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Controlled phage therapy by photothermal ablation of specific bacterial species using gold nanorods targeted by chimeric phages

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The use of bacteriophages (phages) for antibacterial therapy is under increasing consideration to treat antimicrobial-resistant infections. Phages have evolved multiple mechanisms to target their bacterial hosts, such as high-affinity, environmentally hardy receptor-binding proteins. However, traditional phage therapy suffers from multiple challenges stemming from the use of an exponentially replicating, evolving entity whose biology is not fully characterized (e.g., potential gene transduction). To address this problem, we conjugate the phages to gold nanorods, creating a reagent that can be deployed upon use (termed “phanorods”). Chimeric phages were engineered to attach specifically to several Gram-negative organisms, including the human pathogens Escherichia coli, Pseudomonas aeruginosa, and Vibrio cholerae, and the plant pathogen Xanthomonas campestris. The bioconjugated phanorods could selectively target and kill specific bacterial cells using photothermal ablation. Following excitation by near-infrared light, gold nanorods release energy through nonradiative decay pathways, locally generating heat that efficiently kills targeted bacterial cells. Specificity was highlighted in the context of a P. aeruginosa biofilm, in which phanorod irradiation killed bacterial cells while causing minimal damage to epithelial cells. Local temperature and viscosity measurements revealed highly localized and selective ablation of the bacteria. Irradiation of the phanorods also destroyed the phages, preventing replication and reducing potential risks of traditional phage therapy while enabling control over dosing. The phanorod strategy integrates the highly evolved targeting strategies of phages with the photothermal properties of gold nanorods, creating a well-controlled platform for systematic killing of bacterial cells.

Significance

New methods for detecting and killing antibiotic-resistant, Gram-negative bacteria are of prime interest for a wide variety of applications. While phages have long been considered as potential antibacterial agents, many concerns about phage therapy stem from the fact that phages are replicating, evolving entities whose biology is poorly understood in most cases. These concerns could be addressed by destroying the phage immediately upon use. We accomplish this by conjugating phages to gold nanorods, whose excitation by near-infrared light causes localized heating that essentially cooks nearby bacteria. Thus, the phages deliver gold nanorods to the targeted bacteria, and the nanorods destroy both bacteria and phages simultaneously. This strategy transforms phages from an evolving biological entity into a controlled, drug-like reagent.
“biological window” for which soft tissues are somewhat transparent. Other nanomaterials also exhibit nonspecific cytotoxic properties (e.g., nanosilver) through a variety of chemical mechanisms, but a general problem with the application of nanomaterials against bacterial infections is their lack of specificity against bacterial vs. mammalian cells, presenting a general challenge for biocompatibility (25).

To confer specificity to nanostuctures, one may conjugate antibodies that target specific bacterial strains (26), following upon extensive work targeting nanoparticles for cancer cell treatment (27–30). However, phage-based strategies possess several advantages compared to antibody-based strategies. First, greater delivery of nanoparticles per bacterial receptor could be achieved using phages due to the comparatively large surface area of phage, which may accommodate multiple nanoparticles; this property could be useful if bacterial receptors are in low abundance. A related benefit is that the aggregation of nanoparticles with phages on bacteria produces a visible shift in the LSPR spectrum (31), and one might therefore envision applications that combine treatment and detection of bacteria. Second, in addition to the targeting mechanisms evolved by whole phages as described above, chimeric phages can be rationally designed to achieve specificity against different bacterial hosts (8). This potential is largely untapped, as there exists a mostly uncharacterized biological reservoir of phages that could presumably target many different bacterial strains (32). While phages are well known for their host specificity, a number are broad in host range (33), suggesting that the degree of specificity could be tuned depending on the desired application. Third, in practical terms, phages are inexpensive to produce and have typically evolved some hardiness to nonideal environmental conditions. These features make phage-based nanotechnology attractive for biotechnological and biomedical applications.

In this work, we investigated the ability of phage–AuNR bioconjugates (phage–AuNRs; Fig. 1A) to specifically attach to and then kill targeted bacterial cells via the photothermal effect. In this scheme, the phage confers specific targeting while the AuNRs achieve the desired effect. Using chimeric phages we previously engineered (31) to target several bacterial pathogens (two strains of Escherichia coli, Pseudomonas aeruginosa, Vibrio cholerae, and two strains of the plant pathogen Xanthomonas campestris), we first show that the phage–AuNRs can be used to detect specific bacteria through phage-mediated aggregation of AuNRs on the bacterial surface, which causes a red shift of the longitudinal LSPR peak. Next, we use NIR irradiation to induce death of the targeted bacteria via photothermal heating, both in solution and in a P. aeruginosa biofilm grown on a substrate of mammalian epithelial cells. Photothermal lysis was highly selective and resulted in extensive killing of targeted bacteria within minutes, with low rates of damage to nontarget bacteria and mammalian cells (Fig. 1B). After photothermal lysis, the phages are no longer capable of replication, allowing control over dosage in principle. The potential of phage–AuNRs for treatment and diagnosis of antibiotic-resistant bacterial infections is discussed.

