Supplementary Information for “Miniature Gold Nanorods for Photoacoustic Molecular Imaging in the Second Near-Infrared Optical Window”

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Rationales of NIR II Imaging

Photoacoustic contrast agents are strong exogenous optical absorbers that provide enhanced signal in response to laser irradiation.1-5 They are often referred to as molecular imaging agents when carrying ligands to specifically locate molecular targets of diseases.6-8 With molecular specificity, photoacoustic imaging has been demonstrated to diagnose cancer and guide therapeutic procedures, such as thermal and photodynamic therapies.9-12 Although contrast agents can improve the quality and diagnostic value of photoacoustic imaging, background signals from inherent optically absorbing components of tissue can reduce the signal-to-noise ratio of the image by one order of magnitude, resulting in a significantly reduced imaging sensitivity.13, 14 One strategy of further improving the imaging sensitivity is to choose contrast agents with an optical absorption peak away from the spectral regions where tissue absorbs most. In biological tissues, hemoglobin, water, melanin, and fat are the main optical absorbers. In general, tissue absorbs and scatters relatively little within two distinct spectral ranges: 650-900 nm and 1000-1200 nm, called the 1st and 2nd near-infrared (NIR) windows.15 Within the 2nd NIR window, 1050-1150 nm range is where blood-laden tissue generates the lowest photoacoustic background noise, coming from the
relatively low optical absorption of deoxygenated hemoglobin\textsuperscript{16}. Aside from the imaging aspect, low optical absorption also reduces the risk of tissue damage. According to the guideline of the American National Standards Institute the maximum permissible exposure of skin is 100 mJ cm\textsuperscript{-2} at 1064 nm (for nanosecond pulsed lasers),\textsuperscript{17} which is three times higher than that at 800 nm, thus imaging in the 2\textsuperscript{nd} NIR window can relieve potential concerns of thermal damage.\textsuperscript{17} Further, this wavelength range covers 1064 nm – the laser emission line of a Nd:YAG laser, a more stable and cost-efficient light source than an optical parametric oscillator for imaging in the 1\textsuperscript{st} NIR window. Overall, imaging in the 2\textsuperscript{nd} NIR window can significantly improve molecular photoacoustic imaging. However, one significant hurdle to date is the limited choices of contrast agents in this spectral range.

**Materials**

All chemicals in this study were used as received: cetyltrimethylammonium bromide (CTAB, Sigma-Aldrich), gold(III) chloride hydrate (HAuCl\textsubscript{4}, Sigma-Aldrich), sodium borohydride (NaBH\textsubscript{4}, Sigma-Aldrich), silver nitrate (AgNO\textsubscript{3}, Sigma-Aldrich), hydroquinone (Sigma-Aldrich), hydrochloric acid (HCl, Sigma-Aldrich), sodium hydroxide (1N solution, NaOH, Sigma-Aldrich), boric acid (Sigma-Aldrich), O-(2-mercaptoethyl)-O'-methyl-hexa(ethylene glycol) (mPEG\textsubscript{2k}-thiol, MW. 2,000, Creative PEGWorks), Thiol-PEG-Carboxylic acid (MW 2,000, Creative PEGWorks), Cy5-PEG-Thiol (MW 2000, Nanocs), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, ThermoFisher Scientific), N-hydroxysulfosuccinimide (Sulfo-NHS, ThermoFisher Scientific). Phosphate-buffered saline (PBS, 1X, pH 7.4, Gibo).

**Synthesis of Gold Nanorods**
Several Factors need to be considered when designing a synthesis protocol of small AuNRs. These factors include the concentration and type of the surfactant, the pH value of the growth solution, and the concentration of the reducing agent. \( \text{NaBH}_4(aq) \) is a typical strong reducing agent that creates the nuclei by LaMer burst nucleation followed by fast random attachment and intraparticle ripening.\(^{18}\) While using \( \text{NaBH}_4(aq) \) at higher concentrations will create greater quantities of smaller AuNRs, the random nature of the nucleation process can result in a wide range of size distribution (Supplementary Figure 1). Besides the number of nuclei, the pH value is a critical parameter in controlling the AuNR growth. The common ascorbic acid has a pK\(_a\) at 4.1. Decreasing the pH value below 4 significantly decreases its reduction potential, and thus slows down the growth rate, while on the other hand, improving the anisotropy.\(^{19,20}\) Compared to ascorbic acid, the reduction potential of hydroquinone also decreases with the pH value but at a higher rate (57 mV for each pH 1.0-value reduction), and therefore less acid is required to produce high aspect ratio rods. In fact, we observed that AuNRs stop growing when the pH value is lower than 2 in the hydroquinone growth solution. We intricately adjusted the concentration of \( \text{NaBH}_4(aq) \) and the pH value of the growth medium, using the hydroquinone seedless method to reduce the size of long-aspect-ratio AuNRs while maintaining their mono-dispersity. The smallest AuNRs obtained with this method are 8±2 nm by 49±8 nm.

