Electronic Supplementary Material (ESI) for ChemComm.

Supporting information

Gold nanorods with noncovalently tailorable surface for multi-modality image-guided chemo-photothermal cancer therapy

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1. EXPERIMENTAL SECTION

Materials and Characterization

Tetrachloroauric acid ($HAuCl_4 \cdot 4H_2O$), L-ascorbic acid, silver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$), ethanol, 25% glutaraldehyde, MTT, folic acid, amantadine,1-ethyl-3- (3-dimethyl-aminopropyl) carbodiimide (EDC) and N-hydroxy succinimide (NHS) were purchased from Aladin and used as received. N-Cetyltrimethylammonium bromide (CTAB) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Oxaliplatin was purchased from Yuanye biological company (China).

Milli-Q Integral system (Merck) was used to supply Milli-Q water in this work. The morphology of NPs was observed by transmission electron microscopy (TEM, JEOL 2100F, Japan) at 200KV. The Zeta potential of nanomaterials was measured on a Zetasizer (Malvern). NMR spectra were obtained using a Bruker Ultra Shield 600 PLUS NMR spectrometer. Fluorescence spectra were obtained on fluorescence spectrometer (Thermo Scientific Lumina). Fluorescence images were acquired by confocal laser scanning microscopy (CLSM, Leica TCS SP8, German) and inverted fluorescence microscope (Olympus IX73). Fourier transform infrared (FT-IR) spectra and UV- vis-NIR absorbance spectra were measured on IFS-66V/S and Shimadzu UV-1800 spectrophotometers. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were measured using a microplate reader (Infinite F200 Pro, TECAN). Intracellular uptake and cell apoptosis were both quantified by a flow cytometer (Beckman coulter)

Preparation of Au NRs

Gold Nanorods were prepared following a seed-growth method[1-3]. Briefly, the seed solution was formed by mixing $HAuCl_4$ (0.01 M, 250 μL), CTAB (0.10 M, 9.75 mL), and $NaBH_4$ (0.01 M, 600 μL) under stirring for 2 min before aging at 27 °C for 90 min. 120 μL seed solution was added into a growth solution containing CTAB (0.20 M, 50 mL), $AgNO_3$ (0.01 M, 800 μL), and $HAuCl_4$ (0. 1 M, 50.0 mL) and vigorously stirred for another 15 min. L-ascorbic acid (0.1 M, 550 μL) was added and the solution turned to colorless. The Au NRs were grown overnight without stirring at 28 °C. The NRs were washed by water for 3 times to remove excessive CTAB.

Preparation of CB[7]-Au NRs

The mercaptoundecanoic acid was dispersed in ethanol and dropwise added into the aqueous solution of Au NRs. The mixture was sonicated for 1 h before being vigorously stirred for 24 h. Au-COOH NRs were collected by centrifugation and washed with ethanol for 3 times. The $AO_1CB[7]$ was prepared according to previously report[4]. The CB[7]-NH_2 was obtained by thiol–ene "click" reaction between $AO_1CB[7]$and cysteamine and confirmed by ^1H NMR.[5]

EDC, NHS and CB[7]-Au NRs were dissolved in DMSO and stirred for 3 h before adding CB[7]-NH_2. The mixture was magnetically stirred for 24 h at room temperature. The acquired nanocomposites were collected by centrifugation and dispersed in water.

The amount of CB[7] on the surface was calculated by a fluorescence assay. Briefly, an excessive amount of the fluorescent guest molecular AO was used to bind with the surfaced modified CB[7] to form CB[7]·AO. A known quantity of CB[7]-Au NRs was mixed with an excessive amount (amount A) of Ada in water and shake for 5 min before ultra-centrifugation. The Ada (amount B) remain in the supernatants was measured by adding CB[7]·AO and the fluorescence was corresponded to the CB[7]·AO standard curve ($y = -1460.9x + 13197$, Fig. S1). A minus B is the amount of CB[7].

Preparation of OX/FA/CB[7]-Au NRs

CB[7]-Au NRs were surface-functionalized by simple mixing the NRs with functional tags, such as FA-ADA, FITC-ADA and OX, followed by subsequent centrifugation to remove excess ligands. The drug loading content was measured according to Pt content of OX by ICP-MS analysis. FITC/CB[7]-Au/FA NRs was prepared with the same method for studying cellular uptake.