Results

Construction of Phage–AuNR Bioconjugates. The AuNRs were synthesized following a typical seed-mediated protocol (34), resulting in uniform particles with an average aspect ratio of 3.8 (average length, 53.2 nm; average width, 13.7 nm; SI Appendix, Fig. S1A). The UV-vis spectrum of the AuNRs demonstrated transverse and longitudinal absorption peaks at 526 and 800 nm (35), respectively (SI Appendix, Fig. S1B). The capsid of M13KE phage was modified with SATP to introduce thiol groups to primary amines. Thiolation resulted in new signals by Fourier-transform infrared (FTIR) spectroscopy (SI Appendix, Fig. S2A), indicating successful modification of the virions, designated M13KE-SH. The overall morphology of the phage, assessed by transmission electron microscopy (TEM), was not affected by thiolation (SI Appendix, Fig. S2 B and C).

Surface modification of gold nanostructures under aqueous conditions has been widely reported (36–38). M13KE-SH was conjugated to AuNRs by formation of gold–sulfur bonds at room temperature in Tris buffer (pH 3.0) (39). Interaction between

![Fig. 1. Schematic of phage–AuNR bioconjugates for bacterial detection and cell killing. (A) Filamentous phage M13 (gray) was engineered to express the receptor-binding protein from a foreign phage (blue) fused to wild-type g3p to obtain retargeted chimeric phage. Chemical modification by N-succinimidyl-3-acetylthiopropionate (SATP) introduced thiol groups (yellow) along the phage coat, and gold nanorods (AuNRs) were conjugated to the phage via thiol–gold bonds. (B) Phage–AuNR bioconjugates reorganize specific bacteria (blue) in the context of mammalian cells and other nontarget bacteria (green). Attachment of phage triggers AuNR aggregation on the cell surface, resulting in a red-shifted LSPR spectrum (indicated as magenta nanorods) for bacterial detection. Exposure to NIR light induces photothermal heating of AuNRs, leading to highly elevated temperatures localized by the phage, resulting in death of the targeted bacteria.](image-url)
AuNRs and phages during bioconjugation was promoted by the positive charge from trace CTAB on the AuNRs (ζ = 21.9 mV) and the negatively charged capsid protein of phage particles (ζ = −44.3 mV). The formation of Au–S bonds in the bioconjugates was confirmed by X-ray photoelectron spectroscopy (XPS) (SI Appendix, Fig. S1 C and D). Trace CTAB was then replaced by ligand exchange with carboxylated PEG (HS-PEG-COOH) after bioconjugation. Formation of bioconjugates was also confirmed by TEM (Fig. 2A), which indicated ~10 AuNRs per phage. Another approach to estimate the ratio of AuNRs to phage particles is inductively coupled plasma mass spectrometry (ICP-MS) to measure the amount of AuNRs and qPCR to measure the amount of phage in a sample; this approach indicated ~20 AuNRs per phage. Thus, an estimate of the number of AuNRs conjugated per phage particle is roughly 15.

The UV-vis spectrum of the bioconjugates indicates a red shift of ~10 nm compared to AuNRs alone (SI Appendix, Fig. S1B). The negatively charged surface of HS-PEG-COOH-modified M13KE–AuNR (ζ = −28.8 mV) should reduce nonspecific binding to bacteria considering the negatively charged cell surface (ζ = −8.88 mV). TEM demonstrated that while HS-PEG-COOH-modified AuNRs do not attach to E. coli cells in the presence of nonconjugated M13KE (Fig. 2B), phage–AuNRs attach to E. coli cells (Fig. 2C), as expected. To further confirm that the M13KE–AuNR bioconjugates retain the ability to interact with E. coli, M13KE–AuNRs were labeled with a fluorescent dye using fluorescein-5-maleimide (FITC) through thiol-maleimide click chemistry, as M13KE also confirmed by TEM (Fig. 2A) and also confirmed by TEM (Fig. 2C and D), simplifying the detection protocol to a single-step addition of the bioconjugates to the cell sample in appropriate solution. E. coli ER2738 was suspended at varying concentrations in MilliQ water and incubated with M13KE–AuNRs for 30 min. Consistent with prior results using AuNRs, a red shift and broadening of LSPR peaks of the AuNRs were observed in the presence of ≥109 bacterial cells (SI Appendix, Fig. S1B), demonstrating the sensitivity of bacterial detection using phage–AuNRs.

Five chimeric phages recognizing other Gram-negative bacterial strains (I+ E. coli, P. aeruginosa, V. cholerae, and two strains of X. campestris) were propagated in E. coli cells, as previously described (31), and functionalized with AuNRs, as described above. As seen with M13KE–AuNRs targeting E. coli (SI Appendix, Fig. S1B), the sensitivity of detection for these other strains was ~107 CFU using the respective chimeric phage–AuNRs (Fig. 3A and SI Appendix, Fig. S4). To verify the specificity of each of the six phage–AuNRs for its respective host, we incubated each phage–AuNR with the other bacterial strains. For each phage–AuNR, no shift or broadening of the LSPR peaks appeared when nonhost strains were added (Fig. 3B and SI Appendix, Fig. S5), indicating little cross-reactivity among the tested group of Gram-negative organisms. The detection assay was also performed in a mixture of the host strains, and no change of the LSPR peaks was observed unless the heterogeneous mixture contained the targeted host cells (Fig. 3C and SI Appendix, Fig. S6). These results confirm the ability of the chimeric phages to target the AuNRs to their particular bacterial host.

