, and its ability to enhance antibiotic activity, remain to be fully explored.

In this study, we evaluated the ability of GNP-targeted ns-pulsed laser therapy to eradicate MDR *S. aureus* and *Pseudomonas aeruginosa* biofilms and assessed the benefit of combining this therapy with antibiotics. MDR *S. aureus* and *P. aeruginosa* were selected for this study because these pathogens are leading causes of chronic, biofilm-related infections in U.S. military personnel and civilian populations throughout the world and are associated with delayed wound healing, failure of indwelling medical devices, and increased length of hospital stays.

**Methods**

Additional details of the reagents and materials used in this study are provided in Supplementary Materials.

**Preparation of MRSA and *P. aeruginosa* biofilms**

Two MDR clinical wound isolates, namely, methicillin-resistant *S. aureus* (MRSA) SA5120 and *P. aeruginosa* PA 60–65, were received as gifts from a repository at the U.S. Army Institute of Surgical Research (JBSA-Fort Sam Houston, TX). Biofilms were established biofilms, as would likely be present in topical wound infections, and its ability to enhance antibiotic activity, remain to be fully explored.

In this study, we evaluated the ability of GNP-targeted ns-pulsed laser therapy to eradicate MDR *S. aureus* and *Pseudomonas aeruginosa* biofilms and assessed the benefit of combining this therapy with antibiotics. MDR *S. aureus* and *P. aeruginosa* were selected for this study because these pathogens are leading causes of chronic, biofilm-related infections in U.S. military personnel and civilian populations throughout the world and are associated with delayed wound healing, failure of indwelling medical devices, and increased length of hospital stays.

**Methods**

Additional details of the reagents and materials used in this study are provided in Supplementary Materials.

**Preparation of MRSA and *P. aeruginosa* biofilms**

Two MDR clinical wound isolates, namely, methicillin-resistant *S. aureus* (MRSA) SA5120 and *P. aeruginosa* PA 60–65, were received as gifts from a repository at the U.S. Army Institute of Surgical Research (JBSA-Fort Sam Houston, TX). Biofilms were established biofilms, as would likely be present in topical wound infections, and its ability to enhance antibiotic activity, remain to be fully explored.

In this study, we evaluated the ability of GNP-targeted ns-pulsed laser therapy to eradicate MDR *S. aureus* and *Pseudomonas aeruginosa* biofilms and assessed the benefit of combining this therapy with antibiotics. MDR *S. aureus* and *P. aeruginosa* were selected for this study because these pathogens are leading causes of chronic, biofilm-related infections in U.S. military personnel and civilian populations throughout the world and are associated with delayed wound healing, failure of indwelling medical devices, and increased length of hospital stays.

**Methods**

Additional details of the reagents and materials used in this study are provided in Supplementary Materials.

**Preparation of tGNPs**

Spherical n-hydroxysuccinimide (NHS)-activated GNPs (40 nm) were reconstituted at 7 mg/mL in ethanol, sonicated, and stored at −20 °C until use. GNP-antibody conjugates were prepared against SA5120 and PA 60–65 by mixing the reconstituted NHS-activated GNPs with anti-*S. aureus* antibodies or anti-*P. aeruginosa* antibodies, respectively, in PBS. Reaction mixtures were incubated at room temperature for 2 h with shaking and then centrifuged. The GNP-antibody conjugates were re-suspended in PBS and used as the tGNP stock solution. Spherical 40-nm GNPs containing non-reactive methyl groups were utilized in experiments as non-targeted GNPs. Additional details of this method are provided in Supplementary Materials.