Regular-sized gold nanorods (referred to as large AuNRs) were synthesized with hydroquinone based seed-mediated growth.\(^{21}\) Briefly, seeds were made by mixing an aqueous solution of 5 mL of \( \text{HAuCl}_4(aq) \) (0.001 M) and 5 mL of \( \text{CTAB}_{(aq)} \) (0.2 M). Next, 460 μL of \( \text{NaBH}_4(aq) \) (10 mM) in \( \text{NaOH}_{(aq)} \) (10mM) was added to the seed solution under fast stirring at 1200 rpm on a hotplate for 30 seconds. The seed solution was then aged for 5 minutes before adding into the growth solution. A growth solution was made by adding 5 mL of \( \text{HAuCl}_4(aq) \) (1 mM) and 5 mL of \( \text{CTAB}_{(aq)} \) (0.2
M) to 75 μL of AgNO$_3$(aq) (100 mM). Then, 500 μL aqueous hydroquinone (0.1M) was added to the growth solution under gentle mixing. The color of the growth solution turned from orange to clear with a very light yellow muddy color. Finally, various amounts (15-75 μL) of the seed solution were added to the growth solution to study the effect of initial seed concentration on the sizes of AuNRs. The large AuNRs with a width of 18 nm were prepared with a seed concentration of 15 μL. The solution was then aged for 12 hours before being cleaned with two centrifugation cycles at 8,000 g for 30 min.

Small AuNR synthesis method was developed from a hydroquinone-based seedless growth method.$^{22}$ In our study, both the effect of initial concentrations of reduction agents (NaBH$_4$(aq)) and the pH value were studied. Briefly, 0.4 mL of HAuCl$_4$(aq) (10mM) and 10 mL of CTAB$_{(aq)}$ (0.1 M) were added to 22.5 μL of AgNO$_3$(aq) (100mM). Then, 10-30 μL of HCl (1M) and 525 μL of aqueous hydroquinone (0.1M) were added to the growth solution under gentle mixing. The color of the growth solution turned from orange to clear with a very light yellow color. After 15 minutes of stirring, 10-40 μL of freshly prepared ice cold NaBH$_4$(aq) solution was injected into the growth solution using micro-syringe. The mixture was stirred for 15 seconds and aged for 16 hours at room temperature. The AuNR solution was then cleaned by centrifuging at 20,000 g for 1 hour twice.

**PEGylation of Gold Nanorods**

The stabilization agent, CTAB, on the surface of the AuNRs was replaced by PEG-thiol through ligand exchange. The PEG molecules in the initial reaction solution contained 80% of mPEG$_{2k}$-thiol, 10% of carboxylate-PEG$_{2k}$-thiol, and Cy5-PEG$_{2k}$-thiol. Briefly, the CTAB-AuNR dispersion was added to an equal volume of mPEG-thiol (0.2 mM) aqueous solution under vigorous stirring.
The mixture was sonicated for 5 minutes and left to react for 2 hours. Excess mPEG-thiol molecules were removed by centrifugation filtration (Amicon ultra-15, Millipore) at 3,000 g for 10 min. The PEGylated AuNRs were re-suspended in water.

**Peptide Conjugation**

A GRPR-targeting peptide with a sequence of dFQWAVGHStaL-NH2 was chosen as a binding moiety for preparing GRPR-targeting AuNRs (GRPR-AuNRs), because this sequence has a relatively slower dissociation from the receptor and a higher binding rate. Peptides were conjugated to carboxylic groups of PEGs on the AuNR surface through EDC/Sulfo-NHS reaction. In a typical reaction, concentrated PEGylated AuNRs (PEG-AuNRs) solutions were dispersed in sodium borate buffer (pH 9) for adjusting the optical density (OD) to 3 under a gentle agitation. A solution of the GRPR peptide (1 mL, 1 mg/mL, in 0.01 M sodium borate buffer, pH 9) was added to PEG-AuNRs (5 mL) and mixed. Then, aqueous solutions of the coupling reagents EDC (500 μL, 0.2 M) and sulfo-NHS (1 mL, 0.2 M) were added simultaneously. The mixture was stirred at 100 rpm for 24 h at 4 °C. The particles were purified and solvent exchanged by PD-10 Desalting Columns containing Sephadex G-25 resin (GE) and PBS (pH 7.2). For the animal experiment, the GRPR-AuNR solution was concentrated to OD=30 by centrifugation filtration (Amicon ultra-15, Millipore) at 1,000 g for 30 min.