**Photothermal Ablation of Bacterial Cells in Suspension.** The plasmonic resonance of AuNRs converts light into heat, which can be used to damage and kill cells within a submicrometer to micrometer radius (40). Samples containing AuNRs or phage–AuNRs or neither were irradiated by a NIR laser (peak at 808 nm) for 10 min, and the bulk temperature of the solution was measured by a thermocouple (SI Appendix, Fig. S7A). In a control sample of water without AuNRs or phage–AuNRs, some heating (from 24 to 37 °C) occurred from the laser alone. However, all solutions containing AuNRs [AuNRs (equivalent to 3.3 nM AuNRs), M13KE–AuNRs (equivalent to 3.3 nM AuNRs and 1011 phages per mL), or M13KE–AuNRs mixed with E. coli

Fig. 2. Interaction between M13KE, AuNR, and E. coli cells. TEM image of M13KE–AuNR (A) illustrates conjugation of filamentous phage and AuNRs. When E. coli cells were mixed with M13KE and HOOC-PEG–AuNR (nonconjugated), no aggregation or localization of AuNRs to the cells was seen (B), but HS-PEG-COOH-modified M13KE–AuNR bioconjugates attached to E. coli cells did result in visible aggregation of AuNRs on the cell surface (C). Aggregation at one end of the bacterium (in C) presumably occurs near the position of the F pilus; stimulation of retraction by phage attachment may cause accumulation at the root of the pilus (57, 58).
ER2738 (10^6 cells per mL) reached bulk temperatures of 77 to 81 °C. A sample containing M13KE–AuNRs alone reached 81 °C, while a sample containing M13KE–AuNR mixed with E. coli reached a lower temperature (77 °C). This slight reduction is expected due to the aggregation of M13KE–AuNRs on the cells (see preceding section). That is, since aggregation leads to a broadened and red-shifted LSPR peak, absorption at the laser wavelength (808 nm) is reduced (SI Appendix, Fig. S7B) and leads to slightly less efficient heating when phage–AuNRs are aggregated on the target cells. Plating of samples containing M13KE–AuNRs mixed with E. coli ER2738 and irradiated demonstrated that roughly 50% of bacteria were killed by 3 min, ~80% of bacteria were killed by 6 min, and no viable bacteria remained after 10 min (Fig. 4 A and C). Similar results were observed using all six phage–AuNR bioconjugates to kill their respective host bacterial cells (Fig. 4 B and C and SI Appendix, Fig. S8). TEM imaging of M13KE–AuNRs mixed with E. coli ER2738 cells and irradiated demonstrated grossly altered cell morphology (SI Appendix, Fig. S9A). A live/dead cell-staining assay further verified >99% bacterial cell death after 10 min of irradiation by microscopy (SI Appendix, Fig. S10).

In principle, cell death should occur primarily for the targeted host organism bound by the phage–AuNRs. However, nontargeted cells may also die as the temperature of the bulk solution increases or if they are bound nonspecifically by phage–AuNRs. As expected, irradiation of bacteria with nonconjugated AuNRs resulted in gradual cell death (SI Appendix, Fig. S11), presumably from bulk heating of the solution by irradiated AuNRs, and this rate of cell death was substantially slower than the rate of death using targeted phage–AuNRs (Fig. 4C). Irradiated bacteria (without AuNRs) showed no significant cell death compared to nonirradiated bacteria, indicating that irradiation alone is not toxic (SI Appendix, Fig. S12). To test the efficiency and specificity of bacterial cell death in the context of a bacterial mixture, F^+ E. coli cells (ER2738; host for M13KE) that express cyan fluorescent protein (10^6 cells per mL) were mixed with F^- E. coli cells (BL21; lacks receptor for M13KE) that express citrine fluorescent protein (10^6 cells per mL), incubated with M13KE–AuNRs (10^13 phages per mL), and irradiated to induce photothermal lysis (12). Samples were plated and viable colonies were counted. The concentration of F^- E. coli (targeted strain) decreased sharply, with no colony-forming units at 10 min (Fig. 4D and SI Appendix, Fig. S13). In contrast, the concentration of F^+ E. coli (nontarget strain) changed only slightly compared to control (Fig. 4D and SI Appendix, Fig. S13). This confirms that the phage–AuNRs distinguished bacterial strains as expected and selectively killed the targeted cells.

