Hybrid Anisotropic Nanostructures for Dual-modal Cancer Imaging and Image-guided Chemo-thermo Therapies

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ABSTRACT

The multimodality theranostic system, which can integrate two or more different therapeutic modalities and multimodal imaging agents into a nanoentity, shows great promising prospects for the cancer treatment. Herein, we developed an efficient and novel strategy to synthesize hybrid anisotropic nanoparticles (HANs) with intrinsic multimodal theranostic capability [chemotherapy, photothermal therapy, magnetic resonance imaging (MRI), and photoacoustic imaging (PAI)]. For the first time, under the guidance of MRI and PAI, the chemotherapy and thermotherapy induced by administration of multifunctional hybrid nanoprobe were applied simultaneously to the treatment of colon cancer-bearing mice *in vivo*.

1. Introduction

In recent years, despite rapid development in cancer diagnostic and therapeutic regimens, many improvements in survival observed in many cancers have not been observed in colon cancer because it is often diagnosed late and systemic therapy is limited. Therefore, it is urgent to develop novel approaches for colon cancer diagnosis and treatment. With the development of nanotechnology, theranostic nanomaterials are considered to have significant impact and important applications in cancer patient management. Increasing researches have been focused on integrating simultaneously cancer diagnostic and therapeutic functions into a single nanoentity [1-4]. The design of multifunctional nanoplateforms allows for simultaneous delivery of imaging and therapeutic agents [5], which provides unique opportunities for cancer therapy under imaging guidance and serves as truly integrated theranostic systems.

Recently, some researchers have reported that numerous theranostic nanoparticles (NPs) are intrinsically imaging and anti-cancer agents. For instance, anisotropic gold (Au) NPs have been applied to photoacoustic imaging (PAI), optical imaging, and photothermal therapy (PTT) [6]. Further research demonstrates that Au NPs for cancer therapy have full potential for clinical translation [7]. More recently, iron platinum (FePt) alloy NPs have also gained recognition as an excellent theranostic agent. The superparamagnetic property of FePt NP makes it a potential magnetic resonance imaging (MRI) contrast agent [8]. More interestingly, in response to the low extracellular pH in tumors, FePt NP can act as a reservoir to release significant amount of iron ions (Fe^{2+}) inducing a Fenton's reaction in the presence of hydroperoxide. The Fe ions can catalyze H_2O_2 decomposition into reactive oxygen species (ROS) within cells and cause the cytotoxicity [9]. Therefore, FePt NP can also serve as a pH-controlled chemotherapeutic agent. Overall, these

multifunctional theranostic agents are very beneficial to tailor cancer treatment plan and personalize image guided therapy [10].

However, most recent designs of theranostic agents were focused on single-modality theranostic systems, which combined a single-modality imaging component with a therapeutic method. This type of systems may not fully use the power of integration of diagnosis and therapy. Cancer is a complex outcome of multiple pathways, multiple processes and multiple stages. Therefore, combining two or more different therapeutic modalities such as the combination of PTT and chemotherapy into nanoplateforms, is an encouraging approach, which may result in synergistic effects that are more efficient to treat cancer than individual treatment [11]. The well-known “cocktail” therapy (combining two or more anticancer drugs or agents) had actually obtained better antitumor effects in clinical cancer treatment [12]. Additionally, imaging-guided therapy is critically important for cancer treatment. No single-modality imaging can efficiently provide the integrated information about tumor morphology and function. For example, MRI has high spatial resolution, but its sensitivity is rather limited [13]. Photoacoustic imaging (PAI) provides excellent sensitivity but tissue penetration depth is limited [14]. Hence, the multimodal imaging techniques may take advantage of different imaging modalities and help to circumvent the drawbacks of each imaging modality. Therefore, there are urgent demands to integrate two or more single-modality theranostic systems into a multimodality theranostic system. The multimodality theranostic system, literally integrating two or more different therapeutic modalities and multimodal imaging agents into a nanoentity, is expected to show great promising prospects for the cancer diagnosis and treatment. Herein, inspired by organic total synthesis of heterofunctional molecules, we developed an efficient and novel strategy to synthesize hybrid anisotropic nanoparticles (HANs) with intrinsic multimodal theranostic capability (chemotherapy, photothermal therapy, MRI, and

PAI) for simultaneous cancer diagnosis and therapies (Scheme 1). By fusing three nanocomponents [iron-platinum (FePt) alloy and gold nanocrystals] via solid-state interfaces, HANs were constructed in a control and predictable manner and displayed shape-dependent optical characteristics (Fig. 1). The close conjunction of localized surface plasmon resonance between plasmonic metal crystals as well as the synergistic interactions among three components played very important roles on such extraordinary optical and chemical properties, which were then validated by experimental results. As multimodality theranostic platforms, HANs therefore offer great opportunities for integrating distinct imaging modalities and therapy capabilities together. As expected, HANs keep their shape and morphology intact and exhibit great stability in the physiological condition. The resultant HANs not only show excellent shape-control and monodispersity but also provide superior optical and magnetic properties, which make it act as T_2 MRI and PAI contrast agents. It is obviously helpful to therapy under real-time imaging guidance using both MR and PA. Interestingly, as pH-controlled iron reservoirs, HANs exhibited the property of Fe^{2+} ion release in low pH value, which make it act as a potential chemotherapeutic agent for cancer therapy based on the pH differences between tumor tissue (low pH value) and normal cells (pH = 7.4). More importantly, because of their strong and broad absorption in both far red and near-infrared (NIR) region, HANs can result in significant temperature elevation on the desired locations upon laser irradiation. For the first time, under the guidance of MRI and PAI, the chemotherapy and thermotherapy induced by administration of multifunctional hybrid nanoprobe were applied simultaneously to the treatment of colon cancer-bearing mice *in vivo* (Scheme 1).

2. Materials and methods

2.1. Materials. Hydrogen tetrachloroaurate (III) hydrate (HAuCl_4) and platinum (II) acetylacetonate ($\text{Pt}(\text{acac})_2$) were ordered from Strem Chemicals, Inc. *N*-hydroxysuccinimide (NHS), *N*-hydroxysulfosuccinimide (sulfo-NHS), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (EDC) were purchased from Thermo Fisher Scientific. All other chemicals were purchased from Sigma-Aldrich. Triethylamine and dichloromethane (DCM) were distilled prior to use, and *N,N'*-dimethylformamide (DMF) was stored over molecular sieves. Other solvents and chemicals were used as received. Deionized water was obtained from a Millipore Milli-DI Water Purification system. The dialysis membrane tubing (MWCO: 12,000 ~ 14,000, and 100,000) were purchased from Spectrum laboratories.