Photothermal evaluation of Au NRs and CB[7]-Au NRs

For measuring the photothermal performance of CB[7]-Au NRs, 1 mL aqueous dispersion of NRs at Au concentrations of 200 μg/mL was placed in a quartz cuvette and irradiated with an NIR laser (808 nm, 2 W/cm^2 , 300 s). A thermocouple probe with an accuracy of 1 ºC was inserted into the solution to monitor the real-time temperature. The temperature was recorded every 10 s.

The photothermal performance of Au NRs was determined under the same conditions as those for CB[7]-Au NRs.

Release profile of OX *in vitro*

The OX release profile of OX/FA/CB[7]-Au NRs was assessed *in vitro* by using a dialysis method. Phosphate-buffered saline (PBS) solution containing different concentrations of spermine (at room temperature and 70 ºC, respectively) was used as the release medium to study the OX release kinetics. 100 μL dialysate was aliquoted out and replenished at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 5, 10, 22, 30, 36, 40, 48, 52, 60, 70, 80, 100, 120 h. The released OX was quantified by ICP-MS.

Cell Culture and *in vitro* Cytotoxicity Assays

The 4T1 cell line (mice breast cancer cell line) and L02 cell line (human fetal hepatocyte line) were cultured in 25 cm^2 flasks using Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin & streptomycin (PS) at 37 °C with 5% CO_2.

The biocompatibility of NRs was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays using 4T1 and L02 cell lines (1×10^4 cells per well). The cellular viability was calculated following a previously published procedure[6].

Cellular uptake

The 4T1 and L02 cells were incubated with free FITC, FITC/CB[7]-Au NRs and FITC/FA/CB[7]-Au NRs at equal concentrations of FITC for 12 h, respectively. The internalized fluorescence images were acquired on a confocal laser scanning microscope (CLSM) on the cells after washing with PBS. The cellular fluorescence signal was obtained by flow cytometry assay.

Cellular Apoptosis Evaluation

The apoptosis rate of 4T1 and L02 cells after incubation with Au NRs, CB[7]-Au NRs and OX/FA/CB[7]-Au were evaluated by flow cytometry assay. Briefly, the cells after incubation were processed by an Annexin/V-FITC Kit. The fluorescence signal of annexin V-FITC and PI (positive and negative) obtained by flow cytometer reflected the apoptosis rate.

Cytotoxicity and photothermal cytotoxicity

The cytotoxicity of Au NRs and CB[7]-Au NRs were studied via MTT assays. Briefly, the 4T1 and L02 cell line were seeded in 96-well plates with 10^4 cells/well density in 100 μL of DMEM (with 10% FBS and 1% PS), respectively, and were subsequently incubated with various formulations (at Au concentration of 50, 100, 150, 200 and 250 μg/mL and Pt concentration of 0.0156, 0.0312, 0.0469, 0.0625, 0.0781 μM, respectively).

For qualitative evaluation of the photothermal cytotoxicity, Au NRs, CB[7]-Au NRs and OX/FA/CB[7]-Au NRs were used to incubate with 4T1 and L02 cells, respectively for 4 h, followed by irradiation by NIR laser (808 nm, 2W/cm^2, 5 min) before further incubation for 12 h. The cells

were stained with both calcein AM (calcein acetoxymethyl ester) and PI (propidium iodide).

To quantitatively evaluate the photothermal cytotoxicity study, the 4T1 and L02 cell line were treated with OX/FA/CB[7]-Au NRs for 4 h. After the medium was replaced by the fresh medium, the cells were exposed to an 808 nm laser (2 W/cm^2, 5 min). The cells were further incubated for 24 h and 48 h and washed with PBS before adding MTT solution.

***In vivo* Antitumor Study**

In vivo antitumor effect of OX/FA/CB[7]-Au NRs were studied on 4T1 tumor-bearing balb/c mice. 18 Balb/c mice were subcutaneously injected with 10^6 4T1 cells (10^7 cells per mL PBS solution). When the average volume of the tumor reached about 50 mm^3, the mice were randomly grouped into 6 groups (n = 3) and were administered with 200 μmL of (a) PBS control, (b) PBS with laser irradiation, (c) free OX, (d) Au NRs with laser irradiation, (e) FA/CB[7]-Au NRs with laser irradiation, and (f) OX/FA/CB[7]-Au NRs with laser irradiation (400 μg/mL of Au concentration, equal OX concentration in free OX group and OX/FA/CB[7]-Au NRs group, the exact dose information is given in the main text). The PBS and all NRs were injected intravenously into the tumor-bearing mice on day 0. After 9 h of the injection, the tumor regions relevant groups were irradiated for 5 min with an 808 nm laser with an output power density of 2.0 W/cm^2. Tumor size and body weight of mice were measured and recorded every 2 days during the two-week follow up.

