A Sensitive Upconverting Nanoprobe Based on Signal Amplification Technology for Real-Time in Situ Monitoring of Drug-Induced Liver Injury

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1. Synthesis of $NaYF_4$:Yb,Tm@$NaYF_4$ nanoparticles.

$NaYF_4$:Yb,Tm@$NaYF_4$ was prepared by referring to the previously reported method.[1-3]

Synthesis of β-$NaYF_4$:Yb,Tm (20/2 mol%) core nanoparticles. 0.64 mmol of Yttrium(III) chloride hexahydrate (YCl_3•$6H_2O$, 99.9%, Sigma-Aldrich), 0.16 mmol of Ytterbium(III) chloride hexahydrate ($YbCl_3$•$6H_2O$, 99.9%, Sigma-Aldrich) and 0.016 mmol of Thulium(III) chloride hexahydrate ($TmCl_3$•$6H_2O$, 99.9%, Sigma-Aldrich) were added to 6 mL of oleic acid (OA, 90%, Sigma-Aldrich) and 15.0 mL of 1-octadecene (Sigma-Aldrich), the mixture was heated up to 150 °C for 30 min under the argon protection to form a transparent and homogeneous solution. After cooled down to 50 ℃, 10 mL of methanol solution containing 3.2 mmol of ammonium fluoride (NH_4F, Sinopharm) and 2 mmol of sodium hydroxide (NaOH, Sinopharm) was poured to the mixture and the mixture was further stirred for 40 min at 50 ℃, with subsequent heating at 110 °C for 20 min to remove methanol and residual moisture. After the mixture was vacuumed for at last 10 min to completely remove methanol and residual moisture, switch of vacuum and argon for three cycles (1 min for each cycle segment). Subsequently, the solution was heated to 300 ℃ at a heating rate of 10

°C min^{-1} and kept for 1 h under argon protection. After cold to room temperature (RT), the resulting nanoparticles were precipitated with acetone and washed with cyclohexane for twice at 6654 g for 10 min. Finally, they resuspended in 5 mL of cyclohexane and named $NaYF_4$:Yb,Tm.

Synthesis of NaYF4:Yb,Tm@NaYF4 core-shell nanoparticles. 0.8 mmol of YCl_3•$6H_2O$ was added to the solution of 6 mL of OA and 15 mL of 1-octadecene, following, the mixture was heated up to 150 ℃ and kept for 30 min under argon atmosphere to prepare the shell precursor. Next, 5 mL of $NaYF_4$:Yb,Tm cyclohexane solution along with 10 mL of methanol solution containing 3.2 mmol of NH_4F and 2 mmol of NaOH were added after the shell precursor cold down to 50 ℃. The mixture was stirred at 50 ℃ for 40 min and heated to 110 ℃ to remove low boiling point solvents. Subsequently, the solution was heated to 300 ℃ for 1 h under argon atmosphere. After cooling to RT, the resulting nanoparticles were precipitated with acetone and washed with cyclohexane for twice at 6654 g for 10 min by a centrifuge (Eppendorf, 5810R), and resuspended in 5 mL of cyclohexane, named $NaYF_4$:Yb,Tm@$NaYF_4$ (OA-$UCNP_S$).

2. Synthesis of Gold Nanorods

GNR was synthesized by the silver-assisted seed mediated growth method with some adjustment.[4, 5] 52.1 μL of 1% tetrachloroauric (Ⅲ) acid tetrahydrate ($HAuCl_4$•$4H_2O$, 47.8%, Sinopharm) was added to 5 mL of 0.1 M hexadecyltrimethylammonium bromid (CTAB, Sigma-Aldrich), then 0.2 mL of freshly prepared ice-cold 10 mM sodium borohydride ($NaBH_4$, Sinopharm) was cautiously added dropwise under vigorous stirring to form a brownish-yellow colloidal gold seed solution. The seed solution was vigorously stirred for another 2 min and then kept undisturbed at 25-30℃ aging 2 h before use.

To a solution of 50 mL of 0.1 M CTAB, 100 μL of 50 mM silver nitrate ($AgNO_3$, Sinopharm), 1.04 mL of 1% $HAuCl_4$, 200 μL of 1.0 M HCl, 275 μL of 100 mM ascorbic acid (AA, Sinopharm)

and 60 μL of colloidal gold seed solution were added in order to prepare the growth solution, then the mixture was stirred for 15 s and left at 25-30 ℃ overnight undisturbed. The obtained nanorods were collected by centrifugation (12000 g, 5 min) on a centrifuge (Eppendorf, 5410R) and washed with distilled H_2O for three times. Finally, the precipitate was resuspended in 2 mL of distilled H_2O.

3. Thermal treatment of hairpin probes.

Probes H1FB and H2 were incubated in thermal cycler (Labcycler, Germany SENSEQUEST) as following procedures.

H1, H1F and H1FB were treated by the following procedure: 5 min at 94 ℃, 1.5 min at 70.7 ℃, 5 min at 4 ℃;

H2 and H2F were treated by the following procedure: 5 min at 94 ℃, 1.5 min at 60.8 ℃, 1.5 min at 60.8 ℃, 5 min at 4 ℃.