2.2. Synthesis of 7 nm FePt nanocubes. The 7 nm FePt nanocubes were synthesized according to the procedures described in the previous publications [15]. Typically, the platinum precursor [platinum (II) acetylacetonate, or $\text{Pt}(\text{acac})_2$, 197 mg, 0.5 mmol] was dispersed in 20 mL of benzyl ether under nitrogen protection. The resultant mixture was preheated to 70°C until the platinum salt was dissolved and the solution turned bright yellow. The mixture was then heated up to 110°C for 5 min. Iron precursor (iron pentacarbonyl, $\text{Fe}(\text{CO})_5$, 0.5 mmol, 0.13 mL) was quickly injected in the above solution, followed by injections of 2.6 mL of oleic acid and 2.7 mL of oleylamine. The resultant mixture was heated up to 300°C at a heating rate of 5°C/min and then kept at this temperature for 1 hour before it was cooled down to room temperature by removing the heating mental. The particles were precipitated out by adding 70 mL of isopropanol and collected by using a high-speed centrifuge (7000 rtf, 8 min). The resultant particles were re-dispersed in 5 mL of hexane and then precipitated out by adding ethanol. This purification step was repeated twice to remove the extra surfactant and benzyl ether. The final product (7 nm cubic FePt NPs) was dispersed in 10 mL of hexane in the presence of 0.01 mL of oleylamine for further use.

2.3. Synthesis of hybrid anisotropic nanostructures (HANs). The gold precursor (hydrogen tetrachloroaurate, HAuCl_4 , 100 mg, 0.29 mmol) was dissolved in 20 mL of octadecene (ODE) containing 2 mL of oleylamine under nitrogen protection. After stirred at room temperature for 5 min, the solution was heated up to 80°C. Twenty milligram of 7 nm freshly synthesized FePt NPs (dispersed in 1 mL of hexane) were quickly injected in the above solution. The resultant mixture was then heated up to 120 °C for and kept at this temperature for one hour before it was cooled down to room temperature. The solution finally turned to gray-purplish color, indicating the

formation of gold branched nanostructures. The particles were precipitated out by adding 30 mL of isopropanol and collected by a centrifuge (3000 rcf, 5 min). The resultant particles were re-dispersed in 5 mL of hexane and then precipitated out by adding ethanol. This purification step was repeated twice to remove the extra surfactant and ODE. The final product (Au-FePt-Au HANs) was dispersed in 10 mL of hexane in the presence of 0.01 mL of oleylamine for further use.

2.4. Surface modification of HANs. To improve the biocompatibility and physiological stability of HANs, the HANs were coated with polyethylene glycol (PEG). Ten mg of synthesized HANs in 0.5 mL of tetrahydrofuran (THF) was mixed and shaken with 20 mg of DSPE-PEG-NH₂ (MW=5000, laysan Bio, Inc.) for 30 min on the hot plate. The solvent was removed by blowing a slow stream of nitrogen gas. DI water was then added to the dispersed HANs. The aqueous solution (3 mL) of the DSPE-PEG-NH₂ coated HANs were desalted by PD10 column (GE Healthcare, USA). The HANs solutions were then transferred to a MWCO 30,000 Centrifugal Filter Unit (Millipore, Carrigtwohill, County Cork, Ireland) and centrifuged. The final HANs' concentration was measured with inductively coupled plasma mass spectrometer (ICP-MS, Thermo Scientific Xseries 2 Quadrupole, Germany).

2.5. Characterization of HANs. The sizes of the HANs were measured by a dynamic light scattering (DLS) instrument (Malvern Instruments Ltd, Southborough, Massachusetts). The morphologies of the HANs were obtained under transmission electronic microscope (TEM, Tecnai G2, FEI, USA) at 200 kV. Samples were deposited and dried on copper grids covered with a Formvar/carbon support film, followed by plasma cleaning. Ultraviolet-visible (UV-vis) absorption spectroscopy was performed with a Cary UV-Vis (Agilent Technologies, USA). The elemental analyses were performed using ICP-MS. The NP samples were completely dissolved in freshly prepared aqua regia (trace metal grade 70% nitric acid HNO₃:36% hydrochloric acid HCl (Fisher Scientific), 1:3/v:v) before the analysis.

2.6. Determination of Concentration of HANs. The molar atom weight of HANs was calculated based on the ideal models identified by TEM. HANs were modeled as cubic cores (7 nm in length) with two spheres of constant radius of 5 nm. The ratio of gold to platinum weight of tripods was determined by ICP-MS. The weight of single NP was determined by its geometry shape and the density of individual component. The calculated molar atom weight of HANs is 1.53×10^7 g/mol.

The actual weight concentrations of HANs were determined by ICP-MS (gold, platinum, and iron). Based their molar atom weight of HANs, their molar concentrations were calculated accordingly.

2.7. Measurement of MRI relaxation properties in vitro. To evaluate MR contrast enhancement effect of HANs, HANs with various concentrations of iron (1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0 mM) in aqueous solutions were imaged by a 7.0 T small animal MR scanner. T_2 -weight images (T_2 WI) were obtained using a sequence with the following parameters: TR = 4000 ms, TE increased from 20 ms to 140 ms with an increment of 20 ms, FOV = $180 \times 180 \text{ mm}^2$, NEX = 4, slice thickness = 1.5 mm, slice = 4, Matrix size = 256×256 . The region of interests (ROIs) were measured in the T_2 -mapping using imageJ software and the r_2 value was calculated.

2.8. Photoacoustic imaging in vitro. To measure the photoacoustic (PA) signal sensitivity of HANs, we constructed a phantom by filling 5 plastic tubes with the different concentration of HANs aqueous solutions (16, 8, 4, 2, and 0 nM NPs). The aqueous solutions in the phantom mixed with the 1% liquid agarose. The phantom was then suspended inside a water tank and was imaged at a laser wavelength of 680 nm. Three-dimensional ROIs were used to measure the PA signal from each sample. The correlation between the photoacoustic signal and concentration response curve was calculated. PA imaging was performed with a LAZR commercial instrument (Visualsonics, 2100 High-Resolution Imaging System). Ultrasound (US) and PA images were acquired by a MS-250 linear array transducer (21 MHz, 40 dB two-way bandwidth, 256 elements). The laser system (OPOTEK Inc., Carlsbad, CA, 20 Hz repetition rate, 680-950 nm, 50 mJ pulse peak energy, 5 ns pulse width) was used to trigger the system acquisition. The spot size is $1 \times 24 \text{ mm}$, and the full field of view is $14 \sim 23 \text{ mm}$ wide. Acquisition rate of 5 frames per second was used for all the experiments.

