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Theranostic gold-in-gold cage nanoparticles enable photothermal ablation and photoacoustic imaging in biofilm-associated infection models

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Biofilms are structured communities of microbial cells embedded in a self-produced matrix of extracellular polymeric substances. Biofilms are associated with many health issues in humans, including chronic wound infections and tooth decay. Current antimicrobials are often incapable of disrupting the polymeric biofilm matrix and reaching the bacteria within. Alternative approaches are needed. Here, we described a complex structure of a dextran-coated gold-in-gold cage nanoparticle that enabled photoacoustic and photothermal properties for biofilm detection and treatment. Activation of these nanoparticles with a near infrared laser could selectively detect and kill biofilm bacteria with precise spatial control and in a short timeframe. We observed a strong biocidal effect against both Streptococcus mutans and Staphylococcus aureus biofilms in mouse models of oral plaque and wound infections, respectively. These effects were over 100 times greater than those seen with chlorhexidine, a conventional antimicrobial agent. Moreover, this approach did not adversely affect surrounding tissues. We concluded that photothermal ablation using theranostic nanoparticles is a rapid, precise, and nontoxic method to detect and treat biofilm-associated infections.

Introduction

Biofilms, communities of bacteria within an extracellular polymeric substance (EPS), are implicated in many chronic infections (1–3). Once biofilms are established, they are notoriously difficult to eradicate due to the presence of an extracellular matrix surrounding the bacteria and protecting them from antibiotics (4, 5). Hence, it is important to locate the biofilms for rapid and precise action against them. Current therapeutic modalities struggle to disrupt biofilms and often fail to efficiently kill the microbes within (6, 7). Consequently, biofilm-associated infections are responsible for human suffering and massive economic expense (8). The development of therapies capable of killing biofilm-associated bacteria would have a significant impact on the treatment of chronic infections.

Two locations where biofilms are relevant to human health are the skin and the mouth. Dental caries afflict nearly half of the world’s population and are a major public health problem according to the US Centers for Disease Control (9). One of the major oral pathogens involved in dental caries is Streptococcus mutans (S. mutans); its ability to form biofilms is a key aspect of this pathology (10, 11). Over the past decade researchers have devised innovative antimicrobial strategies to inhibit S. mutans biofilm formation (12–14). Unfortunately, antibacterial resistance, a lack of appropriate diagnostic tools, and a paucity of convenient and affordable treatments remain barriers to treating oral biofilms (15, 16).

Wound infections affect 6.5 million people in the USA, with estimated annual costs of $28 billion (17). Bacterial biofilms are a major factor in wound chronicity due to effects on delayed healing and impaired bacterial clearance by antibiotics. In most contexts, Staphylococcus species are the most common bacteria recovered from chronic wounds. There are many strategies to clear wound biofilms, with periodic wound debridement being the most common (18, 19). However, wound biofilms remain a major unsolved and growing problem (20).

Photothermal therapy (PTT) is an antibacterial strategy that utilizes light sources to activate nanoagents and convert the light energy into heat, killing the pathogens (21, 22). This antibacterial strategy is noninvasive and effective against biofilm bacteria, including those resistant to conventional antibiotic drugs (23–25). However, there is still a need to develop antibiofilm agents that are photothermally efficient, biocompatible, and photostable (26–28).

Advances in shape engineering, synthesis methods, and coatings of nanoparticles and their combinations with organic materials have introduced new functionalities for nanostructures and their utility in different bioapplications and therapeutic methods (29, 30). Gold nanoparticles, in particular, have been used to enhance antibiotic efficacy and reduce bacterial viability in vitro (31–33).
Here, we report multifunctional gold-in-gold cage photo-thermal nanoparticles (PTNP), which specifically detect biofilm and kill bacteria with high spatial precision in less than a minute. These nanoparticles were formed by growth of a silver shell over a gold core and subsequent galvanic replacement of the silver with a gold cage. This morphology has a plasmon peak in NIR that provides them an excellent performance in biomedical applications involving optical excitation or transduction. We have evaluated the photothermal properties, biofilm uptake, antimicrobial action, cytotoxicity, and PA ability of these nanoparticles. Our in vitro data demonstrate high potency to eradicate biofilms and eliminate wound and oral pathogens without toxicity to eukaryotic cells. Furthermore, PTNP was examined for its efficacy as an antibiofilm agent to detect and treat in vivo biofilms in wound infections and on teeth using rodent models. These findings demonstrate the potential of these nanostructures for therapeutic applications.

Results

Synthesis of gold-in-gold cage photothermal nanoparticles

Gold-in-gold cage nanoparticles were synthesized through the following method: a modified Turkevich method was used to develop small seeds (47). A seeded growth method was then used to make larger nanoparticles with the equilibrium shape—a truncated octahedron enclosed by 6 square facets and 8 hexagonal facets.
Figure 2. PTNP are taken up by bacterial biofilms. SEM in backscattered electron (BSE) mode showing the morphology of (A) untreated biofilm, (B) PTNP-treated S. mutans biofilm (Scale bar: 10 μm). Red arrows show nanoparticles. (C) ICP-OES measurements on S. mutans biofilms. TEM of S. aureus biofilm uptake of PTNP, (D) untreated biofilm, and (E) S. aureus biofilm treated with PTNP for 24 hours (Scale bar: 1 μm). (F) ICP-OES measurements on S. aureus biofilms, (n = 6). Red arrows show nanoparticles. Statistical analyses used a mixed-model ANCOVA with SAS 9.5 software from SAS Institute. *P < 0.05, **P < 0.005. The data are presented as mean ± SD.
Plasmonic properties in NIR are needed to make them a great candidate for biomedical applications. Therefore, engineering AuNPs characteristics such as shape, size, and composition could help to establish a new agent with the specific purpose of biofilm treatment methods. The plasmonic properties of shape-engineered gold-in-gold cage nanoparticles were measured using UV-vis spectroscopy and are shown in Figure 1F. These nanoparticles have a plasmon peak at 808 nm which is tunable in the NIR region based on its GRR and the thickness of shells and make them a great candidate for biomedical applications (54).