**Characterization of Gold Nanorods**

The optical properties of the AuNRs were characterized by ultraviolet to visible to near infrared (UV-Vis-NIR) extinction spectroscopy. Extinction spectra were collected from a 1 mL AuNR suspension in an Agilent Cary 6000i UV/Vis/NIR spectrophotometer at room temperature with 1 cm of optical path. The fluorescent intensities of both small and large AuNR solutions at the same
OD were different. The ratio of this difference was characterized by epi-fluorescence imaging using IVIS spectrum imaging system (PerkinElmer). Two sets of 100 µL AuNR solutions with matched OD were placed in a black 96 well plate with a clear bottom (Corning).

The fluorescence images after 60 seconds of exposure were recorded with the excitation filter centred at 640 nm and the emission filter centred at 700 nm. The fluorescence intensity was quantified by Living Image® 4.5 software. The extracted fluorescence intensity as a function of OD was plotted and fitted linearly. We used the ratio of the slopes of the linear regression curves as the scaling factor (Supplementary Fig. 9) to compensate the fluorescence intensity difference due to the OD difference between the small and large AuNRs in the bio-distribution test in Figure 5.

To confirm each surface functionalization step, we measured the zeta potentials of the AuNRs. The zeta potential of the AuNRs was measured with a temperature controlled Zetasizer (Malvern) at 25°C. The as-synthesized AuNRs (both large and small) show significant positive zeta potentials (main text, Figure 4a), coming from the positively charged amine group in CTAB. After replacing CTAB with PEG, both solutions show negative zeta potentials because of the acid group in the PEG molecules on the AuNR surface. After functionalization of the GRPR-targeting peptides, the zeta potentials of the AuNRs change to positive again due to the slightly positively charged amino acids.

The shape and morphology changes of the AuNRs were assessed with scanning electron microscopy (SEM) (Zeiss Sigma FESEM, operated at 3 kV) and transmission electron microscopy (TEM) imaging (Hitachi S-5500 FESEM, operated at 30 kV). The size distribution of the AuNRs was calculated using Image J.
To study the size distribution of the AuNRs, we took several wide-field SEM images of the nanorods and analyzed the sizes of one hundred AuNRs. The SEM images show that the seedless method produces relatively more gold nanospheres as a side product, and the size distribution of the small AuNRs was \(8 \pm 2\) nm by \(49 \pm 8\) nm, while the distribution of the large AuNRs was \(18 \pm 4\) nm by \(120 \pm 17\) nm (Figure 1f and Supplementary Figure 2). UV-vis-NIR spectroscopy of both AuNRs shows their extinction peaks at 1064 nm and a similar full-width-at-half-maximum, confirming their similar size distributions. In addition, we also noticed the rise of the spectral baseline at shorter wavelengths for the large AuNRs, which comes from the increased scattering due to their increased size. This spectral behavior at shorter wavelengths indirectly confirms the size difference between the synthesized AuNRs.

**Quantification of Cy5 Dyes and Peptides Per Gold Nanorod**

We quantified the number of Cy5 dyes and GRPR peptide molecules on both small and large gold nanorods. Specifically, we measured gold nanorod concentration (# of nanorod per mL) by nanoparticle tracking analysis (Nanosight). We used potassium cyanide (KCN) to oxidatively etch gold nanorods and release the PEG-Cy5 molecules from the gold nanorods. The Cy5 concentration was measured with fluorescent spectroscopy using standard curve calibration methods. Our measurement shows an average of \(1100 \pm 350\) and \(5900 \pm 980\) Cy5 molecules on each small and large gold nanorods, respectively.

The number of peptides adsorbed on the gold nanorods was determined by subtracting the free peptides from the total peptides in the conjugation solution. In particular, the free peptides in the supernatant were separated from the total peptides bonded on the gold nanorods using centrifuge. The concentration of peptides in the supernatant was measured using the fluorometric peptide assay (Pierce). The assay detects peptides using an amine-reactive fluorescent dye that specifically
labels the N-terminus of peptides. The concentration was measured with fluorescent spectroscopy using standard curve calibration methods. Our measurement shows an average of $310 \pm 110$ and $1130 \pm 240$ peptide molecules on each small and large gold nanorods, respectively.