While the bulk temperature increases upon irradiation, binding of phage–AuNRs to bacterial cells should induce localized heating of the cell. To estimate the temperature of the bacteria, E. coli ER2738 were stained with the temperature- and pH-sensitive dye BCECF, whose fluorescence intensity decreases linearly with temperature (SI Appendix, Fig. S9 B and C). The steady-state fluorescence intensity of BCECF was recorded during irradiation of E. coli ER2738 with M13KE–AuNRs. The apparent cell temperature reached a plateau of ~83 °C after 3 min and rose more quickly than the bulk temperature, being higher than the bulk temperature at all observed time points. The temperature gap between cell temperature and bulk temperature (measured by thermocouple) was observed to be ~13 °C at 3 min (SI Appendix, Fig. S9D). It should be noted that bulk heating observed depends on the concentration of AuNRs as well as heat dissipation properties of the medium and cuvette. The BCECF measurement is also likely to underestimate the true bacterial cell temperature since some dye is also dissolved in the bulk; thus, it should be

Table 1. Chimeric phage bioconjugates and targeted bacterial species

| Bacterial target strain | Source of RBP | Designation of bioconjugates |
|-------------------------|--------------|-----------------------------|
| E. coli (F^-), ER2738   | Wild-type M13| M13KE–AuNR                  |
| V. cholerae 0395        | CTX^ϕ         | M13-g3p(CTX^ϕ)–AuNR         |
| E. coli (I^I), (Migula) Castellani and Chalmers | If1 | M13-g3p(If1)–AuNR |
| X. campestris (pv. camppestris) | qLf | M13-g3p(qLf)–AuNR |
| X. campestris (pv. vesicatoria) | qLxv | M13-g3p(qLxv)–AuNR |
| P. aeruginosa (Schroeter) Migula | Pf1 | M13-g3p(Pf1)–AuNR |

See ref. 31 for additional details.

Fig. 3. Detection of P. aeruginosa. (A) UV-vis spectra of AuNR (black), phage-AuNR (red), and phage–AuNR with P. aeruginosa at 10^2, 10^4, and 10^6 CFU (blue, magenta, and green lines, respectively) are shown; (B) Specificity of P. aeruginosa detection when incubated with different bacterial species (10^4 CFU). Bacterial species shown are E. coli (F^-) (blue), V. cholerae (magenta), X. camppestris (pv camppestris) (green), X. camppestris (pv vesicatoria) (navy), E. coli (I^I) (cyan), and P. aeruginosa (purple). (C) Sensitivity of P. aeruginosa detection in the context of a mixture of bacteria [E. coli (F^-), V. cholera, X. camppestris (pv vesicatoria), X. camppestris (pv camppestris), and E. coli (I^I)]. The target cells P. aeruginosa were present in the amount indicated in the legend (10^2 (blue), 10^4 (magenta), 10^6 (green) CFU); the other bacterial species were present at 10^6 CFU each. The spectra of AuNRs (black) and M13-g3p(Pf1)–AuNR bioconjugates (red) are also shown.
regarded as a lower bound for bacterial cell temperature. In addition, pH is assumed to be constant during irradiation, such that the fluorescence change is attributed to temperature changes. Nevertheless, this measurement validates the qualitative expectation that the targeted cells are heated beyond the level of the bulk solution.

Photothermal Ablation of *P. aeruginosa* in Biofilms. Biofilms present an important obstacle to antibiotics and other therapeutic strategies due to the dense macromolecular network and altered physiological state of the biofilm cells. To determine whether photothermal ablation could be effective against bacterial biofilms, we grew *P. aeruginosa* in a standard biofilm format on glass-bottom plates (41), incubated the biofilm with M13-g3p(Pf1)–AuNRs (10¹³ phages per mL), removed excess liquid by pipetting, and irradiated as described above for 10 min. Live/dead staining of the biofilm showed widespread bacterial cell death (Fig. 5A and B). The green dye SYTO 9 enters both live and dead cells, while the red dye propidium iodide (PI) only enters compromised cell membranes (dead cells). Object-based colocalization analysis was used to determine the fraction of cells (green) that also stained red; this analysis confirmed extensive cell death (Fig. 5A and B and SI Appendix, Fig. S14). In addition, no colonies were obtained after resuspension and plating of the irradiated biofilm (SI Appendix, Fig. S15), indicating that no viable cells remained. The extent of cell death during irradiation was also quantified by the PrestoBlue cell viability assay, which confirmed that viable cells were undetectable after 10 min of irradiation (Fig. 5C).

To assess the effectiveness of phage–AuNRs against more mature biofilms, we grew *P. aeruginosa* biofilms for longer times (24 and 48 h), resulting in increased extracellular polymeric substance (EPS) formation verified by crystal violet staining (SI Appendix, Fig. S16). Cell killing was slightly slower for more mature biofilms, but still there was no detectable viability after 10 min of irradiation (Fig. 5C). To gain a rough estimate of the temperature of the biofilm after NIR irradiation, the biofilms were stained with BCECF. To create a calibration curve, a series of fluorescent images was recorded at different temperatures using a confocal microscope (SI Appendix, Fig. S17A), and the pixel intensity (measured by ImageJ) was plotted as a function of temperature (SI Appendix, Fig. S17B). The average bacterial cell temperature captured a few seconds after 10 min of NIR irradiation was estimated to be 84 °C using this calibration curve (SI Appendix, Fig. S17C), indicating similarly efficient heat transfer from the AuNRs to bacterial cells in the biofilm compared to solution.

