

Supporting Information

Synergistic Photothermal and Antibiotic Killing of Biofilm-associated *Staphylococcus aureus* using Targeted, Antibiotic-loaded Gold Nanoconstructs

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Experimental Details

Chemicals and materials Silver trifluoroacetate (AgTFA), sodium hydrogen sulfide (NaSH), hydrochloric acid (HCl, 99.999%), tetrachloroauric acid trihydrate ($\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$), sodium chloride (NaCl), and dopamine hydrochloride (99%) were purchased from Alfa Aesar. Poly(vinylpyrrolidone) (PVP, M.W.=55,000), phosphate-buffered saline (PBS), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), tris(hydroxymethyl)aminomethane (Tris), citric acid, trisodium citrate dehydrate, bicine, and anti-protein A antibody (catalog number P3775) were purchased from Sigma Aldrich. Ethylene glycol (EG) was purchased from J.T. Baker. Acetone was purchased from EMD. Daptomycin (Dap) was purchased from Cubist Pharmaceuticals. All experiments were performed using 18 M Ω H_2O unless specified otherwise. All chemicals were used as received.

Synthesis of Au nanocages (AuNCs). The AuNCs were synthesized by galvanic replacement reaction between Ag nanocubes and HAuCl_4 as previously described.¹ First, Ag nanocubes were synthesized by the polyol method.² Briefly, 50 mL EG was added to a 250-mL round-bottom flask equipped with a stirring bar and placed in an oil bath at 150 °C. After the temperature equilibrated (30~45 min), EG solutions of 0.6 mL of 3 mM NaHS, 5 mL of 3 mM HCl, 12.5 mL of PVP (0.25 g), and 4 mL of 282 mM AgTFA were sequentially added to the reaction flask. Once the LSPR peak reached ~440 nm (~35 min after addition of AgTFA), the reaction was quenched in an ice bath. Upon cooling, the product was collected by adding acetone to the reaction solution at a ratio of 5:1 and centrifuging at 6,000 rcf for 10 min. The resulting pellet was purified twice with H_2O and collected by centrifugation at 20,000 rcf for 10 min, and resuspended in 10 mL of H_2O for future use.

To synthesize AuNCs, 10 mL of H_2O was heated to boiling in a 50-mL round-bottom flask equipped with a stirring bar. To the boiling liquid, 1 mL of the Ag nanocubes described above

was added and subsequently 1 mM HAuCl₄ was added using a syringe pump at a rate of 45 mL/h until the LSPR maximum was at 750 nm. The AuNCs were purified by saturated NaCl solution to remove by-product AgCl, washed 3 times by centrifugation at 20,000 rcf for 10 min, and resuspended in H₂O at a concentration of 5 nM for future use.

Synthesis of PDA coated AuNCs (AuNC@PDA). The AuNC@PDA were prepared by self-polymerization of dopamine on the surface of AuNCs under basic conditions in the presence of O₂. Briefly, 3 mL of 5 nM AuNC aqueous suspension was diluted to 200 mL using Tris-buffered saline (20 mM Tris and 100 mM NaCl, pH = 9) in a 250-mL, 3-neck, round-bottom flask. The reaction flask was briefly flushed with O₂ and placed in a bath sonicator held at 4 °C with ice. Dopamine hydrochloride (0.2 mmol, 36.0 mg) was added to the flask, the vessel was sealed under 1 atm O₂, and the mixture was sonicated throughout the reaction until the LSPR had red-shifted ~50 nm (~75 min). After this reaction, the product was collected by centrifugation at 6,000 rcf for 10 min, washed with H₂O twice and recovered by centrifugation at 19,000 rcf for 10 min at 4 °C. The AuNC@PDA was resuspended in H₂O at a concentration of 6 nM for characterization and future use.

Loading of Dap to AuNC@PDA (AuNC@Dap/PDA). Dap was loaded to the AuNC@PDA under different conditions to prepare AuNC@Dap/PDA. Briefly, 1 nM AuNC@PDA was incubated with 1 mg/mL of Dap (0.6 mM) in 1 mL of 10 mM citrate buffer (pH = 2.2, 0 mM NaCl or pH = 7.8, 150 mM NaCl). The reaction was allowed to stir overnight at 4 °C in the dark. The product was collected, purified with PBS once and H₂O thrice, and re-collected by centrifugation at 19,000 rcf for 10 min to remove free Dap. AuNC@Dap/PDA was resuspended in H₂O at a concentration of 4 nM AuNCs for characterization and future use.

Conjugation of aSpa to AuNC@Dap/PDA (AuNC@Dap/PDA-aSpa). aSpa was conjugated to the surface of AuNC@Dap/PDA through the N-terminal amine by Michael addition to form AuNC@Dap/PDA-aSpa. Briefly, 1 nM AuNC@Dap/PDA were dispersed in 1 mL of 10 mM bicene buffer (pH = 8.5) and 0.1 nmol of aSpa was added to the solution. The reaction was allowed to proceed at 4 °C for 1 h. The conjugates were collected and washed 3 times with PBS by centrifugation at 19,000 rcf for 5 min at 4 °C. The conjugates were dispersed in PBS for future use and stored at 4 °C.

Characterization of nanoconstructs. Transmission electron microscopy (TEM) images were captured using a TEM microscope (JEOL 100cx) with an accelerating voltage of 100 kV. The hydrodynamic diameters and zeta potentials of nanoconstructs were determined using a dynamic light scattering instrument (Brookhaven ZetaPALS). The concentration of metals was determined using a flame atomic absorption (AA) spectrometer (GBC 932). UV-vis spectra were taken on a UV-vis spectrophotometer (Agilent Cary 50). Fluorescence spectra were recorded on a fluorimeter (Horiba FluoroLog3).