**Photoacoustic Signal Characterization and Phantom Imaging**

To investigate the photoacoustic performance of AuNRs, their photoacoustic response was characterized using a customized photoacoustic system consisting of a nanosecond Nd:YAG laser (OPOTek), a needle hydrophone with a central frequency of 20 MHz (Precision Acoustics), and a computer controlled data acquisition (DAQ) system. The Nd:YAG laser, operating at 1064 nm wavelength, had a pulse width of 7 nanosecond (ns) and a repetition rate of 10 Hz. The laser focus spot size was $\sim 0.5 \text{ cm}^2$. The laser fluence was adjusted by a set of neutral density filters.

We characterized the photoacoustic signals generated from both small and large AuNRs. To do so, we prepared solutions of small AuNRs and large AuNRs with matched ODs. Each AuNR solution was introduced into a 10-cm-long thin wall polyethylene tube (1 mm outer diameter and 0.6 mm inner diameter) mounted on a plastic scaffold. The needle hydrophone was positioned 10 mm above the tube. The laser beam and the hydrophone were positioned perpendicular to each other, and aligned to the same spot of the tube. At each optical fluence, the photoacoustic signal from 200 laser pulses were collected. To study the stability of photoacoustic signal, the peak photoacoustic signal intensity from each pulse was recorded and normalized to the intensity from the first pulse. To compare the photoacoustic intensity of each AuNR solution, we calculated the average and standard deviation of the peak intensities of 200 pulses at each laser fluence.

To rule out the possibility that photoacoustic signal enhancement comes from nonlinearity, we recorded the photoacoustic intensity of each AuNR solution by varying the laser fluences from 1
to 10 mJ cm$^{-2}$ (main text, Figure 2i). The photoacoustic intensity of the small AuNRs was linearly dependent on the laser fluence that indicates no nonlinear photoacoustic contribution to the signal generation. The intensity of the large AuNRs was also linear at low laser fluences and starts to deviate from the linear regression at $\sim$5.5 mJ cm$^{-2}$. As suggested by our photo-damage studies, this signal decay comes from photo-damage of the AuNRs. We linearly fit the intensity data of the small and large AuNRs below 5.5 mJ cm$^{-2}$. The slopes of the linear regression are 0.67 and 0.22 for the small and large AuNRs with a matched OD, confirming the more than 3-fold photoacoustic signal enhancement from the small AuNR solution.

For visualization purposes, we performed photoacoustic imaging of the tube phantoms containing the same AuNRs, but now the four tubes were placed parallel to each other. We used an ultrasound/photoacoustic micro-imaging system (Vevo 2100, VisualSonics, Inc.) with a 40-MHz-array-ultrasound transducer (LZ550, VisualSonics, Inc.). Four tubes filled with AuNRs solutions were mounted on the plastic scaffold from left to right in the following order: 1.0× OD small AuNR, 1.0× OD large AuNR, 1.0× OD small AuNRs, and 1.5× OD large AuNRs (Figure 2g in the main text). We estimated the ratio between the scattering and absorption cross-sections of each AuNR using numerical analysis (Supplementary Figure 5) and our calculations show that, at the same OD, the scattering of the small AuNRs contributes to 1.9% of the total OD, while the scattering of the large AuNRs contributes to 17.5% of the OD. The 1.5× OD of the large AuNRs represent the worst-case scenario.

Laser fluence of 5.5 mJ cm$^{-2}$ was used for the imaging to prevent damaging the large AuNRs. The scanning area was 13.9 mm (width) × 14.9 mm (depth) × 27.6 mm (length) with a step size of 95 $\mu$m. Figure 2g shows the centre area (13.9 mm × 13.3 mm) of the 3D scan (top view).
Size Effect on Gold Nanorod Melting and Shape-Transition Temperatures