Photothermal Ablation of *P. aeruginosa* Biofilm Grown on Mammalian Epithelial Cells. While phage–AuNR-mediated heating was effective for killing bacterial cells, it is possible that heat transfer to surrounding mammalian cells could be deleterious. We grew a *P. aeruginosa* biofilm directly on top of a monolayer of Madin-Darby Canine Kidney II (MDCKII) mammalian epithelial cells (42) and determined the survival of both the bacterial cells and the MDCKII cells after application of M13-g3p(Pf1)–AuNRs with irradiation performed as described above. Microscopy with live/dead staining demonstrated that bacterial cells in the biofilm were killed while MDCKII cells survived, with extensive (~98%)
biofilm adsorbing the M13-g3p(Pf1)–g3p(Pf1) treated and irradiated bacterial biofilm without MDCKII cells negligible fluorescence intensity was observed for an identically for 10 min, could be attributed to the MDCKII cells because of the MDCKII cells before exposure to M13-g3p(Pf1)

Fig. 7B). To further probe the effect of phage–AuNRs on the bacteria and MDCKII cells, we characterized the viscosity of cell membranes using a molecular rotor, a dye whose fluorescence lifetime provides a measurement of local microviscosity. The viscosity of the cell membrane is expected to decrease upon intense heating, leading to destruction of membrane order. We stained the MDCKII/ P. aeruginosa biofilm with the molecular rotor BODIPY C10 and used fluorescence lifetime imaging (FLIM) (43) to assess membrane viscosities after photothermal treatment (Fig. 8 and SI Appendix, Fig. S20). While the MDCKII cells did not exhibit substantial change in fluorescence lifetime after irradiation (2.31 ± 0.17 ns before irradiation; 2.23 ± 0.21 ns after irradiation), the fluorescence lifetime of the dye on P. aeruginosa cells decreased from an average of 2.36 ± 0.12 to 0.92 ± 0.09 ns, corresponding to a dramatic drop in viscosity from 296 to 38 cP (see Eq. 1). This finding is consistent with the idea that the phage–AuNRs directly target the bacterial host cells with relatively little damage to other cells.

To verify whether NIR irradiation destroyed the infectious potential of the phages, M13KE–AuNRs were irradiated for 10 min and then used to infect E. coli for phage propagation. Putative viral DNA was extracted and assayed by qPCR (31). No DNA was detected from propagation of the treated sample (SI Appendix, Fig. S21). During photothermal ablation in solution, αLPS–AuNRs killed cells significantly more slowly than M13KE–AuNRs (50% of cells killed after ∼6 min of irradiation for αLPS–AuNRs compared to <3 min for M13KE–AuNRs) (SI Appendix, Fig. S23A). The cell-killing activity of the conjugates against target cells in the context of a large excess of nontarget cells was also compared for M13KE–AuNRs vs. αLPS–AuNRs. We mixed F+ E. coli (target) with F− E. coli (nontarget) in a frequency of 10−6. In the mixture, irradiation with M13KE–AuNRs eradicated the F+ cells (no detected colonies) while leaving most F− cells alive, as expected. However, αLPS–AuNRs, which are not expected to discriminate between F+ and F− cells, indeed killed both types of cells to the same degree. Moreover, αLPS–AuNRs were not able to eradicate either cell type, with ∼30% of cells surviving (SI Appendix, Fig. S23B).

Comparison to AuNRs Conjugated to Anti-LPS Antibodies. The optical and cell-killing properties of M13KE–AuNRs were compared to those of AuNRs conjugated to a commercially available monoclonal antibody against the lipopolysaccharide (LPS) of E. coli (αLPS–AuNRs; SI Appendix, Methods). In these experiments, the concentration of AuNRs was kept constant between samples with M13KE–AuNRs and samples with αLPS–AuNRs. Attachment of αLPS–AuNRs to E. coli was verified by TEM (SI Appendix, Fig. S22 A and B). During photothermal ablation in solution, αLPS–AuNRs killed cells significantly more slowly than M13KE–AuNRs (50% of cells killed after ∼6 min of irradiation for αLPS–AuNRs compared to <3 min for M13KE–AuNRs) (SI Appendix, Fig. S23A). The cell-killing activity of the conjugates against target cells in the context of a large excess of nontarget cells was also compared for M13KE–AuNRs vs. αLPS–AuNRs. We mixed F+ E. coli (target) with F− E. coli (nontarget) in a frequency of 10−6. In the mixture, irradiation with M13KE–AuNRs eradicated the F+ cells (no detected colonies) while leaving most F− cells alive, as expected. However, αLPS–AuNRs, which are not expected to discriminate between F+ and F− cells, indeed killed both types of cells to the same degree. Moreover, αLPS–AuNRs were not able to eradicate either cell type, with ∼30% of cells surviving (SI Appendix, Fig. S23B).