Two-photon imaging. Cultures of the *S. aureus* strains UAMS-1, LAC, and their isogenic *Spa* mutants were grown to an optical density (OD₅₆₀) of 1.0 in tryptic soy broth (TSB), which corresponds to $\sim 2 \times 10^8$ CFU/mL. This sample (40 μ L) was applied to a glass microscope slide demarcated with a hydrophobic pen and allowed to air dry. Bacteria were heat fixed before adding 90 μ L of PBS and 10 μ L of AuNC@PDA-aSpa or AuNC@PDA suspensions. After 30 min at room temperature, unbound reagents were removed by soaking in PBS 3 times for 10 min each time. The slides were blotted dry before adding 100 μ L of 40 $\times$ diluted 4',6-diamidino-2-phenylindole (DAPI, NucBlue Fixed Cell ReadyProbe Reagent, Molecular Probes) in PBS and incubating for 10 min. This stain was removed and the slides were washed by soaking in fresh

PBS 3 times for 5 min each time. The slides were blotted dry, 10 μ L of PBS was used as a mounting medium, and a #2 coverslip was applied and sealed with nail polish (Saved by the Blue, 230C). Two-photon images were acquired using a customized, four channel, resonant scanning, multiphoton microscopy platform (Thorlabs, USA) and a 40 $\times$ water immersion 0.8 NA objective (Nikon). Illumination was provided by a Mai Tai HP ultra-fast Ti:Sapphire laser (Spectra Physics). DAPI fluorescence was visualized using 10 mW excitation (as measured at the sample) at 700 nm and collecting from blue channel, which utilized a 466 nm center wavelength bandpass with a full width at half-maximum of 60 nm. AuNC luminescence was visualized using 10 mW excitation (as measured at the sample) at 800 nm and collecting from the red channel, which utilized a 607 nm center wavelength bandpass filter with a full width at half-maximum of 70 nm. Images were the average of 200 frames and processed identically using ImageJ (NIH). Quantitative analysis was performed by comparing the ratio of the pixel intensity from AuNC image (800 nm laser, red channel) to the pixel intensity of the DAPI image (700 nm laser, blue channel). The cells (n = 50) were analyzed and the intensity ratios were averaged for each sample.

Release of daptomycin. The release of Dap from the AuNC@Dap_{Lo}/PDA-aSpa and AuNC@Dap_{Hi}/PDA-aSpa was carried out at neutral pH under the near-infrared irradiation. Briefly, each construct was suspended in 200 μ L PBS (pH = 7.4) at a concentration of 0.4 nM on ice. Samples were irradiated by an 808-nm diode laser at a power density of 1.66 W/cm² for different periods of time up to 10 min. After irradiation, the samples were centrifuged briefly at 14,000 rpm for 3 min at 4 °C and the supernatants were collected. The concentration of Dap was then analyzed by ultra-performance liquid chromatography (UPLC, Waters Acquity). As

controls, a solution containing Dap and, if applicable, nanoconstructs digested with KCN, were also evaluated by UPLC.

Elution was performed using a mobile phase consisting of a gradient (90:10 to 10:90) of H₂O and acetonitrile (1% trifluoroacetate) through a phenyl stationary phase (BEH phenyl, Acquity) at a flow rate of 0.2 mL/min with ultraviolet detection at 262 nm. The gradient of mobile phase was as follows: from 0 to 2 min 90:10 of H₂O and acetonitrile; at 3 min 50:50 of H₂O and acetonitrile; at 5 min 40:60 of H₂O and acetonitrile; at 6 min 10:90 of H₂O and acetonitrile; and from 7 to 8 min 90:10 of H₂O and acetonitrile. Dap eluted after 3.5 min and peak integral was linear over the concentrations tested.

Thermal curve assessment. The temperature measurement was performed under the same conditions described above for assessment of Dap release, with temperature changes as a function of time recorded by a thermal couple inserted into the 200 μ L suspension or an infrared sensor.

Western blot analysis. Relative amounts of extracellular protein A (eSpa) was assessed using standardized cell-free supernatants as previously described.³ Briefly, eSpa was detected by Western blot using rabbit anti-Protein A (Sigma Chemical Co., St. Louis, MO) at a 1:4000 dilution as primary antibody. Secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO). Blots were developed using SuperSignal West Femto Chemiluminescent Substrate kit (Thermo Fisher Scientific, Rockford, IL).