Prior studies have shown that the melting temperature of gold nanoparticles decreases when their size reduces.\textsuperscript{23, 24} Although melting can result in shape changes, the temperature at which AuNRs start undergoing morphological changes is not exactly at their melting temperature, but at a temperature called the shape-transition temperature.\textsuperscript{25} The shape-transition temperatures are also size-dependent and generally lower than the melting temperatures. For AuNRs containing fewer than 2,000 gold atoms, the shape-transition temperature is much lower than the melting temperature, but it increases at a faster rate than the melting temperature with respect to the increment of the AuNR volume. If a AuNR contains more than 8,000 gold atoms, both the melting and shape-transition temperatures gradually plateau. Theoretical calculations have shown that the shape-transition temperature of AuNRs (>10\textsuperscript{4} gold atoms) approaches 1,000 K and its melting temperature is about 1,250 K.\textsuperscript{25} Both the small and large AuNRs used in our experiments contain 1.4×10\textsuperscript{5} and 1.6×10\textsuperscript{6} atoms respectively, which are well beyond the size where the shape-transition temperatures are highly size-dependent. Therefore, the difference in the shape-transition temperatures of both AuNRs should be negligible. One hypothesis of the enhanced thermal stability of the small AuNRs is their relatively lower peak temperature than the large AuNRs under the same laser illumination.

Cell Culture

PC3-GFP and DU145-GFP cells were obtained from the American Type Culture Collection (ATCC) and were cultured on collagen-coated flasks (BD Biosciences) in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. The cultures were maintained in a humidified atmosphere containing 5% CO\textsubscript{2}/95% air at 37 °C. The cell lines were authenticated at
Stanford functional genomics facility using Short Tandem Repeat (STR) profiling. The cell lines were tested for mycoplasma contamination upon received, after thawed, and monthly during culture using MycoAlert Mycoplasma Detection Kit (Lonza).

**Animal Studies**

All animal experiments were performed in compliance with the Guidelines for the Care and Use of Research Animals established by the Stanford University Animal Studies Committee, under the protocol APLAC-13024. Healthy male nu/nu mice at age 6 weeks were used in this study. A prostate cancer in mouse model was developed by subcutaneously injecting 100 µL of $5 \times 10^6$ of PC3 mixed with 1:1 volume ratio of growth factor reduced Matrigel into the right flank of each mouse. The tumor was allowed to grow to about 1 cm$^3$ before imaging. Before imaging, mice were anesthetized with 2% isoflurane at 2 liters min$^{-1}$ of oxygen flow and confirmed with tail pinch. Fifty microliters of nanoparticles/phosphate-buffered saline solution (AuNRs, OD = 30 at 1064 nm) were injected to the mice through tail veins.

**In vivo Imaging**

For *in vivo* imaging, photoacoustic imaging and ultrasound imaging were recorded. We used the same 1064 nm, 7 ns, Nd:YAG pulsed laser with a fluence of 25 mJ cm$^{-2}$, and the photoacoustic imaging transducer (VisualSonic LZ250) with a centre frequency of 21 MHz. A volume of 23 mm $\times$ 19 mm $\times$ 16 mm was mechanically scanned with a step size of 63 μm. Given the imaging parameters, each 3D imaging scan thus required 252 frames.

**Fluorescence Imaging of Cells**
Cells after incubating with AuNRs were imaged by a fluorescent microscope (EVOS FL cell imaging system, ThermoFisher Scientific) with 40× objective lens, and its GFP filter set and Cy5 filter set.

**Quantification of Bio-distribution of Gold Nanorods**

AuNR distribution in tissue of main organs at the end point was quantified by epi-fluorescence imaging. For the bio-distribution, mice were sacrificed 48 hours post-injection of AuNRs. Epi-fluorescence imaging of the excised organs was carried out using an IVIS spectrum imaging system (PerkinElmer) with an excitation filter centred at 640 nm and an emission filter centred at 700 nm with 60 seconds of exposure time. Quantitative analysis was performed using the Living Image 4.5 software.

**Blocking Tests**

We used the fifth group of mice to further test the specificity of our GRPR targeting. To do so, we blocked the GRPRs by first injecting 0.5 µmole of GRPR-peptides to each mouse (group 5, N = 3), at one hour before injecting the small GRPR-AuNRs. The photoacoustic imaging of the GRPR-blocked tumor with the small GRPR-AuNRs is shown in Supplementary Figure 10a. For more quantitative comparison, we calculated an average photoacoustic intensity from each tumor of the five groups of mice. We summed up the photoacoustic intensity within each tumor, and then divided it by the volume of the tumor. The quantified photoacoustic intensities show the highest photoacoustic signal from the tumor with small GRPR-AuNRs, which is at least 4 times higher than that from the GRPR-blocked tumor with the small GRPR-AuNRs, as well as from the non-GRPR targeted AuNRs (Supplementary Figure 10b). These results indicate the desired targeting specificity of our GRPR-AuNRs. Further, by comparing the average photoacoustic intensities from the small and large GRPR-AuNRs, we found a 4-fold enhancement from the small AuNRs. This enhancement is slightly higher than our theoretical prediction merely from the size
effect. The additional enhancement is likely attributed to the better tumor penetration and enhanced thermal stability of the small AuNRs.