2.9. Release experiment of Fe^{2+} ion. HANs solutions (1 mL) containing 1 mg of Fe were diluted in 1 mL of PBS buffers (pH 4.8 or 7.4) or two type of cytoplasm mimicking (CB) buffers (CB 7.4, which contains 25 mM HEPES-acetate at pH 7.4, 3 mM Mg acetate, 154 mM potassium acetate, 1 mM EGTA, 300 mM sucrose. CB 5.2, which contains 25 mM MES-acetate at pH 5.2, 3 mM Mg acetate, 154 mM potassium acetate, 1 mM EGTA, 300 mM sucrose.) The HANs solutions were then transferred to dialysis membrane tubing (Spectra/Por, MWCO 3500, Spectrum Laboratories, USA) and the membrane bag was immersed in 20 mL of PBS buffers (pH 4.8 or 7.4) or CB buffers (pH 5.2 or 7.4) with disposable scintillation vials incubated at 37°C ,

respectively. At different time intervals, 1 mL of PBS solution or CB buffer was withdrawn from disposable scintillation vials as samples, and the vials were refilled with the same volume of fresh PBS (pH 7.4 or 4.8) or CB buffers (pH 5.2 or 7.4). The Fe concentration of samples was analyzed by ICP-MS.

2.10. Effect of acid etching on PA and MR signal of HANs. HANs were treated with a neutral buffer (PBS (pH 7.4) or CB buffers (7.4)) or an acidic solution (PBS (pH 4.8) or CB buffers (5.2)) for 24 hours. The treated HANs were sealed in polyethylene capillaries (I.D. = 0.76 mm, O.D. = 1.22 mm, one-inch long, Becton Dickinson Co. MD), and embedded in the 1% agarose gel for photoacoustic analysis. Similarly, the treated HANs were suspended in 1% agarose gel with the final concentration of 0.25 mM in plastic vials. After solidification, samples were scanned using an Agilent Discovery MR901 System with a 72 mm Agilent radiofrequency (RF) coil. The T2-weighted images were obtained using the following parameters: Fast spin echo (FSE) sequence with TE = 40 ms, TR = 3000 ms, Field of view (FOV) = 3×3, matrix size = 256×256, slice thickness = 1 mm. Quantification analysis was performed using ImageJ. The regions of interest (ROI) were drawn over the samples, and the signal intensities of ROI were measured.

2.11. Photothermal heating of HANs in vitro. To study the photothermal effect of HANs induced by NIR irradiation *in vitro*, 50 μ L of HANs solutions at different concentrations (12.5 nM, 25 nM, 50 nM, 100 nM) were irradiated as well as the same volume of control PBS with an 808 nm NIR laser and 0.6 W cm⁻² irradiation power for 150 sec. Thermal images and temperatures of solutions were recorded at every 10 second by a MikroShot thermal camera (Mikron).

2.12. Cell culture. The human colorectal adenocarcinoma cells (HT29 cells) obtained from American Type Culture Collection were routinely cultured in ATCC-formulated McCoy's 5A Medium (Invitrogen) modified containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin streptomycin, 10,000 U mL⁻¹) in 150 mm diameter Primaria dishes at 37°C, saturated humidity, 5% CO₂. The medium was changed every 24~48 h. The mouse embryonic fibroblast cell line (NIH 3T3 cells) obtained from American Type Culture Collection were routinely cultured in ATCC-formulated DMEM (Invitrogen) modified containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin streptomycin, 10,000 U mL⁻¹) in 150 mm diameter Primaria dishes at 37°C, saturated humidity, 5% CO₂. The medium was changed every 24~48 h.

2.13. Cytotoxicity assay in vitro. The cytotoxicity of HANs and photocytotoxicity of HANs under laser irradiation on HT29 cells were examined by employing the MTS assay. HT29 cells with a density of 10^4 cells well⁻¹ were seeded in 96-well plates. After 24 h, the medium of 96-well plates was replaced with 200 μ L of serum-free medium containing different concentration of HANs solutions and incubated. The cells were or were not irradiated by NIR laser with 808nm laser and 0.6 W cm⁻² irradiation power for 5 min. Then cells were incubated for 24 h at 37 °C, saturated humidity, 5% CO₂. The cells in the 96-well were then washed twice with PBS. Cell viability was evaluated by adding 20 μ L MTS assay kit (Promega, Madison, WI, USA) to each well. After 3 h incubation, the absorbance of each well was measured using 490 nm as the test wavelength with a microplate reader (Infinite M1000, TECAN, USA). Cell viability was calculated based on the recorded data.

The cytotoxicity of HANs on NIH 3T3 cells was examined by employing the MTS assay. NIH 3T3 cells with a density of 5×10^3 cells well⁻¹ were seeded in 96-well plates. After 24 h, the medium of 96-well plates was replaced with 200 μ L of serum-free medium containing different concentration of HANs solutions and incubated, followed by the 24 h incubation. Cell viability was calculated based on the recorded data.

2.14. Time course study of cellular uptake of HANs by HT29 cells and NIH 3T3 cells. Both HT29 and NIH 3T3 cells were treated with 25 nM HANs in growth media at 37°C for 1, 2, and 4 h incubation. After incubation, the cell plates were washed with PBS twice. After the cells were lysed with diluted nitric acid, the mixture was further digested with *aqua regia*. The gold, iron and platinum concentration of HANs in cells were measured by Inductively coupled plasma mass spectrometry (ICP-MS) analysis.

2.15. ⁶⁴Cu labeling of HANs. The ⁶⁴Cu labeling procedure was conducted according to the methods previously described [16]. Briefly, the HANs were conjugated with copper chelator NOTA using the p-SCN-Bn-NOTA. The resultant NOTA-HANs were radio-labeled with ⁶⁴Cu by addition 37 MBq of ⁶⁴CuCl₂ in 0.1 N NaOAc (pH 5.5) buffer, followed by 1 h incubation at 40°C. The radio-labeled HANs were purified by a PD-10 column and diluted with PBS for in vitro and in vivo experiment.

2.16. *Tumor models.* All animal experiment's protocols were approved by Administrative Panel on Laboratory Animal Care at Stanford University, and were performed in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care. Female nude mice (5~7 weeks, 18 ± 2 g, Charles River Laboratories) were used for *in vivo* studies. The HT29 colon tumor models were prepared by subcutaneous implantation of 3×10^6 cells in 100 μ L of PBS into the left front flank.

2.17. *In vivo MRI and PAI.* When the tumor volumes reached about 100 mm³, the mice bearing HT29 tumor were intratumorally injected with 50 μ L of 500 nM HANs solutions. The MR images of mice were acquired before the injection as well as 1 h and 24 h post-injection in a 7.0 T animal MRI scanner. *T*₂-weight images were obtained using a 7.0 T MR scanner (TR = 4000 ms, TE = 60 ms, Matrix size = 256×256 , FOV = 30×30 mm, slice thickness = 2 mm). Before the injection as well as 1 h and 24 h post-injection, the photoacoustic and ultrasound images of the mice were acquired by a MS-250 linear array transducer (21 MHz, 20 dB two-way bandwidth, 256 elements). PA images were performed by a LAZR commercial instrument (Visual Sonics Inc., Toronto, Canada) with a 680 nm laser and a focal distance of 10 mm was used to acquire photoacoustic and ultrasound images. Quantification analysis of photoacoustic images was carried out using ImageJ.