**Determination of optimal laser dosage and tGNP concentration**

MRSA biofilms in 96-well glass-bottom plates or on 5-mm glass discs were pre-treated with increasing concentrations of tGNPs from 0 to 100 μg/mL in PBS for 2 h. This 2-h pre-treatment duration was selected as the optimal tGNP incubation time based upon initial pilot data (Figure S3). Following the tGNP pre-treatment, unattached tGNPs were washed away using PBS, and 40 μL of PBS was added to the wells. Biofilms were then irradiated with increasing numbers of laser pulses from 0 to 100 pulses at 532 nm and a fluence of ~1.0 J/cm² using an Nd:YAG laser system (8-ns pulse, 1 Hz; Quantel, Bozeman, MT) and an automated XY–gantry sample positioning system operated through LabVIEW software (National Instruments, Austin, TX). After exposure, samples were analyzed using confocal microscopy or colony forming unit (CFU) assays. Additional details of the method for conducting laser exposures are provided in Supplementary Materials.

**Comparison of tGNPs and non-targeted GNPs for laser therapy**

Biofilms grown on glass discs were pre-treated with tGNPs or non-targeted GNPs at 70 μg/mL in PBS for 2 h (SA5120) or 1 h (PA 60–65). This 1-h pre-treatment duration was selected as the optimal tGNP incubation time based upon initial pilot data (Figure S4). Following the tGNP pre-treatment, samples were rinsed using PBS, and 40 μL of PBS was added to each well. Biofilms were then exposed to 50 laser pulses at 532 nm or sham exposed as described in Supplementary Materials. Biofilms treated with 50 laser pulses alone or PBS alone (controls) were also included. Initial pilot experiments showed that bare GNPs or tGNPs alone (no laser irradiation) had no effect on the bacterial viability of SA5120 and PA 60–65 biofilms (Figure S5), and thus these treatment conditions were not included in subsequent experiments. After treatment, samples were analyzed using confocal microscopy, CFU assays, or scanning electron microscopy (SEM).

**Inductively coupled plasma mass spectrometry (ICP-MS) analysis of GNP binding**

The binding of non-targeted GNPs and tGNPs to the biofilms was quantified using ICP-MS to determine the targeting ability of the anti-*S. aureus* and anti-*P. aeruginosa* antibodies. Biofilms on glass discs were incubated with tGNPs or non-targeted GNPs at 70 μg/mL in PBS for 2 h (SA5120) or 1 h (PA 60–65), rinsed with...
PBS, and then treated with 20 μL of aqua regia (3 parts HCl:1 part HNO₃) spotted onto the surface of the biofilms. Samples were disaggregated by repeated pipetting and then added to a microcentrifuge tube (8 biofilms pooled per treatment group). The surface of the biofilms were rinsed with PBS, and the rinses were added to the tube. Samples were sonicated for 2 min and centrifuged at 16,100 rcf for 10 min at room temperature. The supernatant was transferred into a fresh tube and diluted with PBS to obtain the same final volume in all samples. ICP-MS (NexION 300D, Perkin Elmer, Waltham, MA) analysis was performed according to U.S. EPA SW-846 Method 6020B, using NIST traceable gold standards for calibration and calibration verification. Results are reported as μg of gold per biofilm.

Combination of GNP-targeted laser therapy and antibiotic treatment

Biofilms on glass slides were pre-treated with tGNPs at 70 μg/mL in PBS for 2 h (SA5120) or 1 h (PA 60–65). Samples were rinsed with PBS, 40 μL of PBS was added to each well, and biofilms were then exposed to 50 laser pulses at 532 nm or sham exposed. Biofilms treated with 50 laser pulses alone or PBS alone were also included. Upon completion of laser or sham exposures, SA5120 biofilms were treated with gentamicin, and PA 60–65 biofilms were treated with amikacin. Specific antibiotics and concentrations were based on susceptibilities of the respective planktonic cultures to the antibiotics. Antibiotic solutions were prepared at 2-fold the target concentration using TSB for SA5120 or 20% BHI/0.2% glucose/0.4% NaCl in PBS for PA 60–65, and 40 μL of the appropriate solution was added into sample wells containing biofilms in 40 μL of PBS, resulting in a 1x antibiotic concentration. Samples were incubated for 24 h at 37 °C and then analyzed using CFU assays or SEM.