**Numerical Simulations**

We conducted numerical simulations using finite-difference-time-domain (FDTD, Lumerical) and finite-element-method (FEM, COMSOL) solvers.

First, to obtain the optical absorption of the AuNRs, we used the FDTD solver. In the simulation, we assumed a single AuNR immersed in water, excited by linearly polarized light (plane wave) along the long axis. The simulation domain was truncated with perfectly matched layers to ensure that the outgoing waves are fully absorbed. The optical property of gold followed experimental values in Johnson and Christy.\(^{26}\) In this calculation, we obtained absorption and scattering cross-sections of AuNRs with widths ranging from 8 nm to 18 nm. To ensure accuracy of our calculation, we have completed convergence test, which has set an error limit of \(10^{-5}\). The lengths of these nanorods were also estimated using the same FDTD solver to maintain a resonance at 1064 nm. The simulated lengths of the nanorods matched well with our experimental results, confirmed with SEM imaging. Our calculation shows that the optical absorption cross-section of AuNRs (\(\sigma_a\)) decreases linearly with respective to their volume (Figure 3a and Supplementary Table 1).

Then, we conducted FEM simulations to predict the surface temperature of the AuNRs and the generated photoacoustic signals. This simulation was accomplished by coupling two physics modules in the FEM solver, the heat transfer module and partial difference equation describing photoacoustic signal generation. As the incoming 7 ns long optical pulse was absorbed by the AuNR, the heat from AuNR diffused into the surrounding water at the same time. The temporal profile of the incoming wave was assumed to be Gaussian, as shown in Figure 3b (red curve) of
the main text. Note that we only considered heat conduction from the AuNR to the surrounding water, without taking into account the convection current in the water. Due to elevated optical absorption of AuNRs compared to optical absorption of water at 1064 nm wavelength, during the laser irradiation the AuNR was acting as a heat source. Further, this heating originates from electron-phonon scattering, thus it fell into a femtosecond to picosecond time frame. We used 0.1 ns as our temporal resolution during the simulation, and, therefore, within each time step the absorbed heat inside the AuNR was assumed to be uniformly distributed across the entire volume of the nanorod. Please note that heat was not confined within the AuNR region – during each pulse of the laser illumination, heat diffuses out to the surrounding liquid and it is the heated liquid that generates most of the photoacoustic signal. In COMSOL, heat transfer was modeled using the following equation

\[ \rho C_p \frac{\partial T(\vec{r},t)}{\partial t} + \mathbf{u} \cdot \rho C_p \nabla T(\vec{r},t) + \nabla \cdot \mathbf{q} = Q_{abs}(t), \]  

(1)

where \( \rho \) is the density of the material, \( C_p \) is the heat capacitance at constant pressure, \( T \) is the temperature, which is position (\( \vec{r} \)) and time (\( t \)) dependent. In the second term, \( \mathbf{u} \) is velocity field that describes the effect of a moving coordinate system, which is neglected in this simulation because we assumed no convection or phase-transformation. Here \( \mathbf{q} = -k \nabla T(\vec{r},t) \) describes the Fourier’s law and \( k \) is the thermal conductivity of the material. Further, \( Q_{abs}(t) \) is the time dependent heat absorption, which follows a Gaussian shape with 7 ns pulse width and is proportional to the absorption cross-section and the laser fluence. A temperature of 293.15 K was used as the initial temperature.

The obtained position- and time- dependent temperature profile \( T(\vec{r},t) \) in the nanorod solution was coupled to the time-dependent secondary differential equation to calculate the photoacoustic
signal. The following equation was used to calculate the photoacoustic signal \( p \) in our module,

\[
\left( \nabla^2 - \frac{1}{v_s^2} \frac{\partial^2}{\partial t^2} \right) p(\vec{r}, t) = -\frac{\beta}{\kappa v_s^2} \frac{\partial^2 \tau(\vec{r}, t)}{\partial t^2},
\]

(2)

where \( p(\vec{r}, t) \) is the acoustic signal at a particular location \( \vec{r} \) and time \( t \); \( \beta \) is the thermal coefficient of volume expansion for water and gold in the respective regions; \( v_s \) is the speed of sound; and \( \kappa \) is the isothermal compressibility, which is defined as \( \frac{C_p}{\rho v_s^2 C_v} \), with \( \rho \) being the density of the material, \( C_p \) being the specific heat capacity at a constant pressure, and \( C_v \) the specific heat capacity at a constant volume.