On the 14th day after administration, the mice were sacrificed and their major tissues, including the heart, livers, spleens, lungs, and kidneys, and tumors were excised for histological assays.

The organs and tumors were fixed in 4% paraformaldehyde and the tissue section stained with hematoxylin−eosin (H&E) was observed under an IX73 microscope (OLYMPUS, Japan). All animals used in the procedures were handled in strict accordance with the Guide for the Care and Use of Laboratory Animals, and the procedures were approved by the Animal Ethics Committee, University of Macau.

2. SUPPLEMENTARY RESULTS

Calculation of surface CB[7] on CB[7]-Au NRs

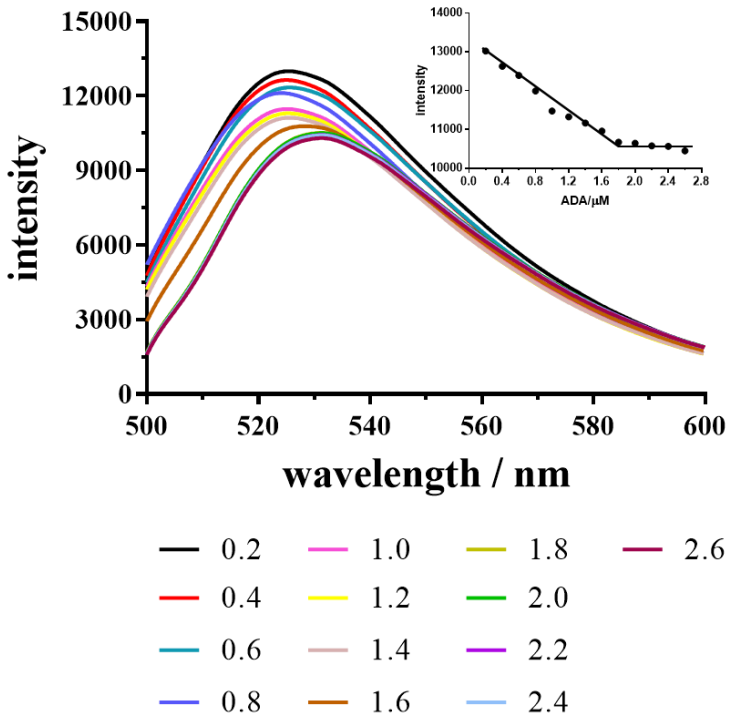

Fig. S1. Fluorescence intensity (λ_{ex} = 450 nm) and standard curve of CB[7]·AO with different amount of ADA (2 mM AO and 1.6 mM CB[7]).

Characterizations of CB[7]-Au NRs

As shown in Fig. S2, the absorption peak at 3500 cm^{-1} corresponding to the N-H stretching vibration of CB[7]-NH_2 disappeared in CB[7]-Au spectra, and the enhanced C-H methylene stretching vibration observed at 2882-2843 cm^{-1} in CB[7]-Au spectra corresponding to the CH_2 of mercaptoundecanoic acid coating on Au NRs, which implied the connection of Au NRs and CB[7] . Moreover, the enhanced C=O stretching at 1750 cm^{-1} indicated the presence of CB[7].

The obtained Au NRs had high-concentration surfactant of CTAB on the surface, showing a high positive charge. The mercaptoundecanoic acid was used to introduce carboxyl group on Au NRs by forming Au-S bond according to a well-established ligand exchange reaction, leading to negative zeta potential of Au NRs. $AO_1CB[7]$ was successfully prepared and further reacted with cysteamine through thiol-ene click reaction under 254 nm UV irradiation to generate amine-CB[7] (CB[7]-NH_2). The amine groups of CB[7]-NH_2 can react with carboxyl group on Au NRs to anchor CB[7] on the surface of Au NRs via a stable amide bond.