The LSPR spectral shift was also compared for αLPS–AuNRs vs. M13KE–AuNRs. αLPS–AuNRs show a spectral shift with a similar limit of detection (LOD ∼ 102 CFU) as M13KE–AuNRs at neutral pH (SI Appendix, Fig. S22). However, M13KE–AuNRs tolerated both acidic and alkaline conditions (pH 3 and pH 10), but the LOD of αLPS–AuNRs increased by 4 orders of magnitude under these conditions. In addition, M13KE–AuNRs tolerated heat treatment (60 °C for 15 min) while αLPS–AuNRs did
not (LOD increased by 2 orders of magnitude). In acidic and alkaline (pH 3 and pH 10) conditions, cell killing by M13KE–AuNRs was maintained at high efficiency while αLPS–AuNRs lost activity (SI Appendix, Fig. S23A). These observations are consistent with the fact that many factors (e.g., temperature, pH, ionic strength, growth media) can disrupt the antigen–antibody interaction (44–46), while phage–host interactions appear to be more robust to environmental factors.

**Discussion**

We present an antibacterial strategy using phages conjugated to AuNRs (phage–AuNRs, referred to in the following discussion as “phanorods,” a portmanteau of “phage” and “nanorods”). The phages attach to targeted bacteria, and irradiation of the nanorods by NIR light causes LSPR excitation. This energy is released as heat, destroying the phage as well as bacteria bound to the phage. The phanorod strategy has important advantages over traditional approaches to phage therapy. First, phage therapy suffers from the major difficulty of managing a replicating and evolvable entity. While the evolutionary capacity of phages is advantageous for overcoming bacterial resistance against a phage, evolutionary potential is an important biocontainment concern in practice. Second, nonlinear replication dynamics mean that dosages cannot be easily controlled, which may be problematic if cell lysis releases endotoxins triggering deleterious host responses (e.g., septic shock). Phanorods are destroyed during irradiation, preventing replication and evolution during treatment and enabling control over dosage. Irradiation could also be used to inactivate excess phanorods after use, avoiding negative impacts, such as evolution of resistant organisms, currently associated with antibiotics in the waste stream. Third, evolution of resistance is an important challenge for any antibacterial strategy, including phanorods. However, because the phage is used only for attachment to cells and downstream events (e.g., replication) are not relevant, bacterial mechanisms for resistance should be limited to alterations of the receptor, presenting a smaller mutational target for evolution of resistance. Fourth, phanorods serve simultaneously as diagnosis and cytotoxic reagents, as the change in the LSPR spectrum can be used to recognize bacterial species. Therefore, although there may be situations in which therapy with phages per se is desired (e.g., if exponential replication dynamics are needed), phanorods may be advantageous for most therapeutic situations.

Conversely, one may consider how phanorods compare to antibody-conjugated nanorods. In addition to unique search mechanisms (see Introduction), phages can possess very high affinities...
Infections or colonization of medical devices, in which the phage cations could be localized topical therapy, particularly for wound treatment of directly accessible tissues or surfaces. Near-term applications are characterized by the ongoing evolutionary thus chemical modification, which characterize phages, are traits under selection by the range of pH tested (pH 3 to 10) as well as heat treatment. We also demonstrated that phanorods were effective in killing a P. aeruginosa biofilm grown on epithelial cell culture (see SI Appendix, Text S2 for further discussion). Some photothermal damage was incurred by epithelial cells, although the viability measured here is likely a lower bound since the biofilm was in direct contact with the monolayer; underlying cellular layers in a physiological context would likely sustain less damage. The phanorods used here absorb in the relatively biologically transparent window of NIR light. In principle, irradiation could be directed only toward areas where activation of the phanorods is desired, reducing potential side effects. Whether iterative phanorod application could be effective in treating thicker and deeper biofilms (e.g., abscesses) without substantial harm to surrounding tissue is an important practical issue. In addition, while excess phanorods could be removed by washing for certain wounds, a consideration for other applications is the in vivo biodistribution of phanorods in the absence of target bacteria. Nevertheless, phanorods and other alternative strategies merit consideration given the current need to develop new antibiotic agents.