2.18. *In vivo PTT.* In order to observe the tumor growth and inhibition *in vivo*, the twenty-four nude mice bearing HT29 tumor models were used. At 2~3 weeks after cells implantation, when tumor volumes reached approximately ~ 100 mm³, the mice bearing HT29 tumors were randomly divided into four groups ($n = 6$ in each group). In the test group (NPs + laser), the mice were intratumorally injected with 50 μ L of 500 nM HANs solutions. At 24 h after injection, tumors were irradiated by the NIR laser with the 808 nm laser at a power density of 0.6 W cm⁻² for 5 min. During irradiation, the temperatures and thermal images of tumor site were taken by a thermal camera at each 30 sec of irradiation as well as before irradiation. For comparison, the mice of NPs group and laser group were intratumorally injected with same volume of HANs solutions. In control groups, mice were injected with 50 mL of PBS. Tumor sizes were measured every 2 days, using a digital caliper, and tumor volumes (mm³) were calculated using the formula (tumor volume = length $\times$ width² $\times$ 0.5).

2.19. *Small-animal PET.* Small animal PET imaging of tumor-bearing injection ⁶⁴Cu-labeled HANs was performed on a Siemens Inveon microPET-CT. Mice bearing HT29 tumors ($n = 4$)

were injected with 0.285 MBq of ^{64}Cu -labeled HANs intratumorally. At different times after injection (1, 1.5, 2, 2.5, 4.5, 12, 24, and 48h), the mice were anesthetized with 2% isoflurane and placed in a prone position near the center of the field of view (FOV) of the scanner. For method comparison study, ^{64}Cu -labeled HANs (3.3 MBq) were injected via tail vein into the HT-29 tumor-bearing mice ($n = 4$) at a dose of 200 pmol/kg of mouse body weight. Five-minute static scans were performed. The PET images were reconstructed with IRW 4.0 software by two-dimensional ordered subsets expectation maximization (OSEM) algorithm. The radioactivity uptake of tumor and major organs were calculated using a region of interest (ROI) analysis over the whole organ region and expressed as a percentage of the injected radioactive dose per gram of tissue (%ID/g).

2.20. Intracellular ROS detection. HT-29 cells were placed on 6-cm dishes at a density of 2×10^5 cell and incubated at 37°C for 24 h. After removing growth medium, the cells were incubated with 20 μM DCFH-DA in a FBS-free medium for 30 min. The DCFH-DA buffer was then replaced with the growth medium after the cells were washed with twice with PBS. The DCFH-DA loaded cells were incubated with Pt nanoparticles [9] and HANs (based on the same Pt concentration: 3.0 μg Pt/ml) for 1 and 2 hours at the standard condition. The cells were washed with twice with PBS after removing the particle solution. The cells were trypsinized, collected and resuspended in 500 μL of PBS, and then filtered through 35 μm nylon mesh to form a single-cell suspension. The cells were then analyzed using a flow cytometry (FACS Aria III, BD Biosciences, San Jose, CA, USA) at the Stanford FACS Facility. The gate was set for the detection of green fluorescent DCF. The excitation and emission wavelengths were 485 nm and 525 nm, respectively. Data were analyzed by FlowJo FACS analysis software (Tree Star, Ashland, OR, USA).

2.21. Histology analysis. Tumor tissues were embedded in Tissue-Tek® O.C.T. (Optimal Cutting Temperature) compound and frozen in -80°C refrigerator for histological examination. Tumors were sliced into 10- μm -thick and stained with hematoxylin and eosin (H&E). In addition, apoptotic cells were measured using immunofluorescent deoxynucleotidyl transferase-mediated X-dUTP nick end labeling (TUNEL) staining, which was performed according to the manufacturer's instructions (in situ cell death detection Kit, Fluorescein, Roche Diagnostics Corp). The slides of TUNEL staining were mounted by cover slips with the vectashield mounting medium containing DAPI (Vector Laboratories, CA, USA) and observed under fluorescence microscope

(Olympus IX81). The percentage of necrotic areas and apoptotic cells were calculated in the tumors.

2.22. Numerical analysis of the optical properties of the HANs. Numerical analysis was conducted employing the same procedure described in our previous publication [16]. Briefly, all the simulations were performed with water as the dielectric medium, with a refractive index of 1.33. A mesh-size of 0.5 nm in all the three Cartesian directions was chosen for the simulation. The centers of FePt cubic cores were placed at the origin of the coordinate system in the simulation box. Gold dielectric data was based on Johnson and Christy, and dielectric data of iron platinum alloy was based on Palik and literatures [17]. The ambient was water with a refractive index of 1.33. The PEG layers were ignored, because their refractive index would depend on the surface coverage, and these layers would lead to less than 10 nm peak shifts to the red. The spectral dependence of the absorption and scattering cross sections for both polarizations were calculated and added. HAN was modeled as one cubic iron platinum core (7 nm in length) with two gold spheres of constant radius of 5 nm.

2.23. Statistical analysis. Measurement data are expressed as the mean $\pm$ SD. Statistical analyses of the data were performed with the SPSS 12.0 software. For cell viability assays, a Student's t-test was applied to identify significant differences. A value of $p < 0.05$ was considered statistically significant.

3. Result

3.1. Construction of plasmonic hybrid anisotropic nanoparticles (HANs)

Recent efforts have been made on the construction of colloidal hybrid nanostructures with two or more components via robust junctions in order to synergistically integrate different chemical and physical properties together. After exploring the seed-mediated nucleation and epitaxial growth of various hybrid metal nanocrystals [16], we focused on the construction of hybrid metal alloy nanocrystals with multiple chemical and physical properties to satisfy our application requirements. Similar to the pure platinum nanocubes, the cubic FePt alloy nanocrystals played an important role on the formation of colloidal hybrid hetero-nanostructures with desired

architectures. The size and shape of FePt alloy nanocrystals are critical to the seed-mediated process and can be easily determined by changing the amount of metal precursors and surfactant [15]. Accordingly, the sizes of cubic FePt nanocrystals with relatively narrow size distribution were finally tuned from 3 to 9 nm. As seen in Fig. S1a - d, the FePt nanocubes (7 nm in size) were synthesized with a very narrow size and shape distribution. The lattice fringe shown in Figure S1d is c.a. 0.195 nm, which is related to (200) planes of FePt in face-centred cubic (fcc) phase. As the core and seeds, the FePt nanocubes could induce the formation of branched hybrid hetero-nanostructures with plasmonic gold nanocrystals. Due to lattice constant matching, the gold nanocrystals prefer to epitaxially grow at the vertices of cubic FePt core. The dumbbell Au-FePt nanostructures were obtained in the presence of 5 nm FePt seeds (Fig. 1a-c). The epitaxial growth of two Au crystals on FePt seeds produced Au-FePt-Au hybrid anisotropic nanostructures (HANs, Fig. 1d). Corresponding to the para and meta configurations of Au crystals surrounding around FePt core, two types of geometrical isomers could be observed in the products (Fig. 1b): one with a linear shape (Fig. 1c); the other in a bent way (Fig. 1d), and they have roughly equal amounts (based on TEM analysis).