**PTNP incorporation within biofilms**

To measure the incorporation of PTNP within biofilm, different concentrations of PTNP (0, 0.1, and 0.25 mg/mL) were coated with 3 different coatings, i.e., polyethylene glycol (PEG), DEX-10kDa and polydopamine (PDA). Supplemental Figure 5 shows DEX-enhanced incorporation of PTNP into *S. mutans* biofilms much more than PDA and PEG. We found that PTNP were incorporated into *S. mutans* and *S. aureus* biofilms while using a low concentration of PTNP (0.25 mg/mL). SEM images of biofilms incubated with PTNP indicated that PTNP bind to *S. mutans* biofilms (Figure 2, A and B), which was confirmed by EDS measurements (Supplemental Figure 6, A-C). To investigate PTNP binding in biofilms of *S. aureus* we also carried out TEM of biofilms (Figure 2, D and E), which showed clear uptake and penetration of PTNP within the biofilm ultrastructure. Figure 2, C and F show that PTNP are significantly taken up into *S. mutans* and *S. aureus* biofilms, as measured inductively coupled plasma optical emission spectroscopy (ICP-OES). These data confirm DEX-enhanced incorporation of PTNP into *S. mutans* and *S. aureus* biofilms, while no binding enhancement was observed on our control sample, a saliva-coated apatite surface (sHA). This polymer promotes selectivity toward biofilms while avoiding binding to host cells. DEX is a component of biofilms and prior evidence suggests that DEX-coated nanoparticles are incorporated into biofilms by the enzyme β-glucosyltransferase (gltFB) (4, 52). These results demonstrate the successful incorporation of PTNP into *S. aureus* biofilms when applied topically, supporting the idea of using PTNP for PA and PTT of biofilms.

**Photothermal activity of PTNP**

Next, we examined the heat generation of PTNP while irradiated with a NIR laser. PTNP solutions and DI water as a control solution were treated with a NIR laser, and the temperature was recorded over time. PTNP structures were found to generate significant temperature increases, higher than the control solution. Figure 3A shows the maximum temperature observed for the PTNP solution was much higher than that for DI water (as much as 50°C). The photothermal conversion efficiency of PTNP was calculated to be 77%, which is comparable with gold nanostars (78%) (56) and gold nanospheres (75.5%) (57), and higher than gold nanocages (53.6%) (58, 59), gold nanorings (42%) (60), and gold nanorods (21.3%) (61, 62). The temperature change of the PTNP and its dependency on laser power and PTNP concentration was also studied. As is shown in Figure 3B, greater laser power density resulted in greater temperature increases. Similarly, increases in the concentration of PTNP led to greater temperature enhancement (Figure 3C).

We found that during 5 cycles of laser on-off irradiation, the PTNP solution reproducibly reached 99.9% of the temperature achieved the first time, indicating the excellent stability of these structures (Supplemental Figure 7A). The TEM of irradiated PTNP samples did not show any difference in morphology, which confirms the photostability of these structures (Supplemental Figure 7, B and C). Here, we chose different laser powers depending on the experiment setting and in agreement with the work of others (60, 63, 64). For only in vitro and ex vivo oral biofilms that develop upon hard surfaces such as hydroxypatite discs or teeth, we used higher laser power (2 W/cm²) and shorter time frames, which is, again, in agreement with recent studies that report powers of 2–2.5 W/cm² (64, 65). For in vitro and in vivo skin studies we used lower laser powers (0.25–0.7 W/cm²) to avoid any possible damage in surrounding tissues, as has been reported in other studies that use powers such as 0.75, 0.78, and 1 W/cm² (56, 66, 67).

To test the biofilm imaging ability of PTNP, we examined the PA signal of PTNP at different PTNP concentrations (0, 0.06, 0.12, 0.25, and 0.5 mg/mL). Increasing PA signal was observed with increasing the concentration of PTNP (Supplemental Figure 8, A and B). We also examined the PA properties of PTNP when...
Figure 4. PTNP efficiently kill biofilm bacteria in vitro. (A) Schematic of photo ablation of biofilm with PTNP in wide area mode and (B) precise spatial control mode. The viability of (C) S. mutans and (D) S. aureus within biofilms treated with PTNP and irradiated in wide area mode (n = 5). The statistical technique used in this experiment is a 2-way ANOVA with Šidák’s multiple comparison correction. *P < 0.05, **P < 0.0001. (E-H) Representative confocal micrographs of treated and untreated S. mutans biofilms exposed to wide area mode (Scale bar: 50 μm). Magnified views of S. mutans biofilms in the boxed areas are shown in the bottom left of each panel (Scale bar: 10 μm). Confocal micrograph images of PTNP-treated S. mutans biofilms exposed to laser in precise control mode for (I) 15 seconds, (J) 30 seconds, and (K) 60 seconds. Live bacteria cells (green), dead cells (red), and EPS (purple) are shown (Scale bar: 50 μm). Biofilm bacteria were irradiated at 808 nm (C-H and I-K were irradiated with laser power 0.5 W/cm² and 2 W/cm², respectively).