Methods

Materials. Reagents were obtained from the following sources: gold(III) chloride trihydrate (HAuCl₄·3H₂O) (99.9%; Sigma), sodium borohydride (NaBH₄) (98%; Fisher Scientific), trisodium citrate dihydrate (99.9%; Sigma), Escherichia coli (Migula) Castellani and Chalmers (ATCC27065, ATCC), E. coli ER2738 (NEB), Xanthomonas campestris pv. campestris (ATCC33931), Xanthomonas campestris pv. vesicatoria (ATCC35937), Pseudomonas aeruginosa ( Schroeter) Migula (ATCC 25102), Vibrio cholerae 0395 (donation from Dr. Michael J. Mahan, University of California, Santa Barbara, CA), M13KE phage (NEB), M13-NotI-Kan construct (12), sodium chloride (NaCl) (99%; Fisher BioReagents), trypticase soy broth (TSB) (98%; Sigma), α-macroglobulin anti-E. coli LPS antibody ([2D7/1] ab35654; Abcam), 2′,7′-bis-(2-carboxyethyl)-5-(and)-6-carboxyfluorescein (BCECF) (Invitrogen), 5-bromosalicylic acid (5-BAA) (>98.0%; Sigma), SYTO 9, PI (Thermo Fisher Scientific), BODIPY C10 (donation from Dr. M. Kuimova from Imperial College London, London, UK), poly(ethylene glycol) (PEO-8000; Sigma), di-alkylation (MWCO 3500 Da; Spectrum Labs), tetraacycline (98%; Fisher Scientific), kanamycin sulfate (98%; Fisher BioReagents), ampicillin (98%; Fisher Scientific), isopropyl-1-thiogalactopyranoside (IPTG) (99%; Fisher Scientific), Mix & Go competent cells (Zymo Research), QIAprep Spin Miniprep Kit (Qiagen), QIAquick Gel Extraction Kit (Qiagen), KpnI-HF/NcoI-HF restriction enzyme and T4 DNA ligase (NEB), and thiol-PEG–acid (HOOC-PEG-5H; Sigma, average M, 5,000; Sigma).

Chimeric Phages. The construction of the chimeric phages used here [M13-g3p(CX)], M13-g3p(P1)], M13-g3p(If1)], M13-g3p(If1), and M13-g3p(P1]) was previously reported (31). Phages were propagated according to standard protocols. Phage concentrations were quantified by real-time PCR as previously described (31). See SI Appendix for more details.

Thiol Functionalization of Phages. Phages were treated with SATP for chemical modification of accessible amine groups of the capsid based on a known protocol (56). See SI Appendix for more details.

Synthesis of AuNRs. The AuNRs were synthesized through a modified method by Murray and coworkers (34). See SI Appendix for more details.

Preparation of Phage–AuNR Bioconjugates. Conjugation of thiolated phage with AuNRs was conducted based on a known bioconjugation method (39).
The AuNRs were resuspended in Tris buffer (50 mM, pH 3). Two hundred microliters of thioloated phage (1 × 10^11 pfu/mL) was added dropwise to 1-mL AuNRs solution (6.8 mM). The suspension was incubated at room temperature for 2 h under moderate rotation. The phage-AuNR conjugates were purified by repeated centrifugation/resuspension cycles (8,000 rpm for 30 min, resuspension in 1 mL of water). The reaction was monitored by UV-vis spectrophotometry (Shimadzu UV-1800) and Zetasizer APS (Malvern) to follow changes of LSPR and zeta potential. The trace amount of residual CTAB was further exchanged with HOOC-PEG-SH (2.0 μM) under the same conditions for 24 h. The phage–AuNR bioconjugates were purified by centrifugation and resuspended in 200 μL of water.

**Visualisation of Binding of M13KE-AuNR to E. coli.** 10^11 M13KE–AuNR bioconjugates (i.e., containing 10^11 M13KE phages) were incubated with 2 ng/mL fluorescein–S-maleimide in 1 mL of PBS buffer (pH 7.0) with gentle stirring at room temperature overnight. The free dye was removed by extensive dialysis (MWCO 3,500 Da) in 500 mL of PBS buffer (pH 7.0), and bioconjugates were concentrated by ultrafiltration to ~200 μL with an Amicon Ultra-4 10,000 filter. The M13KE–AuNR bioconjugates were incubated with 1 mL of Top 10F– cells expressing cyan fluorescent protein at an optical density of ~0.6 for 30 min at room temperature (12). Free bioconjugates were removed by centrifugation at 5,000 rpm and discarding the supernatant. The pellets were washed twice by resuspending in 1 mL of PBS buffer for microscopy. The fluorescence images were recorded on a Leica SP8 confocal microscope (Leica), with excitation at 405 nm.

**Detection of Bacteria with Phage–AuNR.** Bacterial cells (strains of E. coli, V. cholerae, P. aeruginosa, and X. campestris listed above) were grown in liquid culture and cell concentrations estimated by optical density as previously described (31) (SI Appendix, Methods). The cells were collected by centrifugation and resuspended in water at the desired concentrations. Fifty microliters of bacterial solution was added into 100 μL of phage–AuNR bioconjugate solution (~10^11 phage particles per mL) in 1.5-mL tubes. The absorbance of the solutions was recorded by UV–vis spectroscopy (Shimadzu UV-1800) after a 30-min incubation at room temperature. The specificity of detection was tested by adding a different bacterial species or strain from each of the sources of bacteria listed above, mixing 10 μL of each bacterial strain with 10 μL of the AuNR conjugates (prepared as described above) and incubating for 30 min. The mixture was then incubated with M13-g3p (Pf1) at 37 °C for 30 min, and fluorescence signals were measured on a Tecan infinite 200 Pro plate reader (Tecan). The excitation wavelength was 560 nm (bandwidth, 9 nm), and the emission wavelength was 580 nm (bandwidth, 20 nm). Similar results were obtained using a 1-h incubation time. The cell viability was expressed as a percentage relative to the control cells (MDCCKII cells incubated with PBS buffer with no phages or phage–AuNR under the same conditions).