3.2. Characterization of optical and physical properties of HANs for PAI, MRI and PTT

The attachment of two gold nanocrystals on the FePt nanocubes results in the significant change in the optical absorption. Compared to the Au NPs alone and FePt nanocubes, the HANs have a major peak at 540 nm with a broad absorption shoulder across the range of far-red and NIR region (650-1000 nm); an absorption shoulder at 680 nm remains a relatively high level compared to those of gold spheres and FePt nanocubes on a per-weight basis, and importantly, the absorption at 800 nm is still more than one third of that of the major peak (Fig. 2a), all of which make HANs

an excellent photoabsorbing agent for the PAI and PTT. The localized surface plasmon resonance (LSPR) wavelengths of resultant HANs could be optimized within the range of far-red and NIR region by control of the spatial configuration of surrounded Au crystals. The HANs with linear configuration contributes more on the absorption across section in the range of far-red and NIR region than the bent HANs [16], which is consistent with the simulation result (Fig. S3-S5).

To improve the biocompatibility and physiological stability of HANs, the HANs were coated with a monolayer of amphiphilic phospholipid-polyethylene glycols (DSPE-PEG) by a fast, facile self-assembly process [18]. The resultant water-soluble HANs exhibit excellent stability in normal physiological conditions. The hydrodynamic diameter of HANs determined by a dynamic light scattering measurement is about 24.6 nm (Fig. S6).

To further determine the photoacoustic signal sensitivity of HANs, the PA signal intensity of the phantom was quantitatively measured at various concentrations (Fig. 2b). A linear regression analysis with a high R^2 value ($R^2 = 0.989$, Fig. 2c) confirmed that the HANs are ideal candidates for PAI. After the background correction, the detection limitation of PA signal intensity could be as low as 2 nM of NPs. Such nanomolar-level detection sensitivity allows the HANs to be more practicable PAI agents for *in vivo* studies compared to many conventional PAI contrast agents.

To evaluate magnetic property of HANs, HANs of different Fe concentrations in aqueous solutions were scanned by a 7.0 T animal MR scanner. We found that the signal intensity of HANs on T_2 -weighted images decreased significantly with the increase of Fe concentrations (Fig. 2d-inset). Moreover, the T_2 relaxation rate of the HANs was $87.5 \text{ mM}^{-1} \text{ s}^{-1}$ (Fig. 2d). The magnetism of HANs makes it act as a potential MRI contrast agent.

To study the photothermal property of HANs *in vitro*, fifty μL of HANs solution at various concentrations and phosphate buffered saline (PBS) were irradiated. With the increasing

concentrations, the temperature of HANs solutions significantly increased upon the NIR laser irradiation (Fig. 2e). At a concentration of 100 nM, the temperatures of HANs solution raised rapidly, reaching an average temperature of 59.5°C after irradiation for 150 second (Fig. 2f). The temperatures of HANs solution can be easily heated up to above 50°C, which is sufficient to use for the treatment of solid tumors [19, 20]. In comparison, when PBS was irradiated under the same NIR light condition, the temperature increased by only 4.8°C. These results clearly demonstrated that the NIR optical absorbance of HANs can convert to the thermal energy. HANs provide excellent photothermal effect and can be used as efficient NIR light absorbers for the PTT of tumor.

3.3. Validation of HANs for PAI, MRI and PTT

To examine the release of Fe ions from HANs, a dialysis-bag diffusion technique was performed [21]. The cumulative release curves of Fe ions from HANs were shown in Fig. 3a. We found that the release of Fe ions was pH-dependent. Under a physiological condition (pH = 7.4), Fe ions from HANs were still negligible in the solution outside the dialysis-bag in 24 h. At an acidic condition (pH = 4.8), however, HANs have over $31 \pm 3\%$ release of Fe ions in 24 h. Even in cytoplasm mimicking buffers (CB), $24 \pm 4\%$ of Fe ions have been released over 24 h in an acidic pH (5.2). This result demonstrated that HANs were stable in a normal physiological condition (pH = 7.4). Whereas under low pH conditions, the acid etching of the HANs triggers the release of Fe ions. As pH-controlled iron reservoirs, HANs exhibited the pH-triggered release of Fe ions, which make it act as an active chemotherapeutic agent for cancer therapy based on the pH differences between tumor (low pH value) and normal tissues (pH = 7.4).

The cytotoxicity of HANs was determined by employing the MTS viability assay. As shown in Fig. 3b, with the increase of HAN concentrations, the cell viability of HT29 cells treated with HANs decreased. At the same concentration, HANs showed much less cytotoxicity to standard non-cancerous cells (NIH 3T3 cells) compared to HT29 cells (Fig. 3c-3d), indicating their good biocompatibility. Additionally, the cell viability of HT29 cells treated with HANs plus laser irradiation were significantly lower than those of treated with HANs at high concentrations (25, 50, 100 nM). In the absence of HANs, however, there is not significant cytotoxicity observed in HT29 cells irradiated with NIR laser. These results implied that the synergistic effect on cell viability after treatment of both HANs and laser irradiation was higher than that of individual treatment alone, indicating that HANs could act as a dual-treatment (PTT and chemotherapy) agent.

The intracellular ROS levels in HT29 cells treated with PBS, Pt NPs [9] and HANs (based on the same Pt concentration) were measured by FACS flow cytometry using a cell-membrane permeable peroxide-sensitive fluorescent probe (2,7-dichlorofluorescein diacetate, DCFH-DA). The formation of intracellular ROS in response to the nanoparticle treatment was quantitatively measured by monitoring the conversion of nonfluorescent DCFH-DA to fluorescent dichlorofluorescein (DCF). After two ester groups (DA) were cleaved by the intracellular esterase, the DCFH molecules were deprotonated and converted to green fluorescent quinone-like DCF. Flow cytometry with green fluorescent detection can clearly distinguish the DCF staining HT29 cells from non-fluorescent cells. Untreated cells were used as a control. The numbers in each quadrat reflect the percentage of the cells (stained vs. unstained) (Fig. 3e). The fluorescence intensities were evaluated within the population of the cells. There was no ROS produced when there were not particles in the cell cultures. As seen in the Fig. 3e, no significant difference in ROS

production was found between the control cells and the cells treated with Pt NPs, whereas the level of DCFH-DA oxidation by HANs within the HT29 cells was significantly higher than their counterparts from Pt-treated cells. We compared the levels of DCF mediated fluorescence in HT29 cells with different incubation time. The fluorescence intensity from HAN-treated cells increased with increasing incubation time. Thus, all the results confirmed that the hybrid HANs rather than Pt NPs were responsible for significant ROS production in the tumor cells.