Data and Statistical Analysis

For data analysis, MATLAB was used to process the image data acquired using Vevo imaging system. The ultrasound images are shown in dB scale, and the photoacoustic images in linear scale. The two-dimensional (2D) tube phantom images shown in the main text Figure 2 are the maximum intensity projections of the 3D volumetric images to the plane of lateral axis and elevational axis of the transducer.

The photoacoustic signal intensities in Figure 2 were calculated by first summing the photoacoustic intensities inside each tube from each frame, defined by the ultrasound B-mode image, and then taking the average of 290 frames. The error bars are the standard deviation of 290 frames. Data plot, average, and standard deviation were computed in Origin pro 2009.

To test the nanoparticle uptake in the \textit{in vitro} study, we calculated the mean and standard deviation of fluorescent intensities by first summing the fluorescent intensities of Cy5 from 30 cells (red channel) and then normalized it with the cell footprint. This footprint was defined by the area covered with GFP (green channel), indicating the viability of the cell. Image J was used here for
signal analysis. For bio-distribution of AuNRs in the *in vivo* studies, we summed the fluorescence intensities within the region of interest. The region of interest was identified by the footprint of each organ from the photographic images. We then normalized the summation with the footprint to obtain the mean and standard deviation. In both studies, we calculated the two-tailed p-value using an unpaired student t-test to determine the significance. We considered our data significant with p<0.05.
Supplementary Figures

Supplementary Fig. 1. SEM images of AuNRs synthesized with different concentrations of NaBH₄. A higher concentration of NaBH₄ leads to smaller-size AuNRs, but also a smaller aspect ratio and increased concentration of side products (gold nanospheres). These experiments were repeated independently for 5 times with similar results. Scale bars indicate 50 nm.
Supplementary Fig. 2. Wide field SEM images of as-synthesized (a) small and (b) large AuNRs. These experiments were repeated independently for 5 times with similar results.
Supplementary Fig. 3. Photoacoustic intensity of OD-matched small (blue dots) and large (red dots) AuNR solutions irradiated with 200 laser pulses. Laser fluences are (a) 6.1 mJ cm\(^{-2}\), and (b) 8.7 mJ cm\(^{-2}\), respectively. In both panels, the blue lines are the moving average of the photoacoustic intensities from the small AuNRs, and the red lines are the moving average of the photoacoustic intensities from the large AuNRs.
Supplementary Fig. 4. SEM images of small and large AuNRs before and after laser irradiation with 1000 pulses at 25 mJ cm$^{-2}$. Small AuNRs (a) before, and (b) after laser irradiation showing ~50% of the small AuNRs maintain their shape. Large AuNRs (c) before laser irradiation showing their uniform size distribution, and (d) after laser irradiation showing that the majority of the large AuNRs are thermally melted into spheres. These experiments were repeated independently for 3 times with similar results.
Supplementary Fig. 5. Simulations of optical absorption, scattering, and extinction of AuNRs. (a) FDTD simulations of absorption ($\sigma_a$, left y axis, blue) and scattering ($\sigma_s$, right y axis, red) cross-sections of AuNRs with width varying from 8 nm to 18 nm. (b) Theoretical calculations of the amount of scattering cross-section in the total extinction cross-section (in percentage, %) of each AuNR, in the range of 8 nm to 18 nm width.
Supplementary Table 1. Parameters of the AuNRs used for the numerical simulation

| AuNR | Width (nm) | Length (nm) | Surface ($\times 10^2$ nm$^2$) | Volume ($\times 10^4$ nm$^3$) | Surface-to-Volume | Absorption cross-section ($\times 10^{-14}$ m$^2$) | Scattering cross-section ($\times 10^{15}$ m$^2$) |
|------|------------|-------------|-------------------------------|-------------------------------|-------------------|---------------------------------|----------------------|
| AuNR1| 8          | 50          | 1.28                          | 2.43                          | 0.53              | 0.56                            | 0.11                 |
| AuNR2| 10         | 64          | 2.01                          | 4.76                          | 0.37              | 1.01                            | 0.36                 |
| AuNR3| 12         | 76          | 2.87                          | 8.14                          | 0.31              | 1.65                            | 0.97                 |
| AuNR4| 14         | 88          | 3.87                          | 12.83                         | 0.27              | 2.44                            | 2.31                 |
| AuNR5| 16         | 97          | 4.88                          | 18.43                         | 0.23              | 3.24                            | 4.34                 |
| AuNR6| 18         | 108         | 6.11                          | 25.96                         | 0.21              | 4.53                            | 9.58                 |