Confocal microscopy analysis

Biofilms were rinsed with PBS and stained with 50 mM of Concanavalin-A-Alexa Fluor® 488 (Con-A) for 30 min and 0.5 μg/mL of 4',6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature. ProLong™ Gold Antifade reagent was added to the samples and biofilms were imaged using a Nikon Eclipse C1 confocal laser scanning microscope (Nikon, Melville, NY). Images of biofilms were acquired at five non-overlapping fields of view (FOVs) per sample using a 20x objective lens. In biofilms treated with tGNPs followed by laser irradiation, a central damage zone surrounded by an area of residual biofilm was observed; the five FOVs were randomly selected from the central damage zone in these samples. Otherwise, images were acquired from randomly selected FOVs in the central portion of the biofilms. ImageJ software (NIH, Bethesda, MD) was used to quantitate the fraction of the surface area covered by biofilm in the images. Data were expressed as a percentage of the controls and reported as “substratum coverage (% control)” in the results. Additional details of this method are provided in Supplementary Materials.

SEM analysis

Biofilms on 5-mm glass discs were fixed and processed for SEM analysis as previously described. In brief, biofilms were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 1 h at 4 °C and washed thrice with 0.1 M phosphate buffer for 10 min each. Samples were dehydrated by graded ethanol/water mixtures of 50%, 70%, 80%, 90%, 95% and 100% for 10 min each; 100% ethanol three times for 10 min each; and 50% (v/v) hexamethyldisilazane in ethanol for 1 h. Biofilms were air-dried overnight, sputter coated with gold, and imaged using a ZEISS Sigma VP40 field emission scanning electron microscope (Carl Zeiss, Inc., Germany).

CFU assays

Biofilm-containing discs were rinsed in PBS and placed into petri dishes. For SA5120, 20 μL of papain at 100 μg/mL in PBS was spotted onto the biofilm surface, and samples were incubated at room temperature for 30 min. For PA 60–65, 20 μL of β-mannosidase at 0.0225 U/mL in PBS was spotted onto the biofilm surface, and samples were incubated at room temperature for 2 h. Loosened biofilms were aspirated and collected in 1.5-mL microfuge tubes. Discs were rinsed thrice using 20 μL of PBS and the washes were pooled into the microfuge tubes. The pooled fractions were sonicated (Qsonica, LLC, Newtown, CT) at 6–8 W of output power for 2 min, centrifuged to pellet the cells, and re-suspended in 1 mL of PBS. Samples were serially diluted in PBS, plated in duplicate or triplicate on tryptic soy agar (TSA) plates, and incubated overnight at 37 °C. The resulting colonies were enumerated and expressed as CFUs/mL.

For planktonic bacteria, broth cultures of SA5120 in TSB or PA 60–65 in BHI were grown for 16–18 h at 37 °C on a shaker at 250 rpm. The cultures were diluted to an OD₆₀₀ of 0.1 using TSB for SA5120 or BHI for PA 60–65 and incubated at 37 °C for ~1.5 h to reach an OD₆₀₀ of 0.3. Samples were treated with antibiotics in triplicate in 5-mL tubes at 37 °C on a shaker at 250 rpm for 24 h, after which 1 mL of culture from each tube was transferred into a fresh tube and centrifuged to pellet the cells. The cell pellets were washed twice in PBS, re-suspended in 1 mL of PBS, serially diluted in PBS, and plated in triplicate on TSA plates. The plates were incubated overnight at 37 °C, and the resulting colonies were enumerated and used to calculate CFUs/mL.

Statistical analysis

Data are presented as the mean ± standard deviation. For CFU assay results, 0 values were replaced with the lowest observed value in each set of experiments, which was considered the lower limit of detection, and data were log₂ transformed. GraphPad Prism 6 (version 6.04, La Jolla, CA) was used to compare groups via the Student’s t test, and P < 0.05 was considered significant.