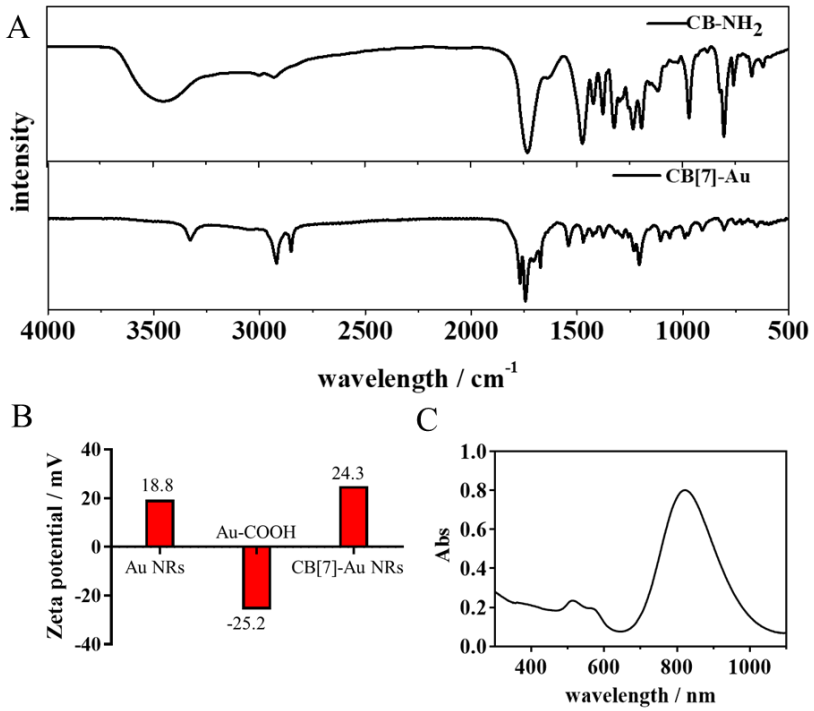

Fig. S2. (A) FT-IR spectra of CB[7]-NH_2 and CB[7]-Au NRs. (B) Zeta potentials of Au NRs, Au-COOH NRs and CB[7]-Au NRs. (C) UV-Vis absorbance spectroscopy of Au NRs.

OCT imaging

The OCT imaging potential was evaluated, referenced with fatty emulsion: water solution at the rate of 10%, 5%, 2.5% and 1.25% at 1300 nm. As shown in Fig. S3, Au NRs and CB[7]-Au NRs showed strong OCT signal between 5% and 10% F/W solution, indicating the promising OCT imaging potential *in vivo*. In fact, Au NRs tend to adhere on the internal wall of capillary tube during imaging, showing a non-uniform imaging effect with stronger signal area around and a weaker signal in the center. However, CB[7] modified Au NRs showed a homogeneous imaging signal likely because the existence of CB[7] improved the stability of Au NRs.

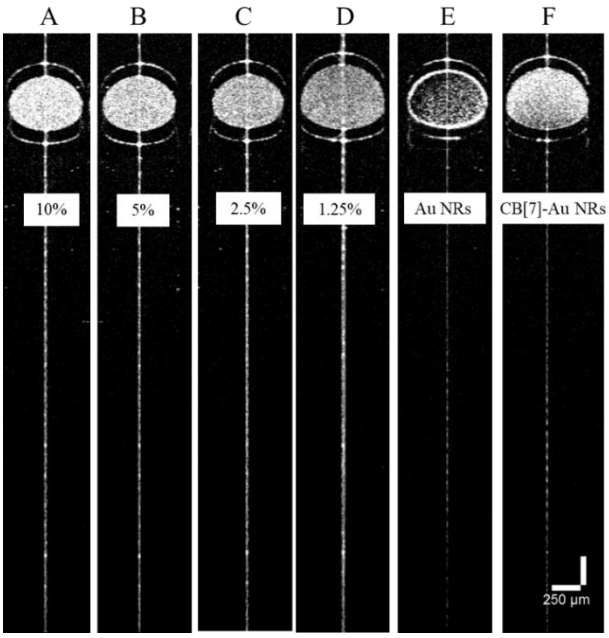

Fig. S3. The OCT imaging of Au NRs (E) and (F) CB[7]-Au NRs (Au concentration 200 μ g/mL). F/W solution (fatty emulsion: water solution) at the rate of 10% (A), 5% (B), 2.5% (C) and 1.25% (D) was used as the standard. The images were acquired at the wavelength of 1300 nm.