Supplementary Table 2. Symbols used in Figure 3.

| Symbols | Unit    | Description                                                                                                                                 |
|---------|---------|---------------------------------------------------------------------------------------------------------------------------------------------|
| $\sigma_a$ | m$^2$   | Absorption cross-section: absorption cross-section of a single gold nanorod (AuNR).                                                         |
| $\tilde{\sigma}_a$ | dimensionless | Normalized absorption cross-section: absorption cross-section of a single AuNR normalized by the absorption cross-section of the 8-nm-width AuNR ($\sigma_{8\text{nm}}$). |
| $\tilde{p}$ | dimensionless | Normalized photoacoustic signal: photoacoustic signal of a single AuNR normalized by the photoacoustic signal of the 8-nm-width AuNR; $\tilde{p}$ is a time-varying function. |
| $\phi_{PA}$ | dimensionless | Photoacoustic quantum yield: emitted acoustic energy divided by the absorbed photon energy of a AuNR. Normalized $\phi_{PA}$ is the quantum yield of each nanoparticle divided by that of the 8-nm-width AuNR. |
| $P$ | pascal | Peak amplitude of photoacoustic signal: peak amplitude of the photoacoustic signal of AuNR samples when all samples have a matched OD. $P \propto \sqrt{\frac{\phi_{PA}}{\sigma_a}}$. The normalized $P$ is calculated by normalizing the peak amplitude of photoacoustic signal from each AuNR sample with respect to that of the 8-nm-width AuNR. Normalized $P = \sqrt{\frac{\phi_{PA}}{\sigma_a} / \phi_{PA,8\text{nm}} / \sigma_{8\text{nm}}}$ |
Supplementary Fig. 6. Calculated maximum surface temperature of 8 nm AuNR illuminated with laser pulses of various energy.
Supplementary Fig. 7. Simulated photoacoustic signals. (a) Simulated photoacoustic signals at 200 nm away from the centre of the AuNRs normalized with the peak photoacoustic signal of the 8-nm small AuNRs, and (b) photoacoustic signals of a AuNR solution normalized to the photoacoustic signals of a OD-matched-8-nm-AuNR solution, predicting that at the same OD, small AuNR solution will generate the strongest photoacoustic signal. In each simulation, only one nanorod is in the simulation domain.
Supplementary Fig. 8. The square maximum of photoacoustic signal as a function of emitted acoustic energy, showing their linear relationship.
Supplementary Fig. 9. Fluorescence intensity of AuNRs as a function of optical density (OD) of the AuNRs. The centre values are the mean fluorescent intensities of 5 wells at each concentration, and the error bars are the standard deviation. The intensity curves are fitted with linear regression. The ratio between the slopes of the two linear fitting curves is a scaling factor equal to 1.36. This scaling factor is used to correct the bio-distribution images in Figure 5 (main text). The insets show representative fluorescence intensity images of (A) large AuNRs, (B) small AuNRs, (C) and (D) are the duplicate of (A) and (B), respectively. Column 1 is a control, and columns 2 to 4 correspond to solutions of AuNRs with ODs from low to high. The fluorescence intensity measurement was repeated 5 times independently with similar results.
Supplementary Fig. 10. Blocking test. (a) In-vivo photoacoustic imaging of GRPR targeted small AuNRs in a murine model of prostate cancer. GRPRs were blocked by injecting 0.5 µmole of GRPR peptides for 1 hour before injecting the small GRPR-AuNRs. The in-vivo blocking photoacoustic imaging is done with one replicate. (b) Quantified photoacoustic intensities of the tumors from the 5 groups of mice, indicating an overall ~4 times of photoacoustic enhancement from the small GRPR-targeted-AuNRs compared to their large counterpart. Compared to the small GRPR-targeted-AuNRs, the GRPR-peptide blocking tumor showed 4 times lower photoacoustic signal, indicating the high specificity of our GRPR targeting. The centre values are
the mean photoacoustic intensities of 252 frames within tumor area (n=252), and the error bars are the standard deviation.
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