Electronic Supplementary Information (ESI)

Charge-conversional polyethylenimine-entrapped gold nanoparticles with ^{131}I-labeling for enhanced dual mode SPECT/CT imaging and radiotherapy of tumors

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Experimental section

Materials

The functional compounds *m*PEG-NHS (Mw = 2 000) and MAL-PEG-SVA (Mw = 2 000) were obtained from Shanghai Yanyi Biotechnology Corporation (Shanghai, China). HPAO was purchased from Sigma-Aldrich (St. Louis, MO). Branched PEI (Mw = 25 000), FI, chloroauric acid ($HAuCl_4$), sodium borohydride ($NaBH_4$), acetic anhydride, triethylamine, and all the other chemicals were provided from Aldrich (St. Louis, MO). $Na^{131}I$ solution was supplied from Shanghai GMS Pharmaceutical Co., Ltd (Shanghai, China). Disposable PD-10 desalting columns were purchased from GE Pharmacia (GE Inc., Fairfield, CT). C6 cells (a rat glioma cell line) were provided from Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and Cell Counting Kit-8 (CCK-8) were provided from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Regenerated cellulose dialysis membranes (MWCO = 14 000) was supplied from Fisher (Pittsburgh, PA).

Synthesis of ^{131}I-APAS-Au PNPs

The functional molecule APAS was synthesized using the same procedure with the previous work.[1-3] Later, refer to our previous literatures, the multifunctional Au PNPs were prepared.[1, 4] In detail, PEI was first reacted with 15 molar equivalents of *m*PEG-NHS under stirring to acquire PEI.NH_2-(*m*PEG). Followed by reacting with 15 molar equivalents of MAL-PEG-SVA through SVA moiety to acquire PEI.NH_2-(PEG-MAL)-(*m*PEG). The prepared 12 molar equivalents of APAS dissolved in water was then added into PEI.NH_2-(PEG-MAL)-(*m*PEG) solution. Through conjugation of sulfydryl in APAS and MAL group in PEI.NH_2-(PEG-MAL)-(*m*PEG), the PEI.NH_2-(PEG-APAS)-(*m*PEG) was generated. 10 molar equivalents of HPAO and 5 molar equivalents of FI were also reacted with functional PEI in sequence to form PEI.NH_2-FI-HPAO-(PEG-APAS)-(*m*PEG). Later, the formed

PEI.NH_2-FI-HPAO-(PEG-APAS)-(*m*PEG) platform was used to entrap Au NPs and acetylate the leftover amine groups of PEI follow the protocols published in our previous work.[5-7] In brief, $HAuCl_4$ solution (200 molar equivalents) was mixed with the PEI.NH_2-FI-HPAO-(PEG-APAS)-(*m*PEG) solution and stirred for half an hour, then added $NaBH_4$ with 5 times molar excess to $HAuCl_4$ under continuous stirring for 2 h to acquire {$(Au^0)_{200}$-PEI.NH_2-FI-HPAO-(PEG-APAS)-(*m*PEG)} PNPs. The leftover amine groups of PEI were then neutralized through acetylation process. Finally, *via* dialysis and lyophilization procedure to acquire the purified {$(Au^0)_{200}$-PEI.NHAc-FI-HPAO-(PEG-APAS)-(*m*PEG)} PNPs (for short, APAS-Au PNPs) according to the previous literatures.[8-10] Meanwhile, the control nanosystem {$(Au^0)_{200}$-PEI.NHAc-FI-HPAO-(PEG-MAL)-(*m*PEG)} PNPs (for short, Au PNPs) without APAS modification were prepared under the consistent conditions.

According to the phenol group of HPAO on the APAS-Au PNPs which can be easily labeled ^{131}I on the ortho-position of phenol, ^{131}I labeled APAS-Au PNPs was synthesized referring to the method illustrated in our previous work.[4, 11, 12] First, chloramine-T (200 μg) was added into the $Na^{131}I$ solution (10 mCi, 100 μL). The 200 μL PBS (0.1 M, pH 7.4) solution of APAS-Au PNPs (200 μg) was then added into the mixture of chloramine-T and $Na^{131}I$ immediately under continuous stirring. Through 30 min incubation at 37 °C, the obtained ^{131}I-APAS-Au PNPs mixture was purified by PD-10 desalting columns. As control, the ^{131}I-Au PNPs without APAS modification were synthesized according to the same labeling strategy.