incubated with S. aureus biofilm. The PA signal was assessed using different PTNP concentrations (0, 0.12, 0.25, and 0.5 mg/mL) which were incubated with biofilm for 24 hours and were imaged at a laser wavelength of 800 nm (Supplemental Figure 9). The PA signal rose with increases in the concentration of PTNP. These findings suggest that PTNP can be used as an agent for both diagnosis and treatment of oral and skin infectious diseases.

In vitro antibiofilm efficacy of the PTNP

Here, we examined how PTNP combined with laser irradiation disrupts biofilms and kills bacteria. Oral biofilms (S. mutans UA159, a strain associated with endocarditis) were formed on SHa discs, while S. aureus biofilms were grown in 96-wall plates as a skin infection model. Biofilms were analyzed using multiphoton confocal microscopy, colony counting (presented as colony forming units or CFU), and computational analysis (COMSTAT and Image J). Biofilms treated with or without PTNP (0.25 mg/mL) were exposed to NIR laser irradiation at 808 nm in both wide-area and precise spatial–control irradiation mode (schematically shown in Figure 4, A and B, respectively), at 0.5 and 2 W/cm² for different time points. Then, bacterial survival studies were conducted on PTNP surfaces using a colony formation assay both with and without NIR irradiation in a wide area mode. The biofilms were removed, homogenized, and the number of viable cells were determined.

We observed an exceptionally strong biocidal effect against S. mutans and S. aureus within biofilms when exposed to NIR laser irradiation and PTNP, causing a greater-than 5-log and greater-than 6-log reduction (elimination of 99.99% of bacteria) of viable oral and skin pathogens, respectively (Figure 4, C and D). This treatment is considerably more effective than the current clinically used oral and skin antimicrobials such as chlorhexidine, alcoholic chlorhexidine, and 0.5% chlorhexidine-10% povidone iodine, which causes around 3-log reduction in the same conditions (68–70). We observed that the dry weight of remaining S. mutans biofilms in the treated samples with NIR light was 41.1% less than the control samples and untreated samples (Supplemental Figure 10). This was surprising, given the short laser irradiation time of this procedure. These results suggest that the treatment can cause EPS degradation as well as killing of the bacteria. These studies demonstrated that PTNP, when illuminated with NIR light, could effectively kill S. mutans and S. aureus, as well as degrade biofilm EPS.

The effects of PTNP and laser treatment at a local and wide area level

To further understand how PTNP can be used as a biofilm treatment, we performed treatment experiments using confocal microscopy. The same treatment procedure was repeated (irradiating the samples with NIR laser in wide area mode, schematic is shown in Figure 4A) in the last section. Representative images of control, PTNP-treated biofilms, exposed control biofilm, and PTNP-treated biofilms to the NIR laser are shown in Figure 4, E-H. We observed almost complete bacteria killing in biofilms incubated with PTNP and after 10 minutes of wide area–mode laser treatment, while few dead cells were observed in other samples. These results confirmed our hypothesis that photothermal ablation PTNP could kill S. mutans bacteria.

To underscore the potential targeted approach of our therapy, we used a modified version of the treatment method described above. Here, the laser beam was focused to an area in the middle of the biofilm covered disc of 2 mm × 5 mm and turned on for 15, 30, or 60 seconds (Figure 4, J and K). The untreated area is on the left and the treated area on the right in Figure 4, I-K. The 15 second NIR laser irradiation is not enough to completely kill the bacteria in the irradiated area; however, 30 seconds of irradiation can completely kill the bacteria, and there is a clear line between live and dead cells (the yellow arrows show the line). The 60 second exposure resulted in bacterial killing even in the unirradiated part of the biofilm, which indicates that, for in vitro localized treatment, 60 seconds of irradiation is too long. Here, we demonstrate how our antibiofilm agent could treat the bacteria locally in less than a minute.

Biocompatibility and cell toxicity of PTNP

In vitro biocompatibility and determining the photothermal activity of PTNP were carried out on different cell types. The cells were treated with DEX-coated PTNP for 4 hours at concentrations of 0.1, 0.25, and 0.5 mg/mL PTNP. The viabilities of the C2BBe1, BJ5IA, gingival, and HaCaT cell lines were determined using the MTS assay. It was found that these structures did not significantly affect the viability of these cell types and are biocompatible under the conditions tested (Figure 5A).