CT and PA imaging

To investigate the CT imaging and PA imaging potential of CB[7]-Au NRs, NRs were imaged at Au concentration of 200, 400, 600, 800 and 1000 μg/mL. As shown in Fig. S4 and Fig. S5, the Au NRs exhibited strong, increased CT and PA signal with increasing concentration of NRs. The signal of CB[7]-Au NRs is not as significant as Au NRs, which may be attributed to surface modification of NRs with CB[7]. However, the CT HU value and PA signal intensity of CB[7]-Au NRs at 1 mg/mL reached 2080 and 4.28×10^5, showing promising CT and PA imaging capability *in vitro* and *in vivo*.

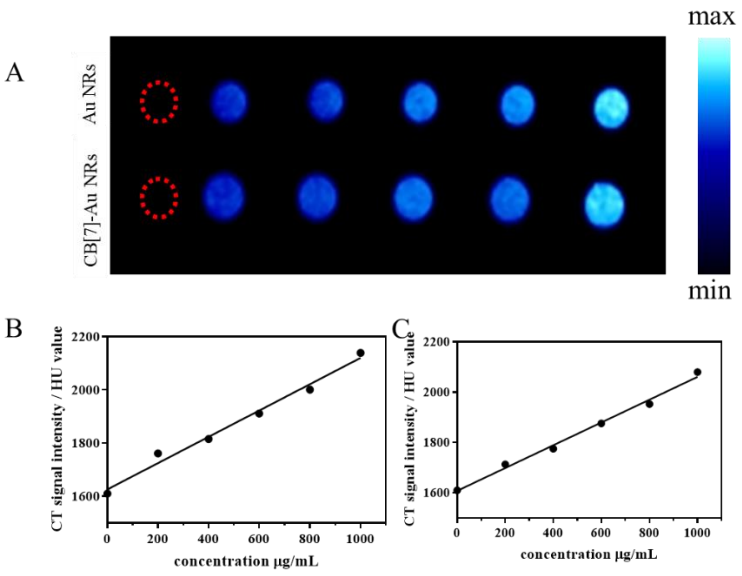

Fig. S4. CT image of Au NRs and CB[7]-Au NRs.

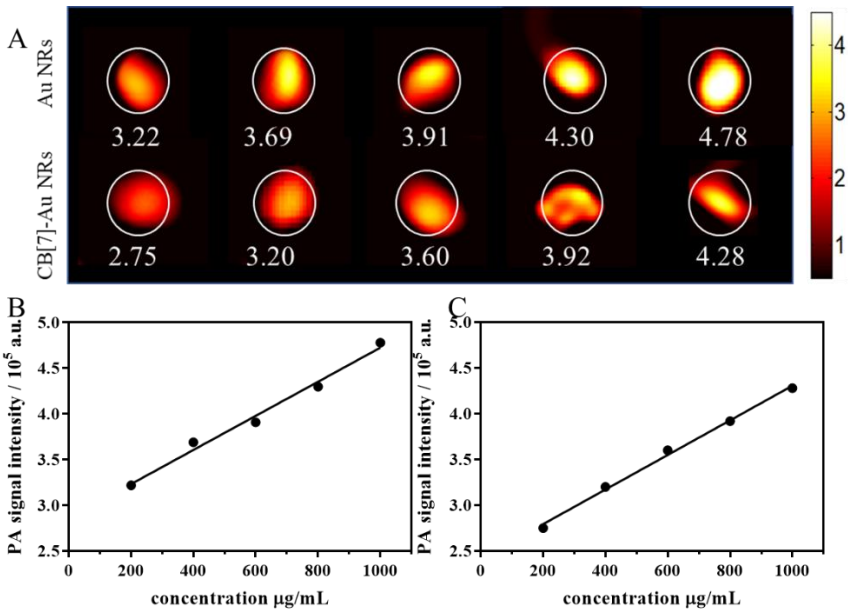

Fig. S5. PA images of Au NRs and CB[7]-Au NRs.

Cellular uptake measured by flow cytometry.