Antimicrobial activity analysis. The *S. aureus* strains UAMS-1 and LAC were grown in TSB and diluted to an optical density (OD₅₆₀) of 0.05, which corresponds to 1×10^7 bacterial cells per mL, using TSB supplemented with 2.5 mM CaCl₂, which is required for the *in vitro* bactericidal

activity of Dap. This suspension (180 μ L) was placed in each well of a 96-well microtiter plate (1.8×10^6 cells per well). Prior to irradiation, the nanoconstruct suspension for each experimental group was sonicated (Bransonic 2800; Branson) and vortexed for 5 s to ensure homogenous dispersion. The appropriate nanoconstruct (20 μ L of 4 nM in PBS) was then added to each well giving a final volume of 200 μ L and a final AuNC concentration of 0.4 nM, which corresponds to 2.4×10^{11} AuNCs per mL ($\sim 2.7 \times 10^4$ AuNCs per bacterium). For irradiated groups, the contents of each well were mixed thoroughly and a Breathe-Easy gas permeable sealing membrane (Diversified Biotech) was used to seal the microtiter plate prior to irradiation, thus preventing evaporation. Plates containing irradiated groups were placed on ice to slow bacterial growth during the experiment and each treatment well was irradiated for 10 min by diode laser (808 nm, 0.75 W) with a 0.30 cm² spot size that covered only the surface area of a well. Immediately following irradiation, the sealing membrane was removed, the contents of each well were mixed thoroughly by pipetting, and a 50 μ L aliquot was removed for bacterial quantification. For non-irradiated groups, a 50 μ L aliquot was removed immediately after mixing for bacterial quantification. After removal of aliquots from each well, plates were re-sealed and incubated at 37 °C with constant shaking (115 rpm). After 24 h, sealing membranes were removed, the contents of each well were mixed by pipetting, and a 50 μ L aliquot was removed for bacterial quantification, which was performed by serial dilution and plate counts to enumerate viable bacteria based on colony-forming units (CFU) per well. The analysis of two time points allowed for simultaneous assessment of PT-mediated killing (quantification at 0 h) and laser-assisted Dap release (quantification at 24 h).

Antimicrobial analysis was also performed with bacteria grown in a biofilm using our previously-published model of catheter-associated biofilm formation with slight modifications.²²

Briefly, 14-gauge fluorinated ethylene propylene catheters (Braun, Melsungen, Germany) were cut into 0.5 cm segments, sterilized, and coated in human plasma as previously described.²⁷ Catheters were then placed in the wells of a 12-well microtiter plate containing 2 mL of TSB supplemented with glucose and sodium chloride (biofilm medium, BFM).²⁷ Each well was then inoculated with the MRSA strain LAC at an OD₅₆₀ of 0.05 and the plate was incubated at 37 °C for 24 h. To treat catheters with our various nanoconstruct formulations, catheters were rinsed in sterile PBS and transferred to the wells of a 96-well microtiter plate. Each well contained 150 µL of BFM supplemented with CaCl₂ and 50 µL of the appropriate nanoconstruct (1.2×10^{11} AuNC/well). Controls included catheters placed in 200 µL of BFM and catheters treated with 5 µg/mL of Dap. Catheters were incubated with their respective treatments for two hours under constant rotation before each catheter was irradiated as described above. Untreated control catheters and catheters exposed to Dap alone were not irradiated. A subset of catheters for each group were harvested immediately and sonicated to disrupt the biofilm. Viable bacteria were subsequently quantified by serial dilution and plate counts. The remaining catheters were allowed to incubate for an additional 24 h at 37 °C in the wells in which they were irradiated, after which they were sonicated and viable bacteria quantified similarly to the planktonic cultures.

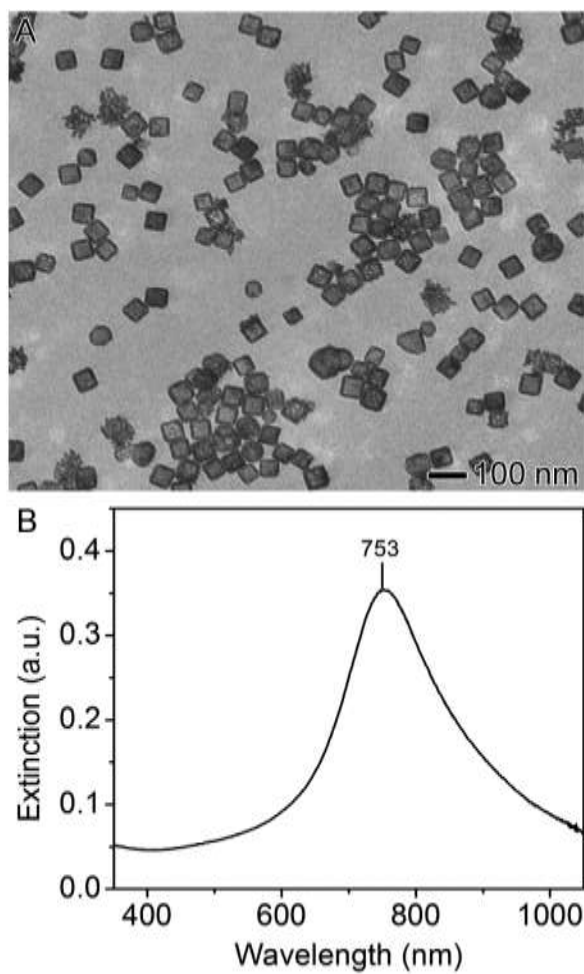


Figure S1. (A) TEM image of AuNCs and (B) UV-vis spectrum of the AuNC aqueous suspension corresponding to the sample shown in (A).

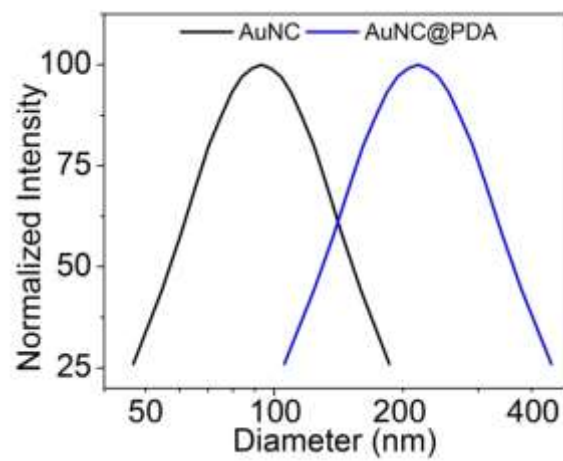


Figure S2. Hydrodynamic diameter of aqueous suspensions measured by dynamic light scattering: (black) AuNCs; and (blue) AuNC-PDA.