To evaluate the precision and selectivity of this treatment method for bacterial cells, we evaluated whether this approach could target biofilms without adversely impacting surrounding tissue. Since the amount of heat generated by PTNP can be controlled by either changing the laser power or concentration of PTNP in the medium, it can provide fine control and homogenous distribution of heat compared with conventional heating probes. For these experiments, HaCaT cells were selected and incubated with different concentrations of PTNP (0.05, 0.12, and 0.25 mg/mL) without light, and their viability were quantified after 24, 48, or 72 hours (Figure 5B). These figures confirm the biocompatibility and non-toxicity of PTNP within different cell lines. In this study, PTNP has been used as an antibiofilm for topical applications where scant uptake is to be expected, and, therefore, persistence and lack of ability to biodegrade is of lesser concern. In parallel experiments, treatment with PTNP was combined with laser irradiation at different powers (0.25 and 0.5 W/cm², 7 minutes) and HaCaT cell viability was tested at 24, 48, or 72 hours after laser irradiation (Figure 5C). Live-dead staining microscopy image of HaCaT cells at 0.5 W...
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laser irradiation at different time points is shown in Supplemental Figure 11. We found that PTNP was well-tolerated by eukaryotic cells and ineffective at killing them via laser irradiation due to lower uptake (Supplemental Figure 12) and greater robustness compared with bacteria. Moreover, the results indicate regrowth of the cells after 72 hours of laser irradiation, which shows that the effects of laser on mammalian cells were negligible, and no cell damage was observed on NIR-irradiated PTNP-treated cells at different powers of 0.25 and 0.5 W/cm² for 7 minutes. This study also suggests the feasibility of carrying out photothermal antibiofilm treatment using PTNP in both oral and wound in vivo models, while keeping the surrounding tissue healthy.

PTNP effects in animal models

PTNP detects and kills pathogenic oral bacteria in an animal model. The antibacterial efficacy of PTNP, with a clinically relevant topical treatment regimen for oral infections, was examined using Sprague-Dawley rats infected orally using an actively growing culture of S. mutans (71). Ex vivo PTT efficacy and PA experiment were performed on rat teeth as is shown schematically in Figure 6A. PTNP nanoparticles (0.25 mg/mL) were applied topically on the teeth, and, after 10 minutes incubation within biofilms, they were irradiated with the NIR light for 1 minute. PTNP absorbs the light and generates local high temperatures that kill S. mutans bacteria. Figure 6B shows the viability of S. mutans within PTNP-treated biofilms after the laser treatments. Here, we found that none of the other groups (control, only PTNP, only laser, and Chlorhexidine) are able to kill the bacteria successfully, and our developed treatment method was more than 99.99% effective in killing the S. mutans bacteria. To evaluate the effectiveness of laser treatment in situ, the viability of bacterial cells within treated biofilms was analyzed by confocal laser scanning microscopy (Figure 6C). As shown in Figure 6C, the biofilm bacteria were alive after treatment in control, PTNP-only, and laser-only groups. But the PTNP-with-laser treatment resulted in a highly effective method that rapidly killed most of the bacteria. We also noticed that, while the biofilm treated by chlorhexidine harbored predominantly dead bacteria in imaging, the bacteria recovered from the jaw were not significantly reduced. This may reflect differences in the location of the imaging analysis and recovery of plaque from both smooth and sulcal surfaces for the CFU counting. For confocal imaging, given the anatomical constraints of the jaw, we could only visualize the live/dead bacteria on smooth surface of the teeth, which could not depict the large number of live bacteria inside the sulcus that are more difficult to kill. Chlorhexidine, a highly cationic molecule, is known for its poor penetration into deeper biofilms. As a result, the bacteria in the deep layers (e.g., sulcal areas) might remain alive while most of the bacteria on smooth surfaces would

Figure 5. PTNP are biocompatible and nontoxic. (A) The effect of PTNP on the viability of several cell lines. (B) The cell viability of HaCaT cells in 24, 48, and 72 hours of incubation with PTNP. (C) Cell cytotoxicity of HaCaT cells incubated with PTNP in different concentrations (0.1, 0.05, 0.1 and 0.25 mg/mL) and time points. Cells were irradiated at 808 nm (0.25, and 0.5 W/cm²). Statics are a 2-tailed t test and mixed-model ANCOVA with SAS 9.5 software from SAS Institute. *P < 0.05. The data are presented as mean ± SD. All graphs are representative of n = 6 experiments.
The feasibility of this treatment strategy to disinfect wounds was also investigated in vivo. A delayed inoculation S. aureus wound model was used to test the applicability of this technique (73, 74).

We next evaluated biofilm imaging and diagnostic potential of PTNP in our oral model. For the oral PA experiment, we imaged jaws incubated with either vehicle (a buffer or solvent as a control) or PTNP (Figure 7, A and B). The PA images reveal strong contrast for the teeth that had been treated with PTNP, while no signal was detected for the control group (Figure 7, C and D). PA image analysis showed that infected rat teeth that were incubated with PTNP had a 72-fold signal increase compared with the control (Figure 7E). PA has recently been used in oral applications in patients (44, 72) and these data confirm the infection on the teeth using PTNP and suggests that PA could also be used to diagnose oral infections.

**Treatment of Staphylococcus aureus–infected wounds using PTNP antibiofilm agents**

The feasibility of this treatment strategy to disinfect wounds was also investigated in vivo. A delayed inoculation S. aureus wound model was used to test the applicability of this technique (73, 74).
As presented in Figure 8A, infection progress was monitored using bioluminescence imaging (BLI). The PTNP-with-laser group was the only group that showed no infections in the BLI images after 5 minutes of treatment (Figure 8B). To confirm the reliability of this technique, animals were euthanized, and wound tissues were extracted for bacterial plate counting (75–77). We also examined our multi-functional nanoagent to image wound infections in vivo. To visualize the infected wounds, 20 μL of 2.5 mg/mL of PTNP solution was dropped onto the wounds area. Then, the PA imaging was performed after 10 minutes, while the mice were still under anesthesia. Similar to oral biofilms, PTNP-treated mice showed high signal intensity localized to the infectious wounds, while no signal was detected in the controls (Supplemental Figure 14).