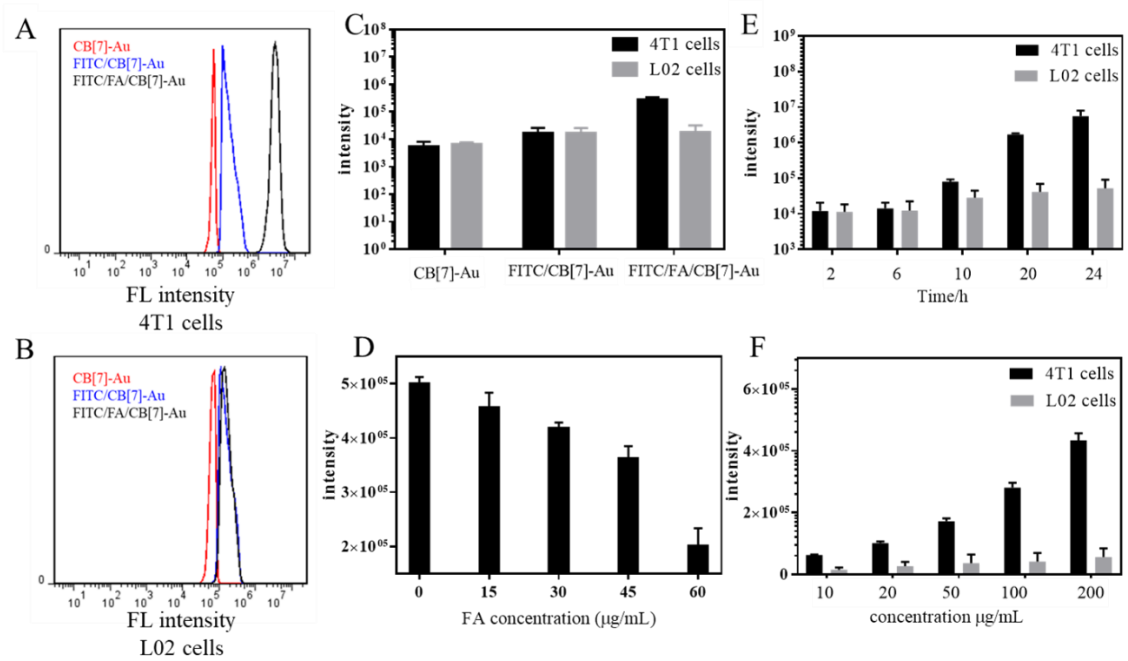

Fig. S6. Cellular uptake measured by flow cytometry. (A) (B) (C): 4T1 cells and L02 cells were cultured with 200 μg/mL CB[7]-Au NPs, FITC/CB[7]-Au, FITC/FA /CB[7]-Au NPs, respectively for 20 h. (D) 4T1 cells were cultured with 200 μg/mL FITC/CB[7]-Au/FA NPs for 10 h, with co-treatment with different concentrations of FA. (E) 4T1 cells and L02 cells were cultured with 200 μg/mL FITC/CB[7]-Au/FA NPs for different time lengths. (F) 4T1 cells and L02 cells were cultured with FITC/CB[7]-Au/FA NPs for 10 h at different concentrations.

Qualitative evaluation of cell viabilities

The cell viability of cells was evaluated qualitatively (Fig. S7) first by using live/dead co-staining assays. Calcein-AM, which may mark living cells with green fluorescence, and PI, which may mark dead cells with red fluorescence, were used to distinguish the live and dead cells upon different treatments. 4T1 and L02 cells were treated with Au NRs, CB[7]-Au NRs and OX/FA/CB[7]-Au NRs respectively for 4 h, followed by irradiation by an 808 NIR laser at the power density of 2 W/cm^2 for 5 min. The cells were further cultured for another 12 h before co-staining with calcein-AM and PI. As shown in Fig. S7, free OX showed weak cytotoxicity, whereas Au NRs, CB[7]-Au NRs and OX/FA/CB[7]-Au (without NIR laser) exhibited similar cell viability both in 4T1 and L02 cells, suggesting good biocompatibility and safety of these materials. Intracellular NRs exhibited photothermal potential and led to cell death under NIR laser irradiation. As expected, the OX/FA/CB[7]-Au NRs, under irradiation, exhibited much higher cytotoxicity y than other groups.

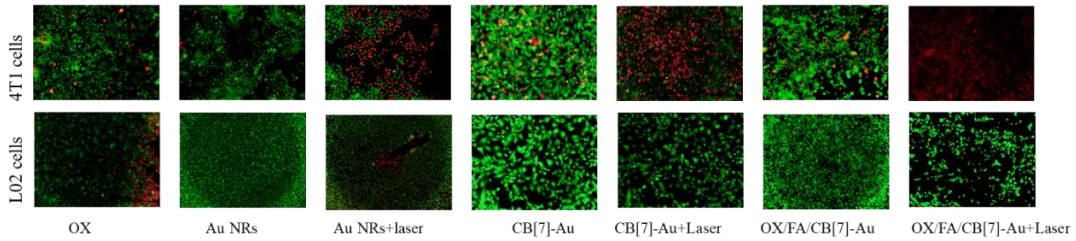

Fig. S7. 4T1 and L02 cells incubated with NPs for 4 h, followed by NIR irradiation (808 nm, 2W/cm^2) for 5 min before further incubation for 12 h. The cells were stained with calcein AM (green, live cells) and PI (red, dead cells) before imaging.