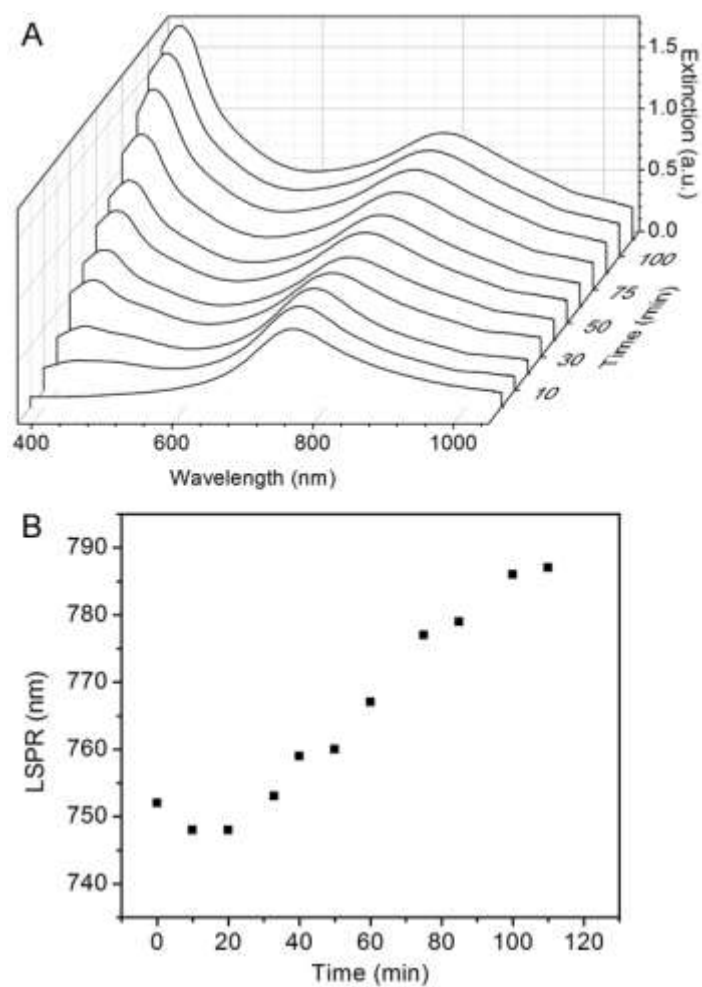


Figure S3. (A) UV-vis spectroscopic monitoring of the reaction solution during self-polymerization process of dopamine on the surface of AuNCs; and (B) LSPR shift of AuNCs as a function of reaction time.