Discussion

Here, we have demonstrated that PTNP are effective theranostic antibiofilm agents, enabling rapid photoablation and biofilm disruption. We show the utility of approach in vitro, against 2 different bacterial species, and in a pair of in vivo preclinical models. This approach represents advancements over existing theranostic agents in demonstrating strong extinction spectra in the NIR region, excellent photothermal conversion efficiency, and capability in imaging of biofilm bacteria and eliminating them rapidly. A variety of gold nanostructures have been used for PTT previously, such nanorods and nanoshells. However, the synthesis of those structures typically involves the use of organic solvents or toxic chemicals, which require cumbersome procedures to eliminate or displace. The synthesis of PTNP, as described herein, avoids the use of such organic solvents and toxic reagents, with the primary reagents used being safe materials, i.e. sodium citrate and ascorbic acid. Therefore, the prospects for scale-up synthesis and clinical translation of PTNP are improved compared with nanorods and nanoshells (58, 59, 61).

PTNP demonstrate several remarkable features that are useful for the treatment of infectious diseases. First, it has a complex morphology and is nontoxic and biocompatible, which provides considerable interest for biomedical applications. Second, it can be used as a dual-use agent for both imaging and therapeutic applications to diagnose, degrade, and disinfect oral and skin biofilms. Third, it can be used in both wide-area irradiation and precise spatial control, which could provide homogenous or localized biofilm treatment to effectively target, activate, and kill bacteria. Fourth, this treatment method using PTNP is antibiotic free, and therefore there is no possibility of antibiotic resistance developing.
Fifth, the biofilm killing using this approach is expeditious and low cost. We find that the amount of the bacteria decreases dramatically by using 3.75 mg of PTNP (when scaling to a human dose of 20 mL), which will only cost about $0.20. The other equipment that is needed to perform the biofilm treatment is a NIR laser that can be used for many years and costs around $200, which is an affordable expense for a clinical office. Alternatively, these agents could be used by patients at home, in combination with low cost, whole surface irradiators, which are already on the market. Our vision is that these PTNP could be incorporated into a variety of daily oral and skin health maintenance products, such as mouth rinses, gel strips, and photothermal bandages.

This study has some limitations. We have assessed biofilm killing but not therapeutic outcomes (e.g., preventing cavities or enhancing wound healing) so future studies will investigate whether PTNP can control cavities or accelerate wound healing. We tested our antibiofilm agent with a single wavelength NIR light. This can be fine-tuned further or tested on other light sources. Exploring drug loading or targeting within PTNP structures to either enhance antibiofilm efficacy or other functionalities (e.g., growth factors and antimineralizing agents) could also lead to improved therapeutic outcomes. Moreover, the use of this treatment method and antibiofilm agent for other biofilm infections including those in catheters, heart valves, lungs, and prosthetic joints could be investigated.

In summary, dual purpose biocompatible PTNP structures, which have photothermal characteristics that detect, target, and kill bacteria within biofilms without the need for broad-spectrum antibiotics, is highly appealing for oral and skin infectious diseases. These properties allow the development of a range of different oral care products and wound disinfections. Here, we showed that this treatment method can accurately and selectively deliver the desired amount of heat to the infected area without damaging the...
healthy tissue and can be carried out in a very short time to target infected bacteria. The results of this research will advance the field of biofilm treatments by introducing new structures of antibiofilm agents that are activated under illumination by NIR light, resulting in biofilm disruption in vitro and in vivo in less than a minute.

Methods

Nanomaterial synthesis and characterization
PTNP were synthesized via the Turkevich method, a seeded growth method, and GRR using chloroauric acid (HAuCl₄). In brief, 16 ± 2 nm seeds were prepared using the Turkevich method, and 78 nm cores were synthesized using the seeded growth method. 3 mL of 1 mM aqueous AgNO₃ (Sigma-Aldrich) was reduced on the 78 ± 3 nm cores using 1 mL of 1 mM L-ascorbic acid (AA) (Thermo Fisher Scientific), making core-shell structures. Then, 10 mL of 500 μM aqueous HAuCl₄ (Sigma-Aldrich), was added at a rate of 1 drop per second to create Au-Au core-cage structures, here termed PTNP (54). Ligand exchange was performed using 5 mL of 0.1 M DEX (Dextran T-10, Pharmacosmos). Next, the mixture of PTNP and DEX was allowed to stir overnight. The final, coated nanoparticles were then collected by centrifugation at 1200g for 10 minutes.

UV-visible spectroscopy
UV-visible spectra were recorded using a UV-visible spectrophotometer (Thermo Fisher Scientific).