Characterization techniques

^1H NMR spectra were recorded using Bruker AV-400 NMR spectrometer. All the samples were dissolved in D_2O for ^1H NMR measurement. UV-vis spectra were acquired on a Lambda 25 ultraviolet-visible spectrophotometer (Perkin-Elmer, Waltham, MA). Dynamic light scattering (DLS)

and zeta potential were performed by a Malvern Zetasizer Nano ZS90 system (Worcestershire, UK). Transmission electron microscopy (TEM) was performed using a JEOL2010F analytical electron microscope (JEOL, Tokyo, Japan) operating at 200 kV. An aqueous solution of APAS-Au PNPs (1 mg/mL) was dropped onto a carbon-coated copper grid and air dried before test. The size distribution histogram of the APAS-Au PNPs was analyzed by ImageJ software (http://rsb.info.nih.gov/ij/download.html). More than 400 NPs from TEM images were randomly selected for the size analysis. The X-ray attenuation efficiency of APAS-Au PNPs is measured by a clinical LightSpeed VCT imaging system (GE Medical Systems, Milwaukee, WI) through comparing with conventional iodine-based contrast agent Omnipaque. Different Au or iodine concentrations (0.01-0.1 M) of APAS-Au PNPs or Omnipaque were prepared and scanned under CT scan equipment with the following parameters: 100 kV, 80 mA, and 0.625 mm slice thickness. The CT images and corresponding X-ray attenuation intensities were acquired by loading the digital CT images in GE AW 4.4 workstation.

Charge conversion measurements

It's essential to investigate the zeta potential of APAS-Au PNPs under various pH conditions for analysis of their charge conversion property. First, the generated APAS-Au PNPs or Au PNPs were dissolved into the phosphate buffer with different pHs (pH 5.0, 5.5, 6.0, 6.5, 7.0, and 7.5) respectively, and the final concentrations of APAS-Au PNPs and Au PNPs were both set as 1 μM. Then the zeta potentials of APAS-Au PNPs and Au PNPs under different pH conditions were recorded by a Malvern Zetasizer Nano ZS90 system (Worcestershire, UK).

Cell culture

C6 cells were utilized as model cell which cultured by DMEM containing 10% FBS, penicillin (100

U/mL), and streptomycin (100 U/mL) under a humidified atmosphere full of 5% CO_2 before cells' experiments.

Cytotoxicity assay

It's crucial to study the cytotoxicity of the formed APAS-Au PNPs and Au PNPs before and after ^{131}I labeling under pH 6.0 or pH 7.4 conditions *via* simulate the microenvironment of normal tissue or tumor tissue by CCK-8 assay. In detail, C6 cells (1.2×10^4 cells/well) were first seeded into a cell culture plate (96-well). After 12 h incubation, the medium in each well was exchanged with fresh medium containing APAS-Au PNPs, Au PNPs, ^{131}I-APAS-Au PNPs, or ^{131}I-Au PNPs at different concentrations with different pHs (pH 6.0 and 7.4, respectively) for 48 h, respectively. The medium with different pHs were adjusted by phosphate buffer respectively. After 48 h incubation, CCK-8 solution (20 μL/well, 5 mg/mL) was added and cultured cells for 4 h. Finally, the absorbance at 450 nm was measured by a Thermo Scientific Multiskan MK3 ELISA reader (Thermo Scientific, Waltham, MA).

***In vitro* cellular uptake assay**

C6 cells (8×10^5 cells/well) were seeded into a 12-well plate and cultured by DMEM for 24 h. Later, the medium in each well was replaced with medium containing APAS-Au PNPs or Au PNPs at the final concentration of 1 μM with different pHs (pH 7.5, 7.0, 6.5, 6.0, 5.5, and 5.0, respectively). The medium with different pHs was adjusted by phosphate buffer. Through 3 h treatment, the cells were washed with PBS and lifted with trypsinization. Then the cells with each treatment was collected through centrifugation and lysed by an aqua regia solution. Finally, the digested cell samples were diluted with PBS before testing by Leeman Prodigy ICP-AES (Hudson, NH).

Flow cytometry analysis

Flow cytometry was also utilized as a vital method to evaluate the cellular internalization of APAS-Au PNPs *in vitro* according to our previous work.[4, 5, 7] Briefly, C6 cells (2×10^5 cells/well) were first seeded into 12-well plate before experiment. After 24 h incubation, the cells were cultured with phosphate buffer adjusted DMEM containing PBS, Au PNPs (1 μM), and APAS-Au PNPs (1 μM) with different pHs (pH 6.0 and 7.4) for 3 h. Later, the cell samples with different treatments were washed by PBS, trypsinized and resuspended in PBS for analysis. The mean fluorescence intensity of each cell simple was acquired by a FACScan analyzer (Becton Dickinson, Mountain View, CA) through collecting 10,000 cells per sample.