Results

Nanoparticle-assisted laser therapy is envisioned as a strategy to disrupt the biofilm architecture and, resultanty, remove a significant barrier that limits diffusion of antibiotics to bacteria in deeper layers of the biofilm. Increasing access of oxygen and other nutrients to biofilm-resident cells via this approach may also increase sensitivity of bacteria to antibiotics. Figure 1 is a schematic illustration of this
approach in which GNP-antibody conjugates selectively bind to the biofilm surface, and when irradiated with 532 nm visible light, amplify laser energy absorption leading to extremely rapid heating (photothermal effect) and generation of acoustic waves (opto-acoustic effect) around the particles. This phenomenon leads to thermal and mechanical damage to the extracellular matrix and bacteria and, ultimately, destruction of the biofilm.

Optimal laser dosage and tGNP concentration

Confocal microscopy analysis of MRSA biofilms in 96-well plates revealed that pre-treatment with 70 μg/mL of tGNPs followed by irradiation with 50 laser pulses resulted in uniform dispersion of the biofilms (Figure 2, A). Treatment with fewer numbers of pulses or lower tGNP concentrations led to incomplete biofilm dispersion, and increasing the number of pulses or the GNP concentration did not significantly increase the extent of biofilm dispersion. Viability assays of MRSA biofilms on glass discs confirmed that treatment of biofilms with 70 μg/mL of tGNPs plus 50 pulses of laser energy led to a significant reduction of CFUs relative to untreated controls, and increasing the doses to > 50 pulses or > 70 μg/mL of tGNPs did not significantly increase bacterial cell killing (Figure 2, B). It was also noted that treatment with up to 100 μg/mL of tGNPs alone (no laser) did not cause noticeable dispersion (Figure 2, A) or decrease in viability (Figure 2, B) of the biofilms. Thus, a tGNP concentration of 70 μg/mL and 50 laser pulses were selected for use in subsequent experiments. In addition, pilot experiments in which bacterial viability was determined in the dosing solutions as well as in the biofilms after treatment confirmed that the decrease in CFUs in biofilms treated with tGNPs plus laser irradiation was mainly due to bacterial killing rather than only dispersal of cells into the culture medium (data not shown).

Comparison of tGNPs and non-targeted GNPs for laser therapy

To confirm targeted dispersion of biofilms was achievable using antibody-conjugated GNPs, biofilms on glass discs were used to evaluate the efficacy of laser therapy on samples pre-treated with tGNPs versus groups pre-treated with non-targeted GNPs. Confocal micrographs of MRSA biofilms showed that control samples were composed of a continuous layer of matrix polysaccharides and bacterial cells (Figure 3, A). Treatment of MRSA biofilms with tGNPs plus pulsed laser irradiation led to the highest dispersion of biofilms as indicated by the sparse green and blue staining in the images (Figure 3, A). ImageJ analysis of the confocal micrographs showed that this treatment resulted in removal of 96 ± 3% of the biofilms (matrix and cells) compared to controls (Figure 3, B). In contrast, treatment of MRSA biofilms with non-targeted GNPs followed by laser or with laser alone removed < 1% of the biofilms relative to the controls (Figure 3, B), indicating successful antibody-targeting of the GNPs to the biofilms. The targeting ability of the antibody was also tested by using ICP-MS to compare the amount of non-targeted GNPs and tGNPs that bound to the MRSA biofilms. In agreement with the confocal microscopy results, the ICP-MS data showed a ~7-fold higher amount of gold in biofilms treated with tGNPs compared to biofilms treated with non-targeted GNPs (Figure 3, C).

For PA 60–65, confocal micrographs showed a robust, continuous layer of biofilm in controls and samples treated with laser irradiation alone (Figure 3, D). Interestingly, treatment with tGNPs or non-targeted GNPs plus 50 pulses of laser irradiation both led to extensive dispersion, suggesting a significant level of non-specific binding of GNPs to the P. aeruginosa biofilms that may be due to the mucoid nature of this bacterial species. ImageJ analysis of the micrographs confirmed that treatment with tGNPs or non-targeted GNPs plus laser irradiation resulted in the removal of 99 ± 0.2% of PA 60–65 biofilms (Figure 3, E). In line with these findings, ICP-MS analysis revealed no difference in the amount of non-targeted GNPs and tGNPs that bound to the P. aeruginosa biofilms (Figure 3, F), indicating mostly non-specific binding of the nanoparticles to these biofilms.