Electron microscopy
Characterization of PTNP was done using a transmission electron microscope (JEOL 1010) operating at 80 kV and scanning electron microscope (SEM, Quanta 600 FEG, FEI). We examined PTNP binding using TEM and SEM to visualize and analyze PTNP distribution within biofilm ultrastructure (78). First, S. aureus biofilms treated either with PTNP or vehicle (Supplemental Figure 3) were washed with DI water to remove unbound materials. Biofilms were fixed with 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4), overnight at 4°C. Prepared samples were fixed in 2.0% osmium tetroxide with 1.5% K₃Fe(CN)₆ for 1 hour at room temperature and rinsed in DI water. Then, samples were dried and were embedded in EMBed-812 (Electron Microscopy Sciences). Several 0.5 μm semithin sections were cut into the sample for pre-view before cutting 70 nm thin sections for TEM. 1% uranyl acetate and SATO lead were used to stain the thin sections of the final samples. TEM images were acquired using a JEOL 1010 electron microscope fitted with a Hamamatsu digital camera and AMT Advantage NanoSprint500 software. EDS was also performed to detect gold and silver from PTNP, with corresponding elemental mapping.

Hydrodynamic diameter and ζ potential measurements
Hydrodynamic diameter and ζ potential of PTNP were examined using dynamic and electrophoretic light scattering (Nano ZS-90 Zetasizer). All DLS data were acquired by diluting 100 μL of 0.1 mg/mL PTNP structures to 1 mL with DI water.

Inductively coupled plasma optical emission spectroscopy
PTNP concentrations were measured using inductively coupled plasma optical emission spectroscopy (ICP-OES, Spectro Analytical Instruments GmbH) to measure the gold and silver concentrations in PTNP samples and also within tissue samples according to a protocol published elsewhere (54).

In vitro heating
The photothermal properties of PTNP were measured using NIR laser irradiation to induce heating of these structures. A milliliter of PTNP (10 μg/mL) was added to a 2 mL quartz cuvette, and a fiber optic thermometer (Nomad, Qualitrol-Neoptix) was inserted 5 mm below the liquid surface. Solutions were then irradiated with an 808 nm laser (OEM Laser Systems) at 2 W/cm² power for 10 minutes. Temperature was recorded every 10 seconds. DI water was used with the same procedure as a control. For the laser power and PTNP concentration dependency experiment, the laser was turned off after reaching a constant temperature (i.e., after 10 minutes). The photostability, shown in Supplemental Figure 5, was measured at 10 μg/mL PTNP and with NIR laser irradiation with 2 W/cm². The laser was turned off after reaching a plateau in temperature and the sample was allowed to cool down to the room temperature before the laser was turned on again to reach the maximum temperature for the next round. The final temperature is given as a Δ to exclude minor variations in baseline temperature and to highlight the differences in temperature gain caused by the conditions used. This experiment was repeated 5 times. All experiments were performed at room temperature with ambient light.

Laser Set-up for in vitro and in vivo experiments
Irradiation using collimated laser beam (Precise spatial control mode): The experiments in which light was collimated and the flux was precisely controlled have been carried out using NIR laser (808 nm, OEM Laser Systems, flux 0.5 or 2 W/cm²). The beam had a rectangular cross-section (2mm × 8mm), and it could cover all of the teeth of a rat. The object was irradiated during different time periods (15 or 30 seconds, or 1, 5, and 10 minutes). This approach can be clinically translated and used for treatment of teeth cavities and biofilm-forming infections in humans.

Wide-area irradiation
The same laser coupled to a fiber (808 nm, OEM Laser Systems) could be used for wide-area irradiation. The beam was focused on the entrance aperture of a multimode glass fiber (Dolan Jenner, 1 m, 4 mm diameter) using a lens (f = 5.5 cm) mounted on a 1D translation stage (Thorlabs) such that the size of the spot on the fiber aperture was approximately 2-3 mm in diameter. Such slight defocusing allowed us to avoid overly high fluxes at the aperture and prevent overheating. The light emerged from the other end of the fiber uncollimated, and therefore by adjusting the distance between the fiber tip and the object, round areas of different diameter could be irradiated. The photon flux across the area was approximately uniform, as judged by visual examination, and it was calculated from the laser power and measured by an optical power meter (Thorlabs) immediately after the fiber and the area diameter.

In vitro oral biofilm model
Streptococcus mutans (S. mutans) UA159 (ATCC 700610) was used as an oral biofilm bacterium and was grown in ultra filtered (10 kDa molecular-mass cutoff) tryptone-yeast extract broth (UFTYE; 2.5% tryptone and 1.5% yeast extract) containing 1% (wt/vol) glucose at
37°C and 5% CO₂ to midexponential phase. *S. mutans* biofilms were formed on sHA discs with surface area of 2.7 ± 0.2 cm² (Clarkson Chromatography Products Inc.), as described previously (75). Each HA disc was coated with filter-sterilized saliva for 1 hour at 37°C and the saliva was prepared as described elsewhere (79). The discs were vertically suspended in 24-well plates using a customized wire disc holder that mimics the smooth surfaces of the tooth. These sHA discs were each inoculated with approximately 2 × 10⁷ CFU of *S. mutans* per milliliter in UFTYE culture medium (pH 7.0) containing 1% (w/v) sucrose at 37°C and 5% CO₂. The culture medium was changed at 19 and 29 hours (twice daily) until 43 hours. Then, the biofilms were collected and analyzed for PTNP binding, biomass reduction, and bacterial killing, as described below.