Confocal microscopy

The cellular internalization property of APAS-Au PNPs towards C6 cells can be further checked by confocal microscopy according to the fluorescence of FI on the APAS-Au PNPs. C6 cells (8×10^4 cells/well) were first seeded in a 12-well plate and cultured for 24 h to allow them adherence. Then the cells were incubated with phosphate buffer adjusted DMEM containing PBS, Au PNPs (1 μM), and APAS-Au PNPs (1 μM) with different pHs (pH 6.0 and 7.4) for 3 h. Later, the cells with treatment were washed with PBS, fixed with glutaraldehyde, and counterstained with Hoechst 33342 referring to the standard protocols published in previous literatures.[1, 5, 7] Finally, the cells were scanned by confocal microscopy with 40 × objective lens (Carl Zeiss LSM 510, Thornwood, NY).

***In vitro* stability study**

Before ^{131}I labeling, the colloidal stability of APAS-Au PNPs under various temperature conditions (4 ^{o}C, 25 ^{o}C, 37 ^{o}C, and 50 ^{o}C, respectively) and time points (day 1, day 3, day 5, and day 7, respectively) can be investigated using the UV-vis spectroscopy referring to the previous method published in the literatures.[1, 6, 7] After ^{131}I labeling, the radiostability of the generated ^{131}I-APAS-Au

PNPs was measured by instant thin-layer chromatography (ITLC). Briefly, ^{131}I-APAS-Au PNPs (100 μL, 1 mCi) was first mixed with PBS (900 μL). After incubation for various time points (1 h, 4 h, 8 h, and 16 h, respectively), the radiochemical purity of ^{131}I-APAS-Au PNPs was assessed by ITLC. The method is also consistent with the previous protocols published in the literatures.[4, 11]

SPECT and CT imaging *in vitro*

Based on the APAS modification, it's essential to investigate the theranostic nanosystem-mediate SPECT and CT imaging of cancer cells *in vitro* through simulating the slightly acidic microenvironment of tumor. C6 cells (2×10^6 cells/well) were first seeded into a 6-well plate and cultured overnight to bring the cells to confluence. Then the cells were treated with phosphate buffer adjusted DMEM containing PBS, ^{131}I-Au PNPs, or ^{131}I-APAS-Au PNPs with various radioactivity concentrations (50, 100, 200, and 400 μCi/mL, respectively) for 3 h at pH 6.0. Later, each cells sample was rinsed with PBS, trypsinized, centrifuged, and resuspended in PBS (200 μL) in a 2 mL Eppendorf tube. Infinia SPECT scanner with an Xeleris Work Station and Low Energy General Purpose Collimators (GE Inc., Fairfield, CT) were utilized to perform the SPECT imaging. As for the CT imaging, similar cells seeding procedure was carried out. After treatment with Au PNPs or APAS-Au PNPs with various Au concentrations (50, 100, 150, and 200 μM, respectively) for 3 h at pH 6.0. The cells were collected and scanned by a clinical LightSpeed VCT imaging system (GE Medical Systems, Milwaukee, WI).

SPECT and CT imaging *in vivo*

Animal experiments were carried out following the typical protocols permitted by the committee for animal care in Shanghai General Hospital. Male BALB/c nude mice (20-22 g) were obtained from Shanghai Slac Laboratory Animal Center (Shanghai, China). Later, the xenograft model was built

referring to the previous literatures.[4, 12] When the tumor grew up to 0.5-1.0 cm^3, the imaging experiments *in vivo* can be carried out.

The obtained tumor bearing mice were fed with KI (1%) contained water to saturate the thyroid for three days before SPECT imaging. The mice were first divided into experimental group (5 mice/group) and control group (5 mice/group). Then a 100 μL PBS solution of ^{131}I-APAS-Au PNPs with 400 μCi radioactive dose was injected intravenously into the mice of experimental group. At the same time, ^{131}I-Au PNPs with the same radioactive dose was injected into the mice of control group. The SPECT images of tumor bearing mice were recorded at various time points post-injection. At 6 h post-injection, one mouse from each group was sacrificed. The major organs and tumors from the sacrificed mice were respectively collected for recording the relative SPECT signal intensities.

As for CT imaging, mice in experimental group and control group were respectively injected with APAS-Au PNPs ([Au] = 0.1 M, 100 μL) and Au PNPs ([Au] = 0.1 M, 100 μL) through tail vein. The scan was then performed before injection and different time points post-injection *via* a clinical LightSpeed VCT imaging system (GE Medical Systems, Milwaukee, WI) equipped with GE AW 4.4 workstation.