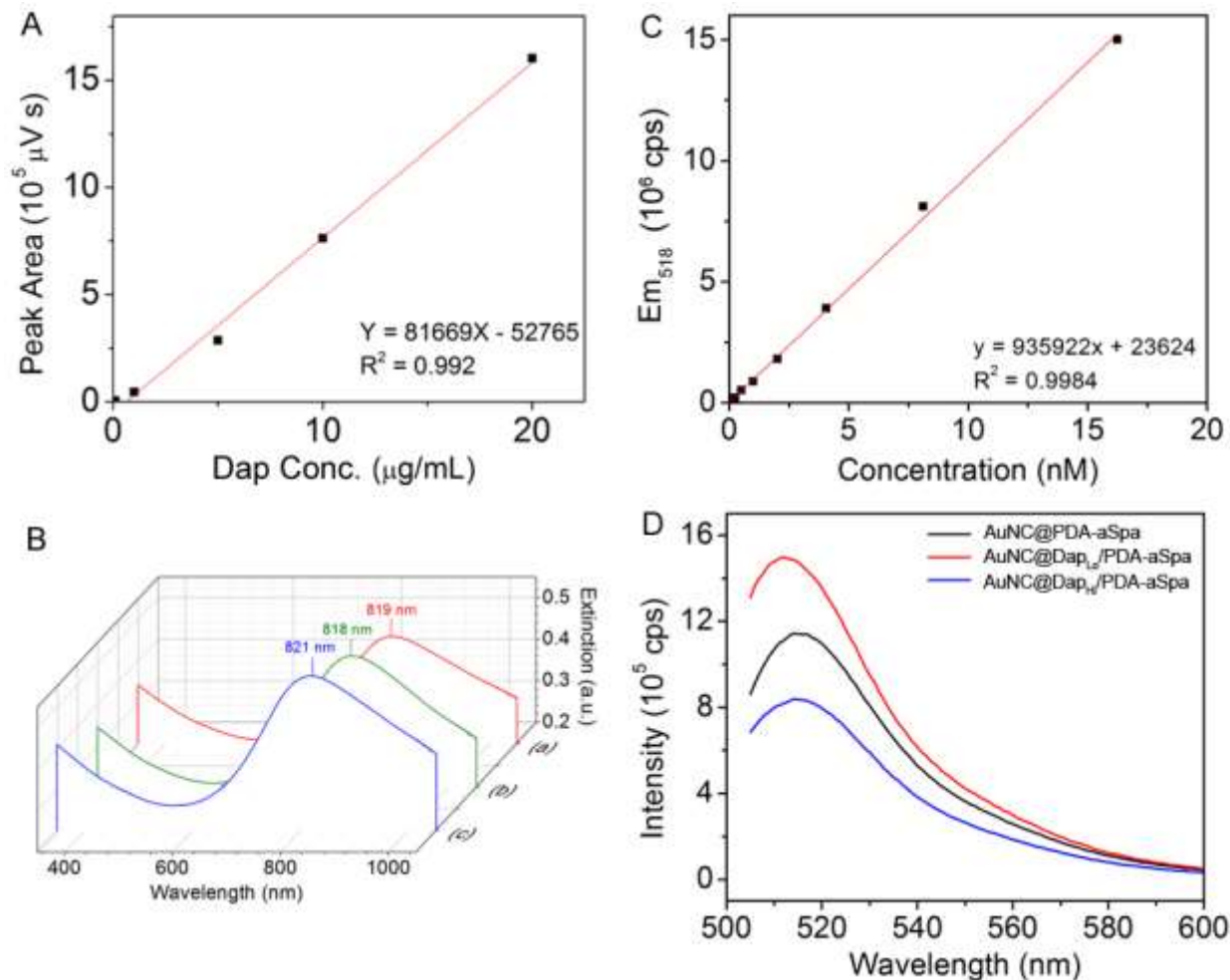


Figure S4. (A) Calibration curve of Dap concentration using UPLC with the data listed in Table S1; (B) UV-vis spectra of AuNC@PDA-aSpa (blue), AuNC@Dap_{Lo}/PDA-aSpa (green) and AuNC@Dap_{Hi}/PDA-aSpa (red); (C) Calibration curve of Dylight488-labeled IgG antibody using fluorescence spectroscopy ($\lambda_{ex} = 493$ and $\lambda_{em} = 518$ nm); and (D) Fluorescence spectra of the Dylight488-labeled IgG antibodies which were attached to AuNC@PDA-aSpa (black), AuNC@Dap_{Lo}/PDA-aSpa (red), and AuNC@Dap_{Hi}/PDA-aSpa (blue) for quantification of aSpa to be ~ 19 , ~ 28 , ~ 13 aSpa per AuNC, respectively.

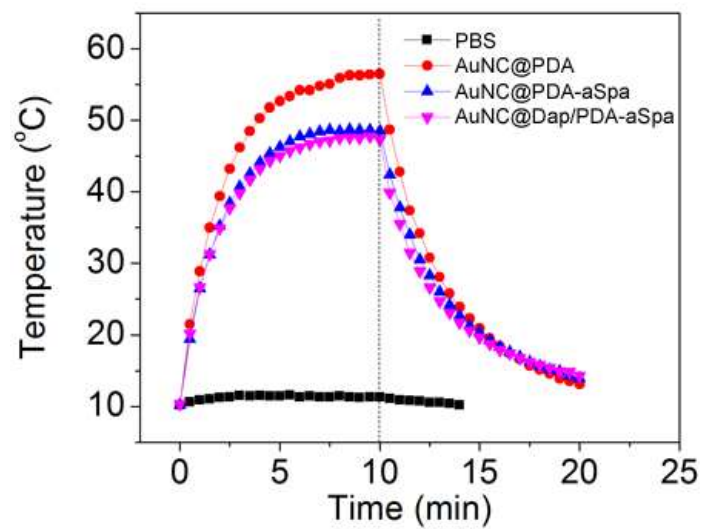


Figure S5. Temperature profile of 0.4 nM suspensions of the indicated AuNC formulations as a function of time of irradiation with an 808-nm diode laser at power density of 1.67 W/cm².

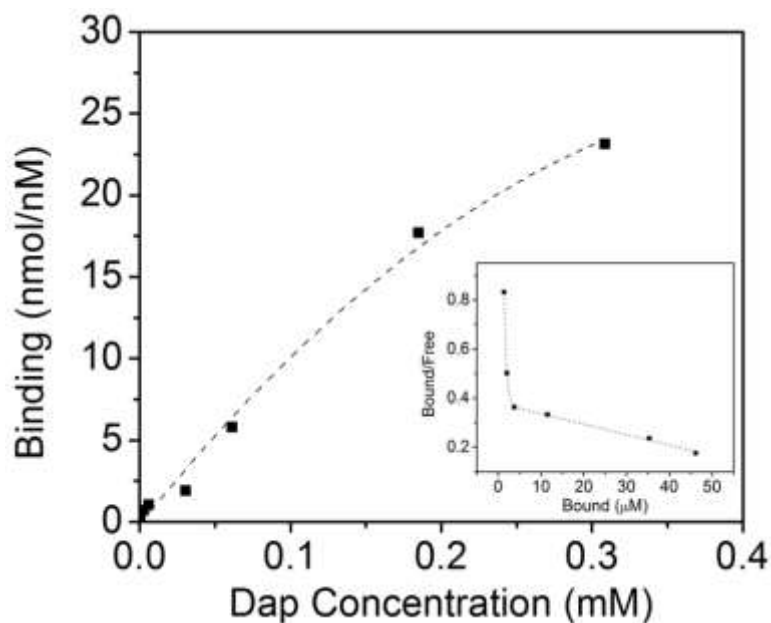


Figure S6. Plot Dap/AuNC@PDA binding versus total Dap. The dash line is the polynomial fitting ($y = -0.17 + 114.69x - 124.19x^2$, $R^2 = 0.989$). The inset is the Scatchard plot of the data by plotting the ratio of bound Dap to free Dap versus bound Dap, suggesting a biphasic binding of strong interaction sites and weak interaction sites.