The apoptosis rate of the 4T1 cell line for 24 h

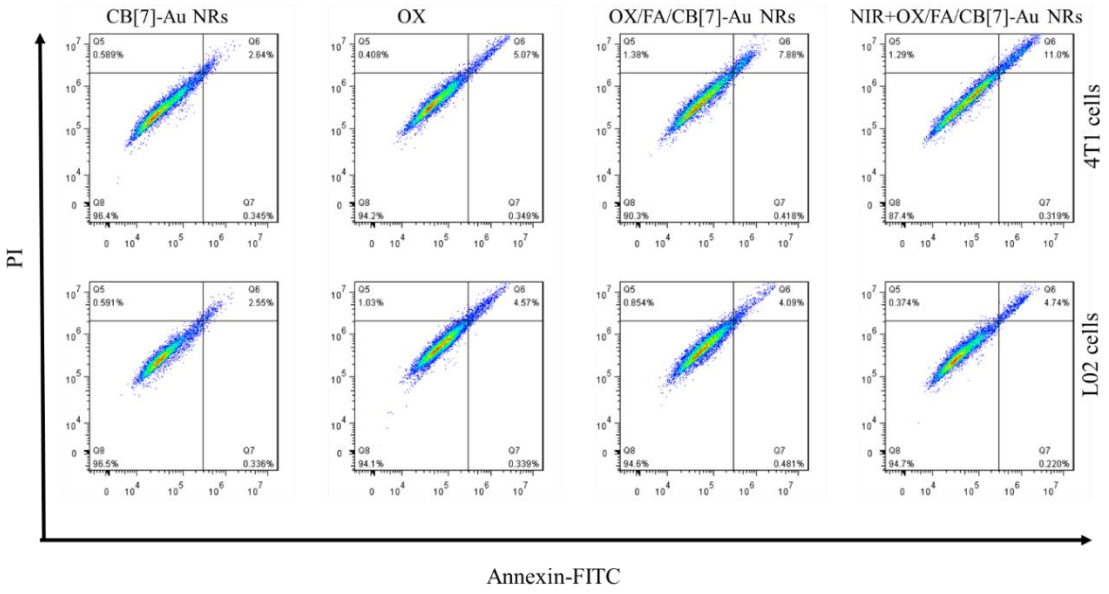

Fig. S8. Flow cytometry analysis of the 4T1 cell line for 24 h. 4T1 cells were incubated with CB[7]-Au NRs, free OX, OX/FA/CB[7]-Au NRs and NIR+OX/FA/CB[7]-Au NRs, respectively for 4 h, followed by NIR irradiation (808 nm, 2W/cm^2) for 5 min before further incubated for 24 h.

***In vivo* imaging of CB[7]-Au NRs in balb/c mice**

FA/CB[7]-Au NRs was *i.v.* injected into balb/c mice to investigate biodistribution *in vivo* under CT. As shown in Fig.S9, enhanced CT signal was observed in the liver region since 4 h after injection (highlighted with white arrows). The CT signal reached to the peak value at 8 to 12 h and dramatically decreased at 24 h. The results indicate the good biocompatibility and fast clearance of Au NRs *in vivo*.