Radiotherapy efficacy *in vivo*

After establishing the tumor model on each mouse, the radiotherapeutic efficiency was then studied. First, the mice were divided into 5 groups (5 mice/group) and respectively injected with 100 μL of saline, Au PNPs ([Au] = 0.1 M), APAS-Au PNPs ([Au] = 0.1 M), ^{131}I-Au PNPs (400 μCi, [Au] = 0.1 M) or ^{131}I-APAS-Au PNPs (400 μCi, [Au] = 0.1 M). The injections were performed every third day. The tumor inhibition efficiency was then assessed *via* analysis of relative tumor volume, survival rate, and body weight in the 21 days' treatment based on the protocols published in the previous literatures.[9,

After treatment, one mouse in each group was randomly selected to be euthanized. The major organs and tumor in each group were obtained, fixed, sectioned, hematoxylin and eosin (H&E) stained and observed according to the protocols published in the previous literatures.[7, 11] Moreover, a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was further utilized to evaluate the apoptosis rate of tumor referring to the standard protocols published in the previous literatures.[4, 12] Briefly, the *in situ* apoptotic detection kit (Roche, Basel, Switzerland) was utilized according to the manufacturer's guidelines. The collected tumor samples were first fixed in paraformaldehyde, dehydrated, paraffin-embedded, and sectioned. The section was then deparaffinized, rehydrated, washed, stained using a TUNEL Kit. Both the H&E stained and TUNEL stained samples were observed with a Leica DM IL LED inverted phase contrast microscope. The number and percentage of TUNEL-positive cells were counted and determined from three random selected fields per mouse.

Statistical analysis

The significance of the acquired data was analyzed by one-way ANOVA statistical method. A value of 0.05 was considered as the significance level and the data were marked with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

Table S1. Radiochemical purity of the ^{131}I-APAS-Au PNPs at different time points.

Time (h)	1	4	8	16
Radiochemical purity (%)	99.4 ± 0.26	97.6 ± 0.61	96.3 ± 0.92	95.1 ± 0.79

Table S2. The hydrodynamic sizes of Au PNPs and APAS-Au PNPs dispersed in PBS buffer with different pH conditions.

pH condition	Materials	Hydrodynamic size (nm)	PDI
pH 7.4	Au PNPs	195.32 ± 12.32	0.36 ± 0.11
	APAS-Au PNPs	205.21 ± 14.71	0.42 ± 0.08
pH 6.0	Au PNPs	203.25 ± 10.64	0.51 ± 0.06
	APAS-Au PNPs	239.28 ± 15.76	0.39 ± 0.13