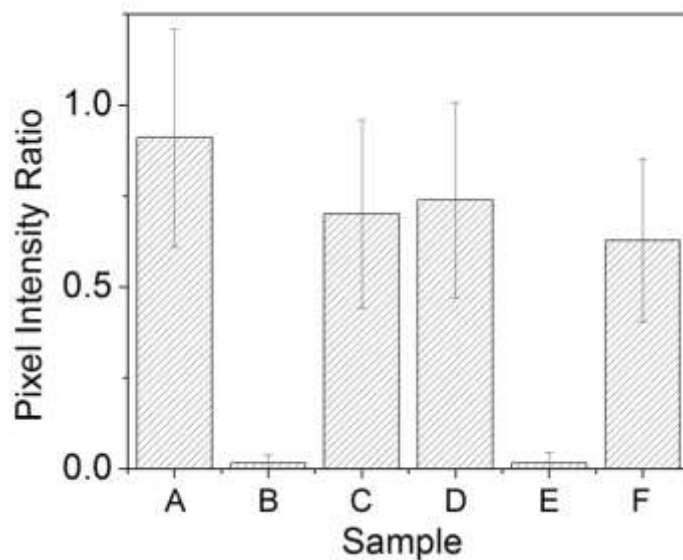


Figure S7. Quantitative analysis of two-photon fluorescence images by pixel intensity ratio of AuNCs (red channel) to DAPI (blue channel) for each sample corresponding to the images in Figure 7, A-F: (A) UAMS-1 exposed to AuNC@PDA-aSpa; (B) UAMS-1 exposed to AuNC@PDA; (C) UAMS-1 *spa* mutant exposed to AuNC@PDA-aSpa; (D) LAC exposed to AuNC@PDA-aSpa; (E) LAC treated with AuNC@PDA; and (F) LAC *spa* mutant exposed to AuNC@PDA-aSpa.

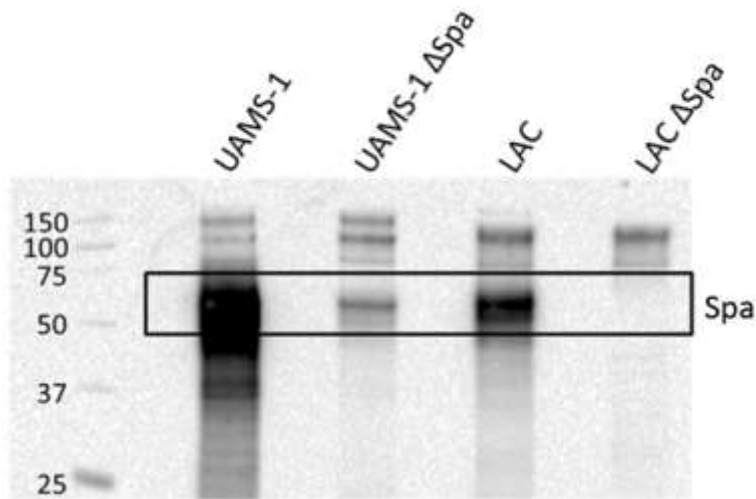


Figure S8. Western blot analysis of Spa mutants. Supernatants from stationary phase cultures of the indicated strains were resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with aSpa antibody. The boxed region indicates the molecular weight range expected of Spa. Although protein A is an LPXTG-anchored surface protein, supernatants were used for this experiment because it is also known to be released into the supernatant in amounts that quantitatively reflect its overall production level in different strains.³