**Bacterial killing and biomass reduction by PTNP with laser irradiation**

To assess the antibiofilm effect of PTNP within biofilms, the bacteria were grown overnight and then 1:100 dilute from overnight culture were added into fresh medium for biofilm assay. For the skin biofilm formation, *S. aureus* isolates (USA300, Newman 502A) were grown at 37°C in liquid luria broth (Thermo Fisher Scientific) at 300 rpm. Then 100 μL of the dilution was added per well in a 96 well dish and the plates were incubated at 37°C with 5% CO₂ for 24 hours. Then the plates were washed 3 times with DI water before incubating with different concentrations of PTNP (0, 0.1, 0.25, and 0.5 mg/mL) for another 24 hours. For oral biofilm formation, the *S. mutans* biofilms were grown on sHA discs as described above. The sHA discs and biofilms were topically treated twice daily by placing them in 2.8 mL sterile PBS twice and replaced with fresh media containing PTNP at different concentrations. The plates were then incubated at 37°C in 5% CO₂ atmosphere for different times (24, 48, and 72 hours). After this incubation, the media was removed, the cells were washed with PBS, then 20 μL MTS reagent and 100 μL of hygromycin B (Sigma-Aldrich) were used as a culture medium for BJ5ta cells. C2BBe1 cells were also cultured in a medium of DMEM, 10% FBS, 45 IU/mL penicillin and 45 IU/mL streptomycin (Gibco), with 10 μg/mL human transferrin (Sigma-Aldrich). Primary human gingival epithelial cells were cultured in keratinocyte serum-free medium (Invitrogen) and HaCaT cells were cultured in DMEM with Glutamax, 4.5 g/L D-glucose, 110 mg/L Na Pyruvate (Gibco/Invitrogen #10569), 5% FBS and mixed with antibiotics streptomycin (100 μg/mL) and penicillin (100 U/mL). The cells were grown at 37°C in a 5% CO₂ humidified incubator. To determine the cytotoxicity of PTNP, 1 × 10⁵ cells per well were seeded in 96-well plates, and then the plates were incubated at 37°C in a 5% CO₂ atmosphere for 4 hours (the cell viability of HaCaT cells were also measured after 24, 48, and 72 hour incubation with PTNP). Then, the media was carefully removed, and the cells were washed gently with sterile PBS twice and replaced with fresh media containing PTNP at different concentrations. The plates were then incubated at 37°C in 5% CO₂ atmosphere for different times (24, 48, and 72 hours). After this incubation, the media was removed, the cells were washed with PBS, then 20 μL MTS reagent and 100 μL cell culture medium was added to each well. The plates were incubated at 37°C in a 5% CO₂ atmosphere for 1 hour, then the absorbance was measured at 490 nm using a plate reader. The percentage of relative cytotoxicity of each concentration of PTNP was calculated compared with control and the data presented as mean ± SD (n = 3).

The effect of PTNP on cell viability was also evaluated while the cells were irradiated with the NIR laser. In these experiments, HaCaT cells were plated in 24-well plates (10,000 cells per well) and incubated at 37°C in a 5% CO₂ atmosphere for 24 hours. Then, the cells were washed with PBS and exchanged with the fresh media containing PTNP with different concentrations (n = 3 wells per condition). The following day, the cells were washed with PBS twice and replaced with fresh media. Immediately after media exchange, wells were irradiated with a NIR laser (808 nm wavelength, 0.25 and 0.5 W/cm²) for 7 minutes. Plates were then incubated at 37°C in a 5% CO₂ atmosphere to be analyzed for laser cytotoxicity of cells at 2, 24, 48, and 72 hours after laser irradiation using the live-dead assay. The media was exchanged with the live-dead reagents consisting of 2 mL PBS, 2 μL ethidium-1 homodimer for dead cells (used from as received stock), and 0.5 μL of stock Calcein AM for live cells. After 20 minutes incubation with the live-dead cocktail, 6 micrographs per well were taken using a Nikon Eclipse Ti-U fluorescence microscope. The images were acquired using 495/515 nm filter for Calcein and 528/617 nm filter for Ethidium Homodimer-1 (EthD-1). The number of live or dead cells were count-
ed in each image to analyze cell cytotoxicity after laser irradiation at different time points.

**In vitro PA imaging**

A Vevo Lazer device (VisualSonics) with LZ550 transducer was used to acquire the photoacoustic images. Twenty μL of PTNP were inserted into 0.5 mm diameter polyethylene tubing which was then covered in 2 to 3 cm of water. PTNP were prepared at different PTNP concentrations (0, 0.06, 0.12, 0.25, and 0.5 mg/mL). The transducer distance from sample was 1.1 to 1.5 cm.

The PA imaging of PTNP within skin biofilms was also carried out using the Vevo Lazer device and LZ550 transducer. The *S. aureus* bacteria were cultured in 96 wells using Bacto Tryptic Soy Broth (TSB) (BD Biosciences) as a growth media. After 24 hours, the biofilm bacteria were incubated with different PTNP concentrations (0, 0.12, 0.25, and 0.5 mg/mL) for another 24 hours. Then, the biofilm treated with PTNP was washed 3 times and was covered in 2 to 3 cm of water. The ultrasound gain and PA gain were adjusted at +27 dB and 22 dB, respectively.