In order to confirm the integrity of the nanoparticles in the harsh physiological condition, we investigated the reliability of the photoacoustic and MR signal of HANs after treated with acidic solutions (Fig. 3e and f). Before the measurements of their PA and MR signals, HANs were pre-treated with either a neutral buffer (PBS (pH 7.4) or CB buffers (7.4)) or an acidic solution (PBS (pH 4.8) or CB buffers (5.2)) for 24 hours. Although the iron release after 24-hour acid etching resulted in a slight increase of MR signal in T₂-weighted images, the photoacoustic intensity roughly remained the same (Fig. 3e).

3.4. In vivo evaluation of image guided photothermal therapy using HANs

Inspired by the promising results *in vitro*, HANs were then used as a theranostic platform for image-guided PTT *in vivo*. Endoscopic intratumoral injection of drugs for treatment of colorectal carcinoma has been applied in clinical therapy [22], which can enhance local drug concentration, maximize drug efficacy, and avoid the systemic side effects induced by the intravenous administration. Therefore, intratumoral injection of HANs was performed in this study. To determine the optimal time point of NIR irradiation, the HT29 bearing tumor mice were intratumorally injected with 50 μ L of 500 nM HANs, then PAI and MRI were acquired before and after the injection. As shown in Fig. 2a, compared to pre-injection images, PA signals after 1 h

post-injection can be clearly detected in the local injection site of tumor. At 24 h after injection, PA signals induced by HANs were evenly distributed in the whole tumors. PAI is a relatively new molecular imaging technique and has recently attracted significant research interests [23]. PAI can also been used to track the uptake, delivery, and distribution of NPs [24]. Similar to PAI, hypointense signal spots on T_2 -weighted MRI at 1 h post-injection can be clearly seen in the tumor. At 24 h post-injection, hypointense signal spots spread to the whole tumor. PAI and MRI can thus dynamically track the distribution of HANs inside the tumor over time, which is helpful to determine the optimal time point of NIR irradiation and can further improve the therapeutic efficiency and reduce side effects of the PTT. After 24 h post-injection, the homogeneous distribution of HANs from the injection incision to the whole tumors was confirmed by both PA and MR imaging (Fig. 4a), thereby guaranteeing their maximum therapeutic effect to the entire tumors.

Based on the above PAI and MRI results, we chose 24 h post-injection as the time point of the PTT *in vivo*. In the test group (HANs + laser), HANs-treated tumors were irradiated by the NIR laser with the 808 nm NIR laser at a power density of 0.6 W cm^{-2} for 5 min. During the irradiation, the temperature of tumor site in the test group showed an increase of 25.3°C during the course of irradiation, eventually reaching an average temperature of 55.1°C . However, the temperature of tumor site injected with PBS only increased approximately 4°C during the course of irradiation (Fig. 4b and c). As shown in Fig. 5a and b, day 2 after the irradiation, the tumors treated with HANs plus laser disappeared and there were only black scars at the initial tumor positions. Moreover, from day 2 to day 20, the volume of tumors remained at ~ 0 and tumor relapse was not found. For the mice group treated with HANs alone, tumors sizes slightly reduced in the initial 4 days. However, tumors in the laser treated group without HANs and PBS-sham treatment group

both showed rapid growth. Successful elimination of tumors by treatment of combination of NPs and laser demonstrated that HANs could promisingly act as a chemo-thermo therapy agents for cancer treatment. By combining chemo-thermo therapies in a single nanopatform, HANs demonstrated excellent synergy in suppressing tumor growth. At last, the haematoxylin and eosin (H&E) staining of tumor sections also demonstrated the high therapeutic efficacy of combination of the HANs and NIR laser irradiation. There was apparent extensive necrosis of tumor tissues in the group of mice treated with HANs plus laser, and the necrotic areas of tumors were significantly greater than those of tumors treated with HANs only, laser only, and PBS ($p < 0.05$, Fig. 5c and d). The cytoplasmic acidophilia, karyolysis, and corruption of the extracellular matrix were shown in necrotic tissues. Nevertheless, the obvious malignant necrosis was not found in the laser group and PBS group. Moreover, TUNEL staining of tumor sections showed that apoptotic cell's percentage of tumors treated with HANs plus laser was significantly higher than that of tumors treated with the HANs, laser, and PBS ($p < 0.05$, Fig. 5c and e). These results highlighted that HANs can be a promising multimodality theranostic system for cancer therapy.

The favorable *in vivo* behavior and biodistribution of HANs are fundamental requirement for development of nanoprobe as theranostic agents [16, 25-27]. We examined the *in vivo* biodistribution of HANs after administration in mice (Fig. 6 and Fig. S7-S8). The uptake of the HANs in liver and spleen reached a maximum after 24 h post-injection and then gradually decreased, suggesting that some HANs were probably being removed from the animal via hepatobiliary excretion. Although additional studies are required to investigate the long-term effects of the HANs and their toxicity when releasing iron upon acid stimulus, our previous results support the idea that gold/platinum-based nanoparticles can be safely used in living subjects [16].

In order to evaluate the bio-compatibility and pharmacokinetics of HANs *in vivo*, we performed the biodistribution study of HANs after intravenous administration, and investigated the effect of the routes of administration on the systemic biodistribution of HANs in the HT29 bearing tumor mice ($n = 4$) (Fig. S10). The high accumulations of the nanoprobe in the liver and spleen were observed after 2-hour post-injection with continuous localization in the liver and spleen over 24 h, indicating that a majority of injected nanoprobe were taken up by the mononuclear phagocyte system (MPS). Even though the majority of accumulation was observed in the liver and spleen, the tumor uptake of the nanoparticles became prominent after 4-hour post-injection, due to the enhanced permeability and retention (EPR) effect. The quantitative biodistribution (%ID/g) of nanoprobe from each organ correlated well with the corresponding PET images (Fig. S11). A significant decrease in liver uptake over 24 h indicated that the particle elimination or clearance could result from the hepatic metabolism and biliary excretion of injected nanoprobe. Similarly, the high kidney uptake at 24 h also suggested that the renal excretion might be involved in the elimination process of injected nanoprobe.