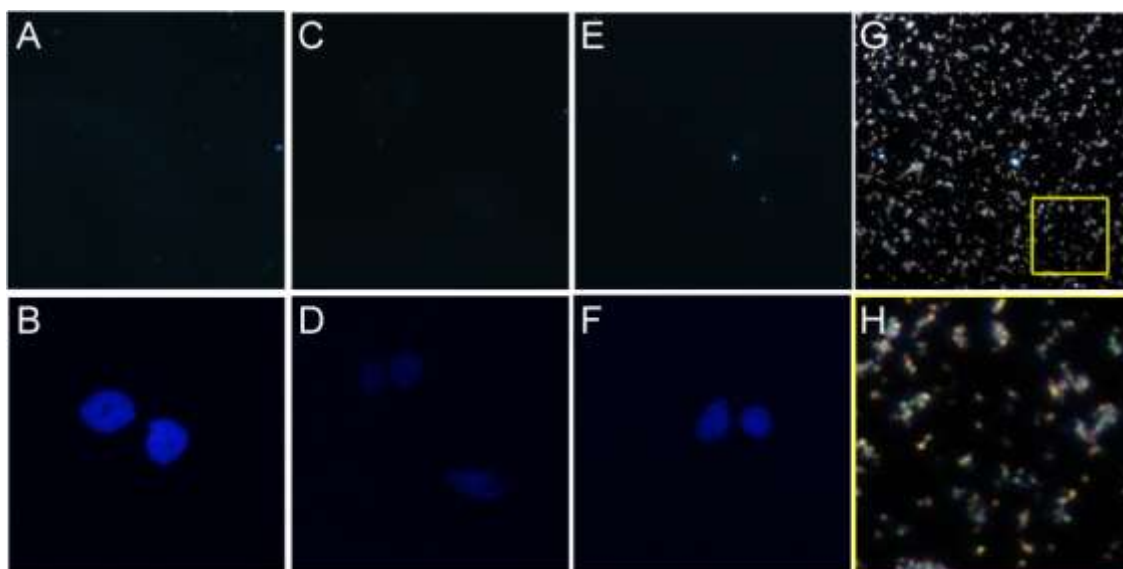


Figure S9. Darkfield images and corresponding fluorescence images of endothelial cells (EA-hy926; ATCC CRL-2922): (A, B) treated with AuNC@PDA-aSPA; (C, D) treated with AuNC@PDA; and (E, F) no treatment. The cells were stained with Hoescht 33452 (NucBlue Live ReadyProbe reagent) and imaged using the DAPI channel on the epifluorescence microscope in the same location as the darkfield (G) Darkfield image of AuNC@PDA on a glass slide fixed in 50/50 carboxyethylcellulose/water. (H) Zoom-in image of an area in (G) labelled using a yellow box. All images were taken using 40 $\times$ objective.

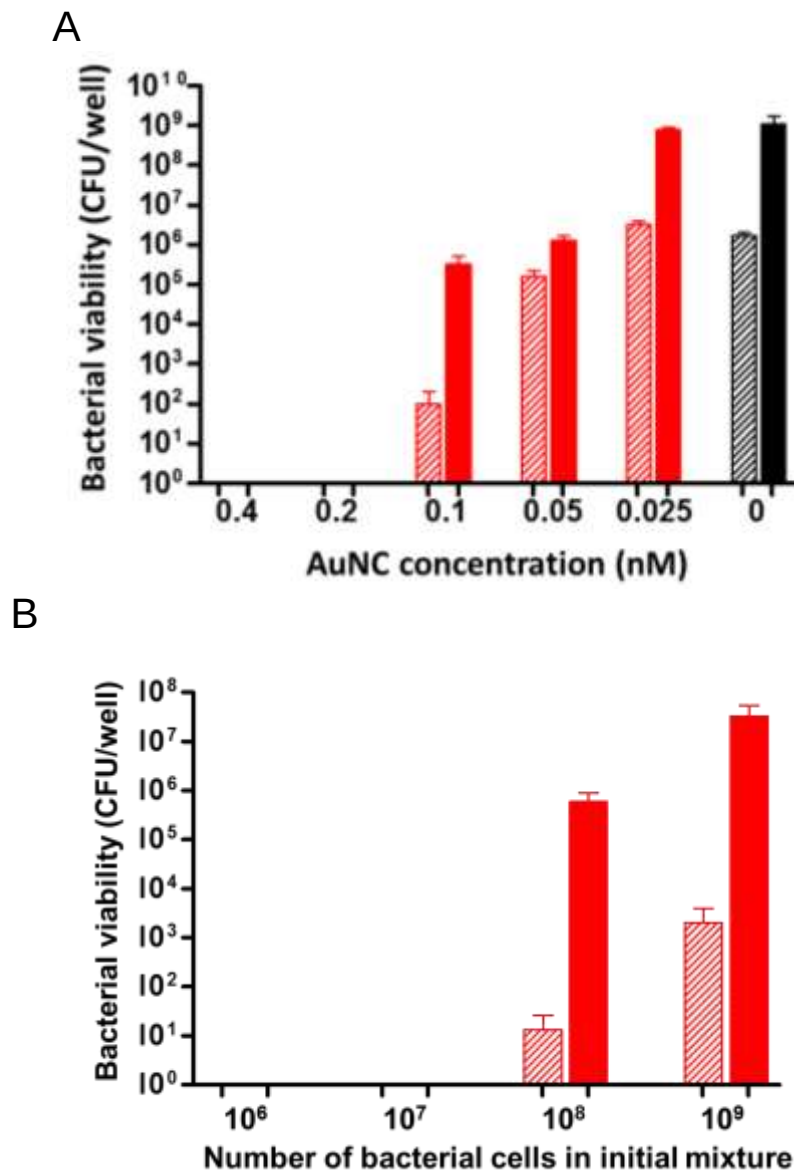


Figure S10. Dose response curves of AuNC@Dap_{Hi}/PDA-aSpa versus number of bacterial cells.

(A) Bacterial viability observed with a decreasing numbers of AuNC@Dap_{Hi}/PDA-aSpa and a constant number of bacteria. (B) Bacterial viability observed with increasing numbers of bacterial cells and a constant number of AuNC@Dap_{Hi}/PDA-aSpa (0.4 nM). All samples were irradiated for 10 min using 808-nm diode laser with a power density of 1.67 W/cm². Killing was assessed at 0 h (striped) and 24 h (solid) after treatment. Black bars indicate non-irradiated groups and red bars indicate irradiated groups.