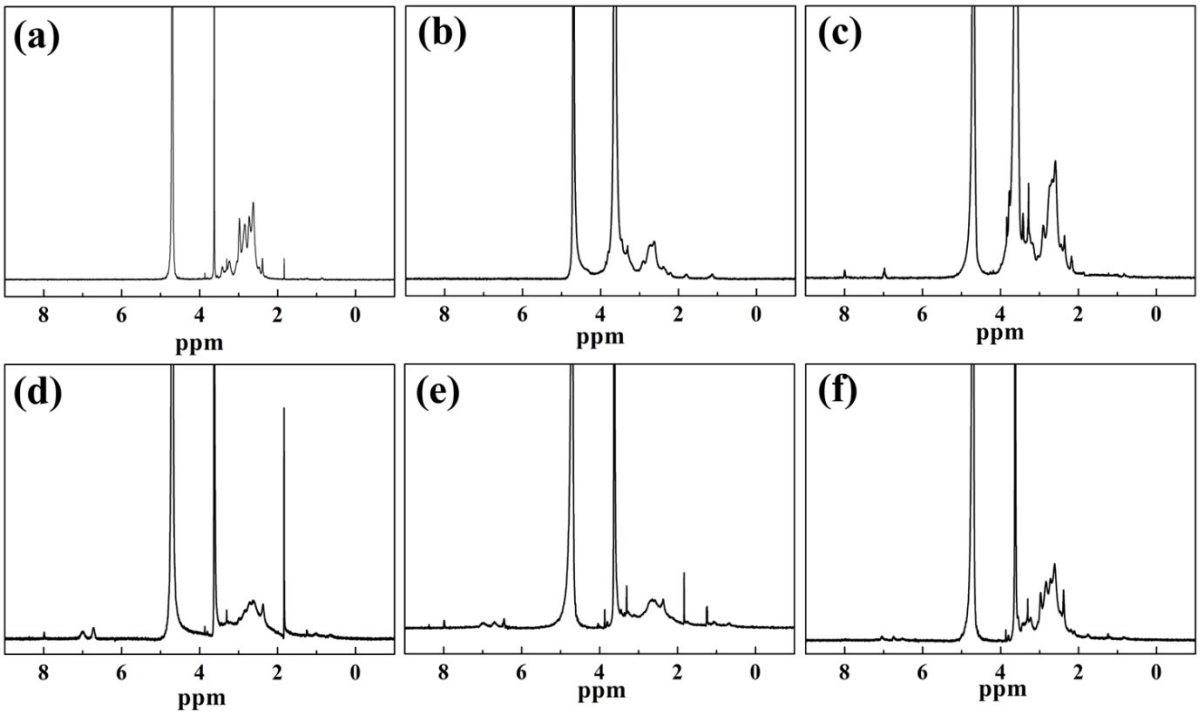

Fig. S1 ^{1}H NMR spectra of PEI.NH_2-(*m*PEG) (a), PEI.NH_2-(PEG-MAL)-(*m*PEG) (b), PEI.NH_2-(PEG-APAS)-(*m*PEG) (c), PEI.NH_2-HPAO-(PEG-APAS)-(*m*PEG) (d), PEI.NH_2-FI-HPAO-(PEG-APAS)-(*m*PEG) (e), and PEI.NH_2-FI-HPAO-(PEG-MAL)-(*m*PEG) (f). All the intermediate products above were dissolved in D_2O.

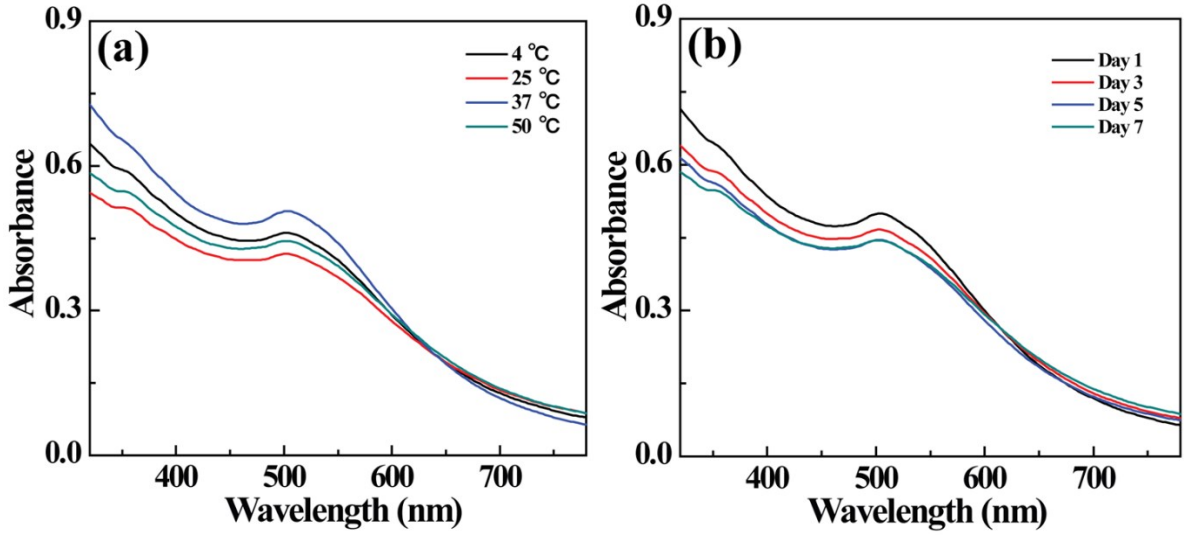

Fig. S2 UV-Vis spectra of APAS-Au PNPs dissolved in water at different temperatures (a) and different time points (b).