Effect of GNP-targeted laser therapy on biofilm viability and morphology

CFU assays showed that treatment of MRSA biofilms with tGNPs plus laser irradiation led to ~1-log reduction in bacterial viability (90% cell killing), while treatment with laser irradiation alone or non-targeted GNPs plus laser did not cause a significant
decrease in biofilm viability relative to controls (Figure 4, A). Treatment of \( P.\ aeruginosa \) biofilms with tGNPs plus laser resulted in a 1.6-log reduction in viability, and treatment with non-targeted GNP plus laser led to a 1-log reduction in viability, both of which were statistically significant relative to controls (Figure 4, B). Thus, data from the CFU assays were in agreement with the results of the confocal microscopy analysis shown in Figure 3.

SEM micrographs of MRSA biofilms at 50× magnification revealed that control samples and samples exposed to laser irradiation alone exhibited intact biofilms with no apparent damage (Figure 4, C, top row of images). In contrast, treatment with GNP plus laser irradiation led to significant dispersion of the biofilm matrix and detachment of bacterial cells from the glass surface as shown by a cleared ‘damage zone’ surrounded by residual biofilm on the outermost edges of the glass discs. This pattern is likely due to the Gaussian distribution of energy in the laser beam that is expected to cause maximal biofilm disruption in the area of highest laser energy deposition. When visualized at 30,000× magnification (Figure 4, C, bottom row of images), no overt cell wall damage was observed in the bacteria remaining on the discs after exposure to GNP-targeted laser therapy, suggesting the residual cells were viable (Figure 4, C). However, these biofilms did show areas in which cells and extracellular matrix appeared to have been dispersed (red arrows in Figure 4, C), in contrast to controls and samples treated with laser irradiation alone that exhibited more extensive amounts of extracellular matrix material.

\textit{GNP-targeted laser therapy synergized with gentamicin against MRSA biofilms}

The benefit of combining GNP-targeted laser therapy with antibiotic treatment was determined by assessing the effects of combination therapy on bacterial viability and dispersion of biofilms. Treatment of MRSA biofilms with GNP-targeted pulsed laser therapy followed by 24-h gentamicin treatment at 100 \( \mu \)g/mL caused a 4-log reduction in viable bacteria (99.99%), whereas treatment with gentamicin alone or GNP-targeted laser therapy alone resulted in only a 1-log reduction in viability compared to untreated controls (Figure 5, A). Thus, combining GNP-targeted laser therapy and gentamicin treatment led to a synergistic enhancement of effect against the MRSA biofilms. Notably, this 4-log reduction in biofilm viability was similar to that observed in planktonic MRSA SA5120 cultures treated with gentamicin (Figure 5, B), suggesting that the GNP-targeted laser therapy effectively dispersed the biofilm matrix allowing the antibiotic to access and kill the bacteria. Treating MRSA biofilms with gentamicin at 100 \( \mu \)g/mL for 48 h instead of 24 h following GNP-targeted therapy did not increase the antibacterial effect of the combination treatment (Figure S7). It was also noted that GNP-targeted laser therapy enhanced the antibacterial activity of 24-h vancomycin treatment at 100 or 1000 \( \mu \)g/mL against MRSA biofilms, though the effect was less pronounced than with gentamicin (Figure S8).

SEM imaging confirmed that treatment of MRSA biofilms with the combination of GNP-targeted laser therapy and gentamicin resulted in dispersion of the biofilm matrix and bacteria from the glass surface, particularly in the ‘damage zone’ that is presumed to be the site of highest laser energy deposition (Figure 5, C). In contrast, biofilms treated with gentamicin alone showed no apparent alterations in the integrity of the biofilms (top row of images in Figure 5, C), as indicated by a lack of a damage zone on the disc (50× magnification) and presence of extracellular matrix (30,000× magnification), and resembled the SEM image of the control biofilm in Figure 4, C.