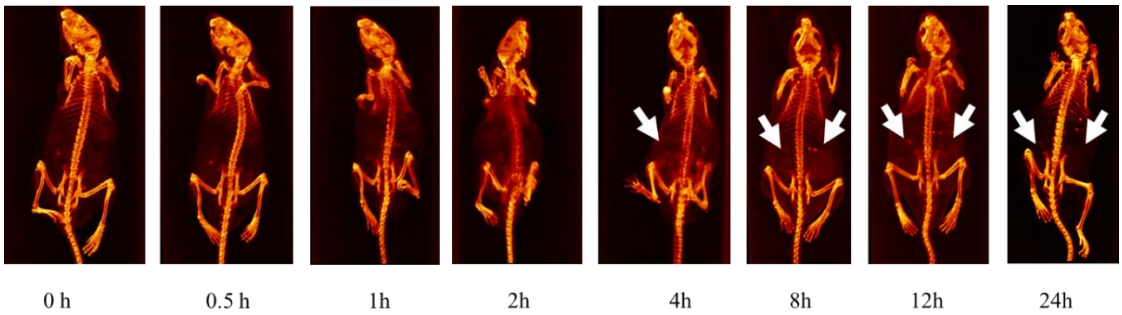

Fig. S9. CT imaging of balb/c mice at different time points after *i.v.* injection of FA/CB[7]-Au NRs (Au concentration at 1mg/ml, 100 uL).

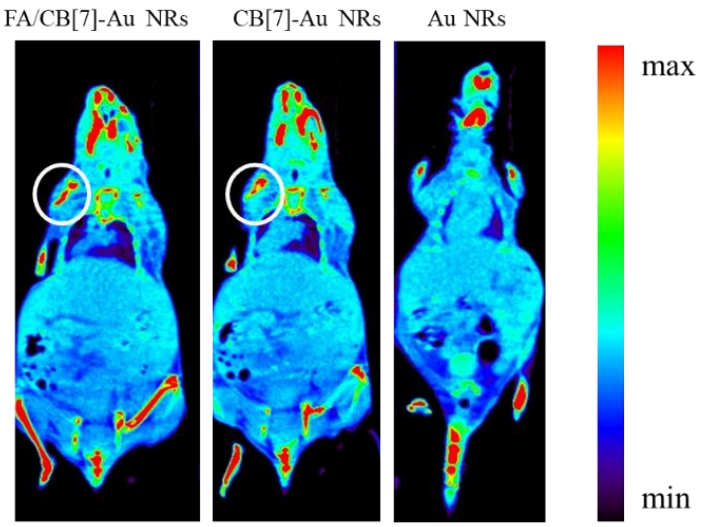

Fig. S10. FA/CB[7]-Au NRs, CB[7]-Au NRs and Au NRs were respectively *i.v.* injected in 4T1 tumor bearing balb/c mice. The CT signal intensity in tumor at 12 h after NPs injection reached 31.83%, 20.21% and 13.73% of tumor accumulation, respectively.

Main organs' histology analysis

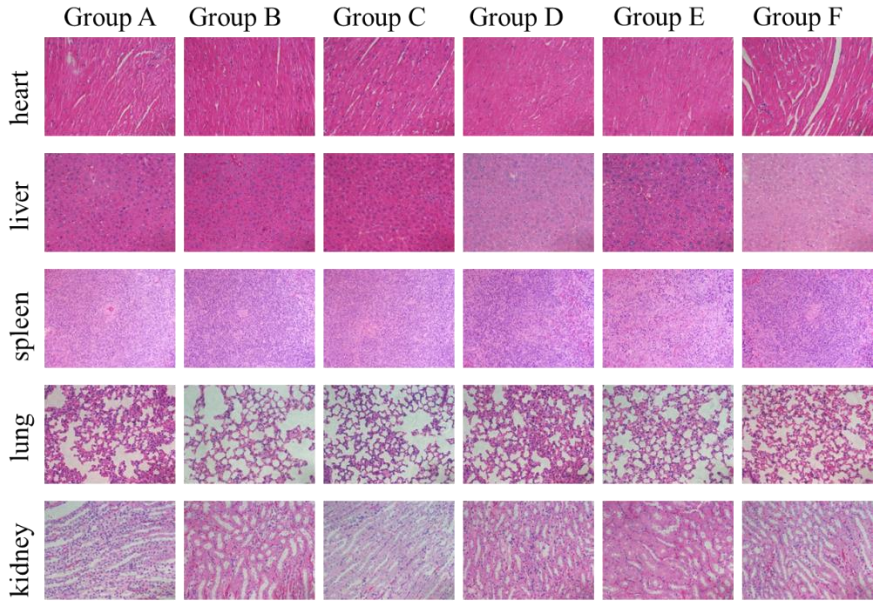

Fig. S11. H&E stained images of tissue slices of major organs and the tumor from different groups: (A) PBS control, (B) PBS control with laser irradiation, (C) free OX, (D) Au NRs with NIR laser, (E) FA/CB[7]-Au NRs with NIR laser and (F) OX/FA/CB[7]-Au NRs with NIR laser, after treatment for 14 days.

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