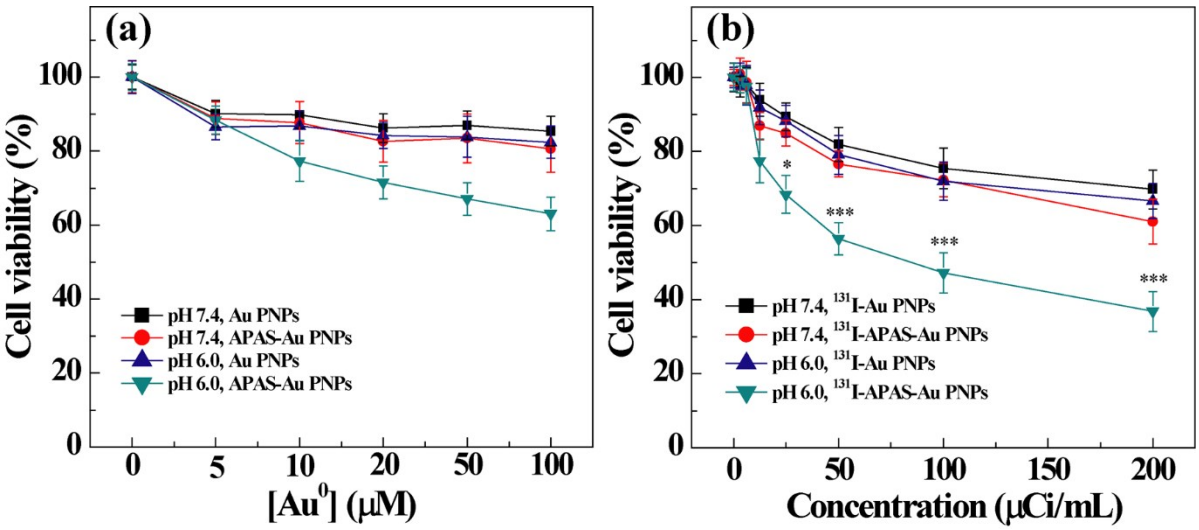

Fig. S3 (a) CCK-8 assay of C6 cells treated with APAS-Au PNPs or Au PNPs at different Au concentrations under pH 7.4 or pH 6.0 condition for 48 h, respectively. (b) CCK-8 assay of C6 cells treated with ^{131}I-APAS-Au PNPs or ^{131}I-Au PNPs at different ^{131}I radioactive dose under pH 7.4 or pH 6.0 condition for 48 h, respectively.

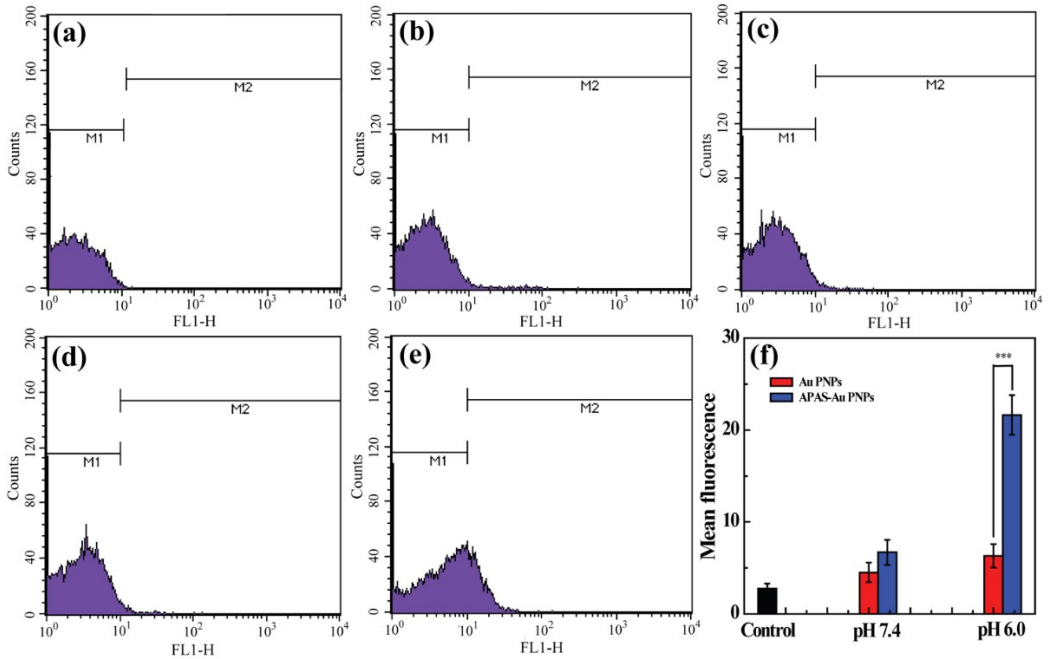

Fig. S4 Flow cytometric analysis of C6 cells treated with PBS (a), Au PNPs (b), APAS-Au PNPs (c) under pH 7.4 condition for 3 h. And C6 cells treated with Au PNPs (d) and APAS-Au PNPs (e) under pH 6.0 condition for 3 h, respectively. The concentrations of both Au PNPs and APAS-Au PNPs were set as 1 μM. (f) shows the comparison of the fluorescence intensity of different Au PNPs with C6 cells under different pH conditions.