4. Discussion

Colon cancer is a deadly disease. Many colon cancer patients die of their malignancy despite radical surgical treatment, radiotherapy and chemotherapy, probably because they are often diagnosed late and systemic therapy is limited at an advanced stage. The occult blood testing and colonoscopy have been established as routine for colon cancer diagnosis and monitoring, yet still suffer from limited diagnostic ability and poor reliability [28]. Surgery, followed by systemic chemotherapy and radiotherapy, is recommended for the subset of colon cancer patients who have been fortunate to have their disease completely resected. Chemotherapy kills actively dividing cells including

cancer cells, but its administration is limited by toxicity to non-cancer cells. Thus it is an urgent need to develop new techniques and tools for augmenting the diagnosis of colonic cancer and better adjuvant therapies in order for significant improvements in survival.

Our design of multifunctional hybrid anisotropic nanostructures aims to combine the benefits of seamless integration of both dual-modal molecular imaging and image-guided chemo-thermo therapies, with the ultimate goal of improving overall treatment of colon cancers. We introduced a novel strategy to manipulate constitutional nanocrystals at the nanometer-scale to build ‘all-in-one’ multifunctional nanoprobe which can combine the advantages found with the specific nanomaterials and anisotropic nanostructures, and provide complementary diagnostic information and synergistic treatment effects. To the best of our knowledge, this work is the first integral use of both MR and PA imaging via an anisotropic nanostructure as a theranostic probe for both chemotherapy and photothermal therapy in living subjects.

Engineering nanoprobe with magnetic and optical properties is a vital step to make them possible as ‘all-in-one’ multifunctional probes for simultaneous cancer diagnosis and treatment. In this study, we constructed Au-FePt-Au hybrid anisotropic nanostructures by fusing an FePt alloy nanocube with two Au nanocrystals together via solid state interfaces. The characterization of those nanoprobe using TEM, DLS and ICP-MS confirmed that we had successfully prepared monodisperse nanoprobe with a narrow size distribution and uniform shape. It is very important that each individual nanoprobe has nearly identical physical and chemical properties for controlled biodistribution, contrast and therapeutic effects. In such an anisotropic nanostructure, the cubic FePt nanocrystal plays a critical role for rendering the nanoprobe with desired magnetic, optical, and chemical properties. First, the cubic FePt alloy nanoparticles are intrinsically superparamagnetic. Even after conjugated with gold nanoparticles, the resultant HAN showed a

relatively high T_2 relaxivity rate compared to commercial MRI contrast agent Ferumoxytol [29]. Second, the FePt cubes were used as cores to fabricate the linear- and bent-shaped anisotropic metallic nanostructures, generating the surface plasmon hotspots at the junctions between plasmonic metal crystals. The increases of the surface plasmon hotspots in terms of their locations and intensities resulted in a red-shift of the absorption of anisotropic nanostructures [16]. Compared to individual compositional components on a per mass basis, the HANs have a much stronger and broader absorption in both far red and NIR regions. It makes HANs very promising not only as contrast agents for *in vivo* photoacoustic imaging but also as photosensitizing agents for *in vivo* photothermal therapy. The excellent photothermal effect of HANs is also a direct result of their relatively high absorption at the laser wavelength. Third, the ROS induced by FePt cores of HANs in the acidic environment caused the cell apoptosis, especially for tumor cells. HANs were normally inactive in the circulation, and then activated in response to the low extracellular/intracellular pH in tumors [30]. When the nanoprobe was quickly internalized by tumor cells via the endocytosis process and taken into the acidic endocytic organelles, the acidic environment further induced the Fe releasing from the FePt cores of HANs. The released Fe ions cause the Fenton reaction: Fe ions catalyze the hydrogen peroxide (H_2O_2) decomposition into ROS that are highly reactive to lipid membrane and DNA chains [9]. Compared to normal tissue cells, the tumor cells produce large amounts of H_2O_2 [31]. Therefore, the accumulation of Fe ions in tumor cells dramatically induced the formation of ROS in the tumor cells and eventually led to cellular damage and cell death. Although the difference of nanoprobe's cellular uptake between tumor cells and normal cells was not very significant in *in vitro* experiment, the production of large amounts of hydrogen peroxide by tumor cells was responsible for a disproportionate number of cells' deaths after cells were treated with HANs.

The ‘all-in-one’ multifunctional HANs possess both intrinsic imaging and therapeutic properties, which can be optimized accordingly. The MRI and photoacoustic signals produced by the HANs *in vitro* were highly linear and correlated with each other. The HANs as MRI and PAI contrast agents have excellent magnetic and optical properties, and can chemically withstand normal physiological conditions without property degradation in the detection or treatment time period. The very high spatial resolution with MR imaging provides detailed anatomic information and probe distribution, and PA imaging of probes with high sensitivity can guided where PTT might best be directed. Such complementary information from the dual MR/PA probe dramatically improves diagnostic and therapeutic performance, particularly in regions of high clinical suspicion. With detailed information from MR and PA imaging, the HANs were injected to achieve efficient tumor elimination through chemo- and photothermal- therapies at a very low dose.

The ideal multimodality theranostic probes can detect many cancers including metastatic lesions even at an early stage, release anticancer agents at the targeted sites, and provide a real-time feedback of therapy response. Recently, the rapid development of the miniaturized photoacoustic endoscope significantly facilitates the applications of theranostic nanoparticles for diagnosing and treating the gastrointestinal tract diseases [32, 33]. Advanced endoscopes integrated with imaging and therapy modules could improve the diagnostic, treatment and surgical techniques for detecting and treating bleeding, polyps and cancerous growths within the gastrointestinal tract. Multifunctional theranostic nanoprobe with endoscope systems could significantly reduce the procedure time and have a great potential for simultaneous detection, delineation and minimally invasive surgical procedures for colon cancer treatment.

5. Conclusion

In summary, we have successfully fabricated novel HANs in a control and predictable manner. The HANs show small sizes and special structures. The HANs integrated two different single-modality theranostic systems into a multimodality theranostic system via simple methods, which can combine desired functions of FePt NPs and Au NPs into a nanoentity. As T_2 MRI and PAI contrast agents, the HANs could act as dual-modal imaging probes for image-guided therapy. Furthermore, as pH-controlled Fe ions reservoirs, HANs possess chemotherapeutic function in response to the low pH of tumor. More importantly, the HANs also have a strong and broad absorption in the far red and NIR regions, under NIR light irradiation the HANs can induce significant temperature elevation and locally eliminate tumor efficiently. These results demonstrate that Au-FePt-Au HANs hold great promise as a multimodality theranostic agent for image-guided cancer therapy.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/xxx>.

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Scheme and Figures

Scheme 1. Schematic illustration of the design of heterostructure HANs as a multimodality theranostic platform.