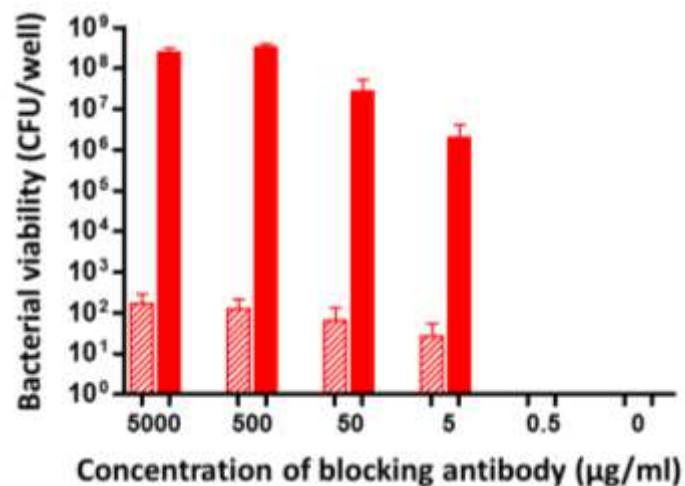


Figure S11. Blocking PT and antibiotic killing effects using unconjugated antibody. Experiments were repeated using the number of AuNC@Dap_{Hi}/PDA-aSpa, bacterial cells, and laser irradiation parameters used in our primary experiments after pre-incubation of bacterial cells with the indicated concentrations of unconjugated aSpa. Killing was assessed at 0 h (striped) and 24 h (solid) after treatment.

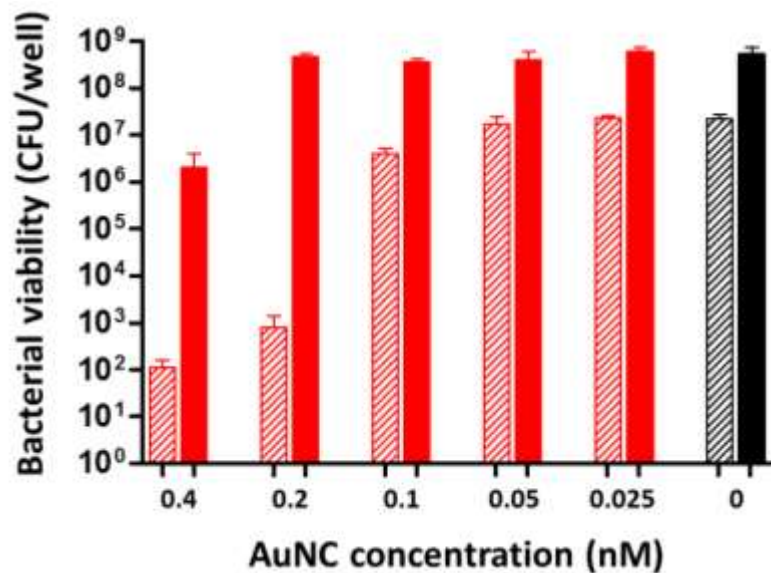


Figure S12. Dose response curves of AuNC@Dap_{Hi}/PDA-aSpa versus *S. epidermidis*. Bacterial viability observed with a decreasing numbers of AuNC@Dap_{Hi}/PDA-aSpa and a constant number of bacteria. Killing was assessed at 0 h (striped) and 24 h (solid) after treatment. Black bars indicate non-irradiated groups and red bars indicate irradiated groups.

Table S1. UPLC data for analysis of Dap loading and release.

	Integration Area ($10^5 \mu\text{Vs}$)	Concentration ($\mu\text{g/mL}$)
AuNC@Dap _{Lo} /PDA	3.02	4.3
AuNC@Dap _{Hi} /PDA	12.5	15.9
AuNC@Dap _{Lo} /PDA-aSpa	2.63	3.9
AuNC@Dap _{Hi} /PDA-aSpa	7.15	9.4
AuNC@Dap _{Lo} /PDA release data:		
2 min release	0.17±0.08	0.9±0.4
4 min release	0.82±0.15	1.7±0.3
6 min release	1.13±0.11	2.0±0.2
8 min release	1.53±0.18	2.5±0.3
10 min release	1.73±0.44	2.8±0.7
AuNC@Dap _{Hi} /PDA release data:		
2 min release	0.49±0.24	1.2±0.6
4 min release	1.04±0.43	1.9±0.8
6 min release	1.77±0.38	2.8±0.6
8 min release	2.65±0.27	3.9±0.4
10 min release	3.26±0.07	4.6±0.1

References:

- (1) Chen, J.; Yang, M.; Zhang, Q.; Cho, E. C.; Cobley, C. M.; Kim, C.; Glaus, C.; Wang, L. V.; Welch, M. J.; Xia, Y. Gold nanocages: A novel class of multifunctional nanomaterials for theranostic applications. *Adv. Funct. Mater.* **2010**, *20*, 3684-3694.
- (2) Zhang, Q.; Li, W.; Wen, L.-P.; Chen, J.; Xia, Y. Facile synthesis of Ag nanocubes of 30 to 70 nm in edge length with CF₃COOAg as a precursor. *Chem. Eur. J.* **2010**, *16*, 10234-10239.
- (3) Mrak, L. N.; Zielinska, A. K.; Beenken, K. E.; Mrak, I. N.; Atwood, D. N.; Griffin, L. M.; Lee, C. Y.; Smeltzer, M. S. saeRS and sarA act synergistically to repress protease production and promote biofilm formation in *Staphylococcus aureus*. *PLoS ONE* **2012**, *7*, e38453.
- (4) Weiss, E. C.; Zielinska, A.; Beenken, K. E.; Spencer, H. J.; Daily, S. J.; Smeltzer, M. S. Impact of sarA on Daptomycin Susceptibility of *Staphylococcus aureus* Biofilms In Vivo. *Antimicro. Agents Chemother.* **2009**, *53*, 4096-4102.
- (5) Beenken, K. E.; Blevins, J. S.; Smeltzer, M. S. Mutation of sarA in *Staphylococcus aureus* limits biofilm formation. *Infect. Immun.* **2003**, *71*, 4206-4211.