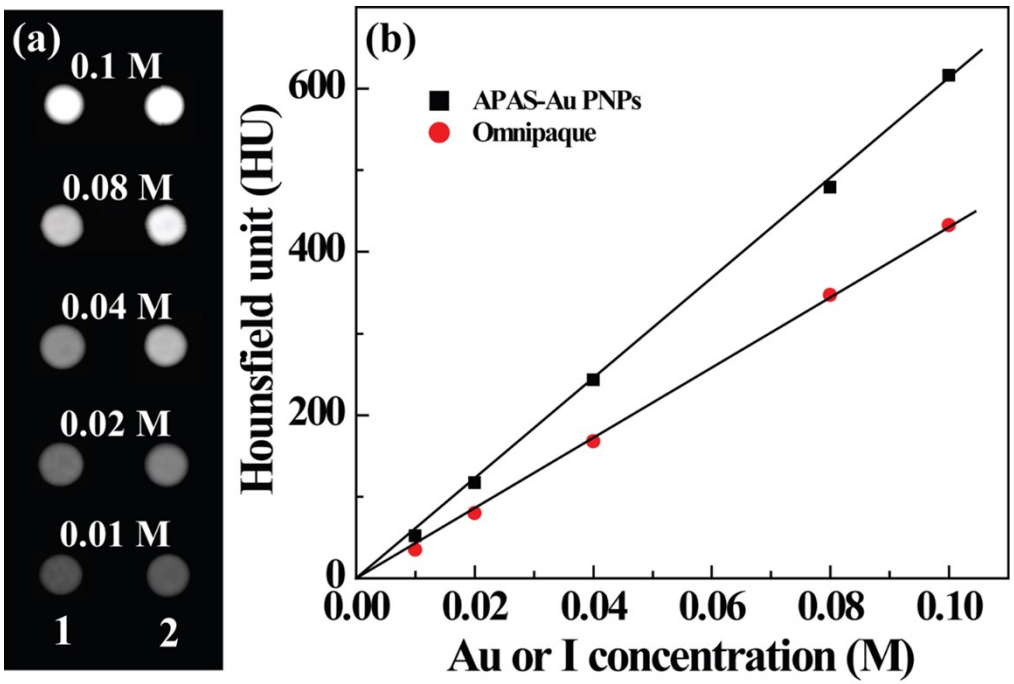

Fig. S5 (a) CT images of Omnipaque (1) and APAS-Au PNPs (2). (b) X-ray attenuation (HU) of APAS-Au PNPs and Omnipaque as a function of the molar concentration of the radiodense element (Au or iodine).

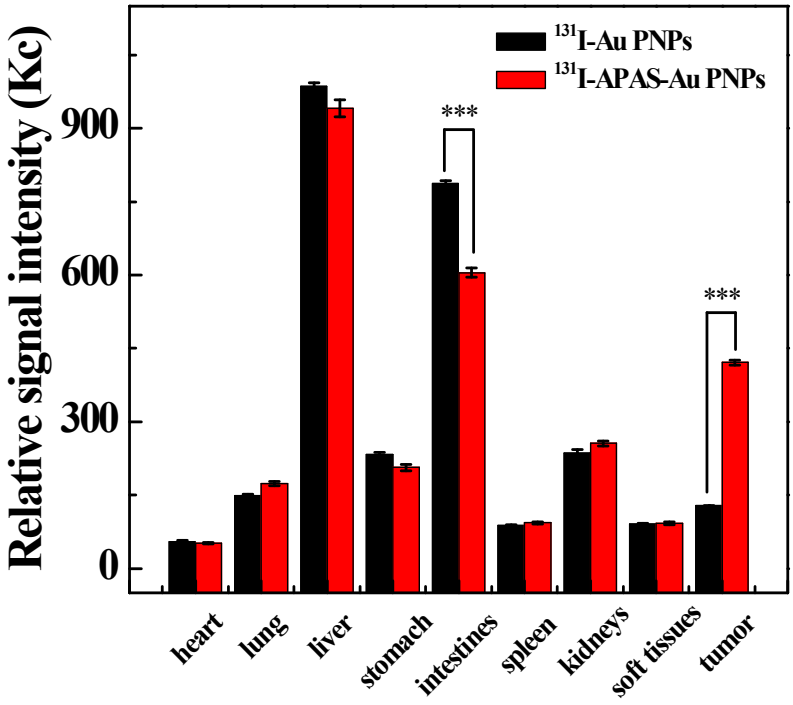

Fig. S6 Relative SPECT signal intensity of different organs at 6 h post-injection of ^{131}I-Au PNPs and ^{131}I-APAS-Au PNPs, respectively.