Fig. 1. Construction of plasmonic hybrid anisotropic nanoparticles (HANs). **a-c**, Representative TEM images of dumbbell Au-FePt nanoparticles in different magnifications. HRTEM images of dumbbell Au-FePt nanoparticles are shown in **b** and **c**. The top-right inset in HRTEM image reveals the 0.195 nm spacing between (002) planes of FePt alloy particle and 0.235 nm between (111) planes of Au particle. The structural diagram of Au-FePt dumbbell nanoparticle is shown in the inset of **c**. **d-f**, Representative TEM images of Au-FePt-Au HANs nanoparticles HANs (linear vs. bent shape) in different magnifications. **e**, HRTEM image of linear HANs. The inset shows the structural diagram of linear HANs. **d**, HRTEM image of bent HANs. The top-right inset shows the structural diagram of bent HANs. The lattice fringe of Pt component in the inset is 0.197 nm and related to (002) planes of Pt in the fcc phase. The lattice fringe of Au component in the inset is 0.236 nm and related to (111) planes of Au in the fcc phase.

Fig. 2. Characterization of optical and physical properties of HANs for PAI, MRI and PTT.

a, UV–vis–NIR absorbance spectra of FePt nanocubes, Au spheres, and HANs in aqueous solution based on the same weight. **b**, PAI of phantom at various HANs concentrations *in vitro*. **c**, The linearity of concentration dependent PA signal intensities of HANs. **d**, T_2 relaxation rate as a function of Fe concentrations of the HANs. The inset: T_2 -weighted MR images of HANs at various concentrations of iron at 7.0 T MR scanner. **e**, Thermal images of HANs solution at the various concentrations after 2 min of NIR laser irradiation at 808 nm. PBS is used as a control. **f**, Temperature evolution curves at the various HANs concentration under continuous NIR laser radiation.

Fig. 3. Toxicity and stability of HANs for PAI, MRI and PTT. **a**, The cumulative release of iron ions from HANs in PBS buffers (pH = 4.8 and 7.4) and Cytobuffer (pH = 5.2 and 7.4). **b**, Cell viability of HT29 cells incubated in the different concentrations of HANs with or without laser

irradiation. **c**, The time course of cellular uptake of HANs by HT29 cells or NIH 3T3 cells. Both HT29 and NIH 3T3 cells were treated with 25 nM HANs at 37°C for 1, 2, and 4 h incubation. All results, expressed as percentage of cellular uptake, are mean of triplicate measurements $\pm$ SD. * $p < 0.05$ (two-sided Student's *t*-test). **d**, Cell viability test of NIH 3T3 cells treated with different concentrations of HANs over 24 hours without laser irradiation. **e**, Intracellular ROS detection. The intracellular ROS levels in HT29 cells treated with PBS (Ctl), Pt NPs [9] and HANs (based on the same Pt concentration: 3.0 μ g Pt/ml) were measured by FACS flow cytometry using the peroxide-sensitive fluorescent probe DCFH-DA. The HT29 cells were treated with PBS (1), DCFH-DA alone (2), Pt NPs for 1 h (3), Pt NPs for 2 h (4), HANs for 1 h (5), and HANs for 2 h (6). The lower left quadrant (Q4) represents viable, nonstained cells, while the lower right (Q3) shows viable cells with high fluorescent intensity (high intracellular ROS). The detection of green fluorescent DCF was measured at FITC channel (485 nm excitation and 525 nm emission). **f**, Quantification of the intracellular ROS levels in HT29 cells after treated with PBS, Pt NPs and HANs. * $p < 0.01$, ** $p < 0.001$ (two-sided Student's *t*-test). **g & h**, The effect of acid etching on PA and MR signal of HANs after treated with a neutral buffer (HANs) or an acidic solution (Acid-etched HANs) for 24 hours. **g**, Maximum intensity projection PA images of HANs after treated with a neutral buffer (HANs) or an acidic solution (Acid-etched HANs). PA signal intensities of HANs and acid-etched HANs measured at 680 nm are shown in a column graph. **h**, MR images of HANs after treated with a neutral buffer (HANs) or an acidic solution (Acid-etched HANs). MR signal intensities of HANs and acid-etched HANs are shown in a column graph. * $p < 0.05$ (two-sided Student's *t*-test).

Fig. 4. *In vivo* evaluation of image guided photothermal therapy using HANs. **a**, Ultrasound images of B-mode, photoacoustic image, overlaid photoacoustic and ultrasound images of B-mode, and T_2 -weighted MR images of HT29 tumor-bearing mice before and 1 h and 24 h after intratumoral injection of the HANs. **b**, Representative infrared thermal images in the test group were showed at the different time points as well as the PBS control. The color bar relates the relative temperature values. **c**, Temperature evolution curves of the HT29 tumor mass as measured over the 5 min course of NIR laser treatment.

Fig. 5. Image guided photothermal therapy for HT29 tumor model. **a**, Tumor growth curves of different groups of HT29 tumor-bearing mice after treatment. **b**, Representative photos of tumor-bearing mice after various treatments before and day 4 after the treatment. **c**, Histological sections of tumor tissues were H&E and TUNEL stained ($\times 10$). Necrotic areas (arrow) were seen by H&E staining. Nuclei are stained blue (DAPI staining, $\times 10$), apoptotic cells are stained green (TUNEL staining, $\times 10$). Scale bar = 100 μ m. **d**, The necrotic area as a percentage of the tumor tissue is shown in the bar graph. Necrotic areas of tumors treated with HANs plus laser were significantly greater than that of tumors treated with HANs, laser, and PBS. **e**, Quantitative analysis of apoptotic cells in tumors treated with HANs plus laser, HANs, laser, and PBS is shown in the bar graph. Apoptotic cells of tumors tissue in the test group were significantly more than that in the HANs, laser, and PBS group. Asterisk (*) indicates p -value < 0.05 .

Fig. 6. Pharmacokinetic and bio-distribution in a mouse model after administration of ^{64}Cu labeled HANs. Small animal PET images of mice bearing the HT29 tumor intratumorally injected with 0.285 MBq of ^{64}Cu labeled HANs ($n = 3$ per group, data represent means $\pm$ standard deviations (SD)). **a**, Decay-corrected whole-body coronal PET images of nude mice bearing human HT29 tumor at 1, 1.5, 2, 2.5, 4.5, 12, 24, and 48 h after intratumoral injection of 0.285 MBq of ^{64}Cu labeled HANs (100 pmol / kg of mouse body weight). **b**, Decay-corrected whole-body coronal PET/CT images of mice bearing human HT29 tumor at 2, 4.5, 12, and 24h after injection. **c**, PET quantification of tumors after intratumoral injection of 0.285 MBq of ^{64}Cu labeled HANs (100 pmol / kg of mouse body weight).