\textit{GNP-targeted laser therapy synergized with amikacin against \( P.\ aeruginosa \) biofilms}

To assess the applicability of GNP-targeted laser therapy against gram-negative bacteria, the effect of combination...
therapy with amikacin against *P. aeruginosa* biofilms was evaluated. Treatment of *P. aeruginosa* biofilms with GNP-targeted laser therapy alone or 8 μg/mL of amikacin alone for 24 h led to ~1-log reduction in cell viability relative to controls (Figure 6, A). When the biofilms were treated with GNP-targeted laser therapy in combination with 8 μg/mL of amikacin, a 5-log reduction in viability was observed, indicating synergism between the two treatments. Increasing the amikacin concentration from 8 to 16 μg/mL did not further reduce cell viability in biofilms treated with the combination therapy. For comparison, planktonic PA 60–65 cultures treated with 16–64 μg/mL of amikacin for 24 h showed ~4-log reduction in viability compared to controls (Figure 6, B). Taken together, the findings revealed that the combination therapy was as efficacious against biofilms as amikacin alone against *P. aeruginosa* planktonic cultures, further suggesting that GNP-targeted laser therapy effectively disrupts the barrier properties of biofilms and allows antibiotics to access the resident bacteria.
Discussion

Achieving therapeutic efficacy against recalcitrant wound infections is largely predicated on overcoming the survival mechanisms of biofilms that significantly reduce the effectiveness of many commonly used antimicrobials.8,9 Indeed, treatment regimens that remove biofilms using physical or chemical debridement have been shown to improve clinical outcomes in patients with chronically infected wounds and diabetic ulcers.8,28 Most of these techniques, however, do not specifically target infectious material in a wound and may result in significant trauma to vital host tissue. In this report, we investigated GNP-mediated ns-pulsed laser therapy as a novel strategy for targeted destruction of MDR biofilms and enhancement of antibiotic efficacy, an approach that may be useful for limiting collateral host tissue damage.

A major finding of this study is that GNP-targeted laser therapy was able to rapidly disperse 96–99% of the extracellular matrix and cells (Figure 3) and kill up to ~90–98% of the resident bacteria (Figure 4, A and B) in MRSA and MDR P. aeruginosa biofilms. Confocal microscopy and SEM analysis revealed the most extensive removal of the MRSA and MDR P. aeruginosa biofilms within the main damage zone, which is likely the site of highest laser energy deposition. The images also showed removal of mostly extracellular matrix and fewer of the bacteria on the outer edges of samples, which is expected due to the Gaussian nature of the incident laser beam and possible distortions of the laser beam at the edge of the sample well (Figure 4, C). Thus, the data collectively indicate highly effective removal of biofilm infections in areas subjected to the full laser dosage.

Prior studies with planktonic cultures showed that antibody-targeted GNPs irradiated with ns laser pulses generated highly localized photothermal phenomena at the surface of bacteria.22 It is likely that the anti-biofilm effects observed in the current investigation were the result of similar photothermally-induced damage mechanisms such as heat- and pressure-induced denaturation, degradation, and
disruption of the extracellular matrix and biofilm-associated bacteria. The finding that tGNPs (Figures 2, A and S5) or pulsed laser exposure alone (Figures 3 and 4) caused no significant changes to biofilm integrity or viability further indicates the anti-biofilm effects observed in samples treated with the combination of tGNPs and ns-pulsed laser irradiation were due to induction of photothermal phenomena rather than the individual effects of the antibodies, GNPs, or laser irradiation against the biofilms. In addition, no increase in temperature was detected in the dosing solutions in our experiments (Figure S9), which supports the assertion that use of ns-pulsed laser irradiation generates thermal energy on a ns timescale localized around the GNPs and limits heat diffusion into the surrounding medium.
Another significant finding of the current investigation is that the combination of GNP-targeted pulsed laser therapy with 24-h gentamicin or amikacin treatment led to a synergistic 4- to 5-log reduction in MRSA and P. aeruginosa biofilm viability, whereas antibiotics or GNP-targeted laser therapy alone caused only a 1-log reduction in viability (Figures 5 and 6). Notably, the level of killing induced by the combination therapy against the biofilms was similar to the reductions in viability observed in planktonic MRSA and P. aeruginosa cultures treated with the antibiotics alone. These data suggest that GNP-targeted laser therapy was effective in eradicating the occlusive properties of the extracellular matrix that limit the diffusion of antibiotics to the bacteria within the biofilm. Because drug tolerance has also been attributed to reduced growth rates of nutrient-starved bacteria in deeper layers of the biofilm, it is possible that GNP-targeted therapy enhanced antibiotic susceptibility of the biofilms by increasing penetration of oxygen and other nutrients to the underlying bacterial cells.