**Photothermal Lysis of Bacteria with Phage–AuNRs.** To test thermolysis in aqueous solution, the mixture of bacterial solution and phage–AuNRs (10^11 phages, and 10^6 cells in 1 mL solution) was irradiated for various time periods (0 to 10 min) using an 808-nm diode laser (3.0 W/cm²; Q-BAIHE Company) ~8 cm from the top of the solution. After irradiation, a 10-μL aliquot of the resulting solution was diluted into PBS buffer (0.1 M, pH 7.4) to a ratio of 1:1,000 and cultured on plates for colony counting using ImageX. X. campestris (pv campestris) and X. campestris (pv vesicatoria) were cultured on YPD agar plates with no antibiotics; the other cells were cultured on LB plates. After the bacteria were cultured on the plates containing 10 μg/mL tetracycline. The E. coli strains expressing cyan or citrine fluorescent proteins (12) were cultured on LB plates containing 1 mM IPTG and 100 μg/mL ampicillin.

To test thermolysis of a P. aeruginosa biofilm grown on solid support, the biofilm was prepared using a protocol modified from the literature (41). A single colony of P. aeruginosa was selected and grown in LB overnight at 37 °C in a shaker incubator. The overnight culture was diluted 100-fold into fresh medium. One hundred fifty microliters of the dilution was added to Lab-Tek plates (culture area, 0.7 cm²) and incubated overnight at 37 °C. After incubation, the liquid was removed by turning the plate over and shaking out the liquid. The remaining biofilm was washed by submerging the plate in a small tub of water and shaking out water twice. Three hundred microliters of M13-g3p(Pf1)–AuNR bioconjugates (10^11 phages per mL) were added into the biofilm and incubated for 30 min. Unbound bioconjugates in suspension were removed by pipetting. The biofilm was irradiated with the NIR laser for 10 min as described above. Cell viability was studied by growing the resuspended bacteria on LB plates (1 μL of cell suspension was diluted in 1 mL of PBS buffer, and 5 μL of the dilution was plated onto LB plates) for colony counting, and by confocal microscopy with live/dead cell viability staining with SYTO9 and PI. To test thermolysis of a P. aeruginosa biofilm grown on mammalian epithelial cells, wild-type MDCCKII epithelial cells in suspension were seeded at 5.0 × 10^4 cells per well in eight-well chamber slide Lab-Tek plates and grown to confluency. Dulbecco’s modified Eagle’s medium (DMEM) (Thermo Fisher; 11885076) was supplemented with 10% FBS (Thermo Fisher; 10437036) and 1% penicillin–streptomycin (P/S) (Thermo Fisher; 15140122), and kept in an incubator at 37 °C. After 48 h, the cells were washed twice with PBS buffer to remove traces of P/S. The biofilm of P. aeruginosa was cultured on the top of the MDCCKII epithelial cells in 15 mM Hepes buffer (pH 7 with DMEM and 10% FBS) as described above. Planktonic cells were removed from the biofilm by careful pipette. Incubation with M13-g3p(Pf1)–AuNR (10^11 phages per mL) and NIR irradiation of the bacterial cells was performed as described above. Cell viability of the MDCCKII cells was assayed by the PrestoBlue cell viability assay, and bacterial and mammalian cells were assessed by confocal microscopy with live/dead cell viability staining with SYTO9 and PI.
equation, which was obtained by measuring the fluorescence lifetime of BODIPY C10 in different solutions of methanol/glycerol mixtures with known viscosities (43):

\[
\log \tau = \log T + 0.75614 + 0.4569
\]

Here, \( \tau \) is viscosity (in centipoises), and \( T \) is fluorescence lifetime (in nanoseconds).

**Concentration of AuNRs.** Single-particle ICP-MS was performed with an Agilent 7900 ICP-MS (Santa Clara) to determine the concentration of the AuNRs. The phage–AuNR bioconjugates were incubated with 5% nitric acid for a week to degrade the virus before the measurement. The analysis was carried out in a time-resolved analysis mode with an integration time of 100 µs per point and no settling time between measurements. The data analysis was conducted with the Agilent ICP-MS MassHunter software (version C01.04 Build S44.3) via single-nanoparticle application module.

**TEM.** TEM was performed on a Tecnai FEI G2 Sphera microscope (Materials Research Laboratory [MRL], University of California, Santa Barbara) as previously described (31). M13KE–AuNR samples were prepared as described above (Methods, Visualization of Binding of M13KE–AuNR to E. coli). The AuNR size was calculated by measuring 200 AuNRs.

**Zeta Potential Measurements.** Zeta potentials were measured by using a Malvern Zetasizer Nano ZSP operating a 4 mW He–Ne laser at 633 nm as previously described (31). Data from three or more individual samples were averaged, and each sample was measured five times (10 runs each).

See SI Appendix, Methods for description of additional optical characterisation, synthesis of AuNRs, colocalization analysis, antibody–AuNR experiments, chimeric phases, phage and bacterial propagation, and EPS quantitation.

**Data Availability.** All data are included in the manuscript and SI Appendix.

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