**Animal and infectious disease models**

Ex vivo efficacy of PTNP for oral biofilms. An established rodent model that mimics dental caries, including *S. mutans* infection of rat pups, was used for ex vivo experiments for oral infectious disease (80, 83). Briefly, 15-day-old female Sprague–Dawley rat pups were purchased from Harlan Laboratories. First, the animals were infected orally with actively growing (midlogarithmic) culture of *S. mutans* UA159, and their infection was confirmed via oral swabbing. The animals’ diet was based on the NIH cariogenic diet 2000 (TestDiet) and 5% sucrose water ad libitum. The animals were categorized into 5 treatment groups. The treatment groups were: (a) control (0.1 M NaAc buffer, pH 4.5), (b) PTNP only (0.25 mg/mL), (c) laser only, (d) PTNP and laser irradiation, and (e) chlorhexidine. The animals’ physical appearance was monitored daily, and the body weights were recorded weekly. The experiment proceeded for 3 weeks (21 days). At the end of the experimental period, the animals were sacrificed and their jaws were surgically removed and aseptically dissected. Jaws with infected teeth were placed in a solution of PTNP (0.25 mg/mL), chlorhexidine or vehicle for 10 minutes and washed in DI water (the jaw was dipped in DI water 3 times), which was immediately followed either by NIR laser irradiation (at 0.7 W/cm²) for 5 minutes. Other groups were treated with the same condition, but without the NIR laser irradiation. Animals were euthanized after the treatment and in vivo imaging. For the infected wound with the associated nanoparticles, and laser only group were exposed to the laser treatment (808 nm NIR laser, at 0.7 W/cm²) for a duration of 5 minutes. Other groups were treated with the same condition, but without the NIR laser irradiation. Animals were euthanized after the treatment and in vivo imaging. For the infected wound with the associated nanoparticles, and laser only group were exposed to the laser treatment (808 nm NIR laser, at 0.7 W/cm²) for a duration of 5 minutes. Other groups were treated with the same condition, but without the NIR laser irradiation. Animals were euthanized after the treatment and in vivo imaging. For the treated and untreated wounds were extracted for bacterial plate count. The surface temperature of wounded mice was also monitored before treatment, after 1, 2, 3, 4, and 5 minutes of treatment using an FLIR 1 thermal imaging camera (FLIR Systems).

Ex vivo imaging

BLI. BLI was performed by using a Lago X imaging system (Spectral Instruments Imaging). Mice infected with luciferase-expressing XEN36 *S. aureus* were anesthetized with 2.5% isoflurane, and bioluminescent signals were quantified by using Aura software (Spectral Instruments Imaging). The acquisition parameters were adjusted as follows; exposure time 30 seconds, binning low (2), f/stop 1.2, and FOV 25. The luminescence data was analyzed using Aura software.

In vivo PA imaging. PA was performed with a VisualSonics Vevo 2100/LAZR high-frequency ultrasound/PA scanner with 256 array transducers. The experiment was carried out with skin infected male 8–12-week-old C57BL/6J mice (n = 3). The procedure was performed under anesthesia with isoflurane (1.8%–3%, 2 L/min O²) before imaging on control and PTNP treated mice (20 μL of 0.25 mg/mL) for 10 minutes. The images were acquired using 800 nm NIR light, with ultrasound gain of +27 dB, PA gain 35 dB, and priority 95%.

Ex vivo PA imaging

Ex vivo PA imaging was captured using a Vevo Lazer @2100 device (VisualSonics). PA imaging was performed on infected wounds (n = 3), which were exposed to PTNP (0.25 mg/mL) for 10 minutes, and infected wounds, which had no incubation with PTNP (control). The jaws were adhered to a dish with glue and were covered with 1-2 cm water. An LZ550 transducer, 800 nm NIR light, with ultrasound gain of +27 dB, and PA gain 35 dB, were used to acquire PA images. Image analyses were performed by placing ROIs on the images of control and biofilms incubated with PTNP. Then, signal-to-background ratio (SBR) values were calculated from these measurements (from 6 images per group).

**Statistics**

Statistical analyses for the experimental data were performed using a 2-tailed t test, mixed-model analysis of covariance (ANCOVA), and SAS 9.5 (SAS Institute). For the in vivo experiments, an analysis of outcome measures was done with transformed values of the measures to stabilize variances. The data were then subjected to ANOVA for all sets of data. *P* values of less than 0.05 were considered statistically significant.

**Study approval**

The reported studies in animals were approved by the Stanford University Institutional Animal Care and Use Committee (Palo Alto, Cal-

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For the subsequent 48 hours, mice were monitored at least daily for wound condition and signs of distress. Meanwhile, the infected wounds were monitored with BLI to visualize the infection rate in each wound. After 48 hours, the treatment procedures were carried out. For the PTNP-only and PTNP + laser groups, 20 μL of 0.25 mg/mL of nanoparticles were applied to an inoculated skin wound. After an incubation time of 10 minutes, while the mice were under anesthesia, the infected wound with the associated nanoparticles and laser only group were exposed to the laser treatment (808 nm NIR laser, at 0.7 W/cm²) for a duration of 5 minutes. Other groups were treated with the same condition, but without the NIR laser irradiation. Animals were euthanized after the treatment and in vivo imaging. For the treated and untreated wounds were extracted for bacterial plate count. The surface temperature of wounded mice was also monitored before treatment, after 1, 2, 3, 4, and 5 minutes of treatment using an FLIR 1 thermal imaging camera (FLIR Systems).
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