Overall, our findings agree with prior reports that biofilm-associated bacteria exhibit up to 3 orders of magnitude lower sensitivity to antibiotics than planktonic cultures and decreased penetration of gentamicin and amikacin into biofilms may reduce the antibacterial efficacy of these agents. Furthermore, our data are in agreement with previous observations that dispersal of biofilms rapidly restores susceptibility of bacteria to antibiotics, including gentamicin. Reduced penetration of vancomycin has also been proposed as a mechanism of drug tolerance in S. aureus biofilms, and GNP-targeted laser therapy did enhance the antibacterial effect of this antibiotic against SA5120 MRSA biofilms. However, the efficacy of GNP-targeted laser therapy combined with vancomycin against SA5120 biofilms (2-log decreased viability) did not reach the level of antibacterial activity of vancomycin against planktonic SA5120 cultures (4-log decreased viability; Figure S8). This indicates mechanisms other than reduced diffusion through the extracellular matrix may be responsible for the decreased susceptibility of SA5120 biofilms to vancomycin, or the biofilms may have started to regrow during the 24-h vancomycin treatment following dispersal by GNP-targeted laser therapy. Vancomycin-induced formation of biofilms has been observed in some strains of MRSA, and thickened biofilms were observed in some samples treated with vancomycin in the current study (Figure S8, C).

The majority of previous investigations of GNP-targeted laser therapy for antimicrobial applications involved the use of planktonic cultures and/or CW laser systems. A limited number of studies have reported the anti-biofilm activity of photothermal or acoustic wave effects generated by pulsed laser systems in combination with GNP, Au@Ag nanoparticles, or antibiotics. For example, Ding et al. found that near-infrared femtosecond-pulsed laser irradiation had no effect on S. aureus biofilm viability when combined with GNP, but caused an 85% reduction in viability in combination with Au@Ag nanoparticles (combination of photothermal and silver effects). Using a ns-pulsed laser to generate non-thermal shockwaves, Yao et al. observed a 1-log reduction in Staphylococcus epidermidis biofilm viability when this therapy was combined with 24-h gentamicin treatment. In comparison, our results revealed that GNP-targeted ns-pulsed laser therapy alone was as effective as these other methods in that it caused a 1-log reduction in bacterial biofilm viability, and in combination with antibiotics, our approach achieved a greater anti-biofilm effect of up to a 5-log decrease in viability.

In conclusion, our findings demonstrate that GNP-targeted laser therapy potentiates the activity of antibiotics against in vitro MRSA and P. aeruginosa biofilms via photothermal destruction of the matrix and cellular components of the biofilm. These results are a first step towards developing a non-invasive and efficacious adjunct therapy for the treatment of chronic topical wound infections. Additional investigation is required to determine whether this targeted approach will indeed induce selective eradication of biofilms while minimizing collateral host tissue damage in vivo.

Acknowledgments

The authors gratefully acknowledge Dr. Tao You of the U.S. Army Institute of Surgical Research for assistance with SEM imaging, Mr. Justin Bequette of the Naval Medical Research Unit San Antonio (NAMRU-SA) for assistance with schematic design, Ms. Virginia Lowry of NAMRU-SA for assistance with biofilm experiments, and LT Noel E. Dickens of NAMRU-SA for assistance with statistical analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2019.102018.

References