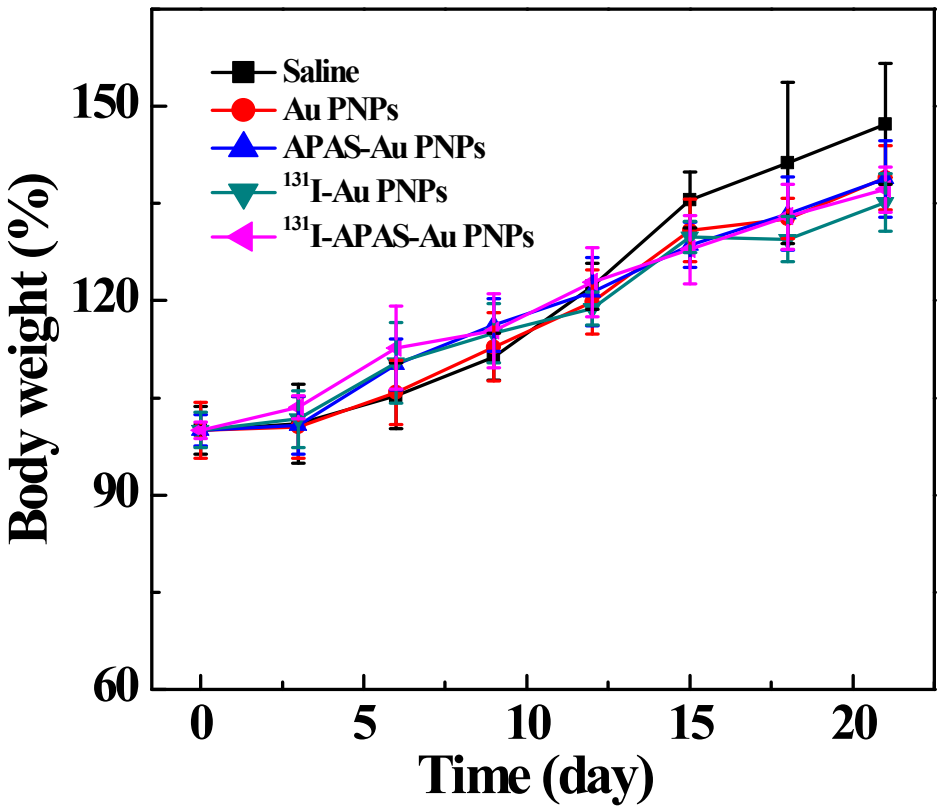

Fig. S7 The body weight of C6 tumor-bearing mice after treatment with saline, Au PNPs, APAS-Au PNPs, ^{131}I-Au PNPs, and ^{131}I-APAS-Au PNPs respectively. Saline was used as control. The relative body weights were normalized according to their initial weights (Mean ± SD, n = 5).

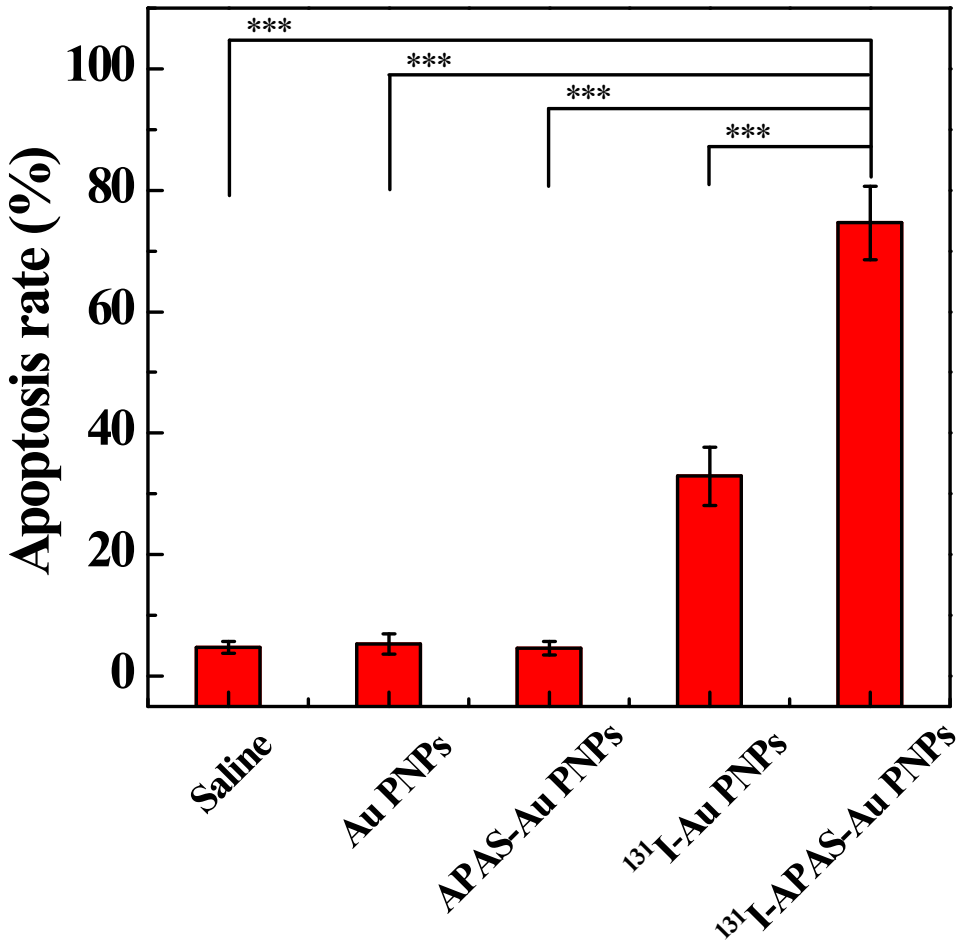

Fig. S8 Apoptosis rate of tumor tissue measured by TUNEL assay.

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