Oligonucleotide sequences were commercially synthesized by Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China), and they were listed in Table S2,

4. Cell culture

HL7702 cells and RAW 247.6 cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai) and cultured in Dulbecco's modified Eagle's medium (DMEM, Jiangsu Keygen Biotechnology Co., Ltd.) containing 1.5 g L^{-1} $NaHCO_3$ and 5.5 mM glucose, supplemented with 10 % fetal bovine serum (FBS, Gibco, South America), 100 U mL^{-1} penicillin, and 100 U mL^{-1} streptomycin maintained at 37 ℃ in a humidified incubator with 5% CO2. As their concentration reached 80%, the next cell experiments could be done. Cell density was determined using a hemocytometer prior to each experiment.

5. Cytotoxicity of the as-prepared nanoprobe

100 μL of HL7702 cell suspension (5×10^3 cells $well^{-1}$) was dispensed in a 96 well plate. The

cells were pre-incubated overnight. Next, different concentrations of UCNPs-H1/H2-GNR (0, 12.5, 25, 50, 100, 200 μg mL^{-1}) were diluted with cell-culture medium and added to wells, respectively. After 24 h incubation, 20 μL of 5 mg mL^{-1} methyl thiazolyl tetrazolium (MTT, Beyotime Biotechnology Co., Ltd., Shanghai) culture medium solution was added, these cells were incubated for another 4 h. Then the medium was discarded and 100 μL of DMSO (Sigma-Aldrich) was added. The absorption value of each well was measured by microplate reader (Bio-Tek Synergy, America) after the cell plate was incubated at RT for 2 h.

6. The stability of hairpin structure of H1 and H2

HL7702 Cells were seeded and cultured in 4-well glass bottom confocal dishes for 12 h. To form the hairpin probe-Lipofectamine 3000 complex, solvent A and B were prepared ahead respectively according to the operating manual of Lipofectamine™ 3000 Transfection Reagent (Thermo).

Solvent A: 1 μL of FAM modified H1 (H1F, 10 μM) or FAM and fluorescence quencher BHQ modified H1 (H1FB, 10 μM) was diluted with 100 μL of opti-MEM and gently mixed;

Solvent B: 3 μL of Lipofectamine 3000 was diluted with 100 μL of opti-MEM and gently mixed.

Next, solvent A and solvent B were mixed together and incubated at RT for 30 min. Subsequently, the mixture was added to cells and incubated for 4 h, then the HL7702 cells were washed with cold PBS for three times to remove the extracellular H1F and H1FB. Confocal imaging of the cells was acquired using a 60× objective on a laser scanning confocal microscope (Olympus 3000).

Same assays were applied to investigate the stability of H2 by using H2F and H2FB.

7. The cellular localization of nanoprobe

HL7702 cells or RAW247.6 cells were seeded in 35 mm confocal dish and incubated for 12 h. The medium was replaced with fresh one containing UCNPs-H1/H2F-GNR or UCNPs-H1/H2F-GNR

at a concentration of 100 μg mL^{-1}, respectively. After incubation for 2 h, the cells were washed with PBS for 5 times. Lysosomes was specifically stained with Lysotracker deep red (Thermo) according to the manufacturer protocol. The fluorescence images of cells were taken using a 60× objective on laser scanning confocal microscope.

8. Cell Transfection

Gene overexpression and gene knock down techniques were applied to regulate the miR122 expression in HL7702 cells. Cells were seeded on 6-well plate or 35 mm confocal dish and cultured for 12 h. The medium was replaced by serum-free medium before transfecting the plasmid which carries miR122 gene or small interfering RNA (siRNA) targeting miR122 gene. To form the DNA-Lipofectamine 3000 complex, solvent A and B were respectively prepared ahead according to the operating manual of Lipofectamine™ 3000 Transfection Reagent (Thermo).

Solvent A: 0.5 μg of plasmid and 2.5 μL of P3000 were diluted with 50 μL of opti-MEM and gently mixed.

Solvent B: 2.5 μL of Lipofectamine 3000 was diluted with 50 μL of opti-MEM and gently mixed.

Solvent A and solvent B were mixed together and incubated at RT for 15 min to form the plasmid-lipofectamine 3000 complex. Then, the plasmid-lipofectamine 3000 complex was added to the culture plate. After the cells were cultured for 4 h, the old medium was replaced and the cells were cultured for another 24 h.

9. Quantification of miR122 expression by RT-qPCR:

The MiPure Cell/Tissue miRNA Kit (Vazyme) was used to extract total miRNA from HL7702 cells and mice liver by according to the manufacturer protocol. Briefly, RNA isolator was added to lyse cells or mice liver, then the chloroform was added and the mixture was centrifugated to remove

protein and DNA. Next, the supernatant was mixed with ethanol and transferred to RNA-spin column to remove macromolecular RNA. Finally, the filtrate was added to miRNA column and centrifugated, then the total miRNA was eluted with RNase-free water and its concentration and quality was determined by Nanodrop. The first strand cDNA was reversely transcribed from miR122 RT-primer sequence using miRNA 1st Strand cDNA Synthesis Kit (Vazyme) according to the manufacturer protocol. The miRNA Universal SYBR qPCR Master Mix (Vazyme) was used according to the protocol provided by the manufacturer. The qPCR reactions were conducted on a full-automatic fluorescent quantitative PCR system (Light Cycler96). The primers used for RT-qPCR were listed in Table S1.

10. In vivo toxicity studies

The mice were randomly assigned into 5 groups: the experimental group were administrated with UCNPs-H1/H2-GNR (20 mg kg^{-1}) for 1, 3, 7 and 14 days, respectively, then the blood samples, liver and kidney were harvested; the control group were administrated with physiological saline (China resources double-crane Pharmaceutical) and sacrificed at last day.

Three important hepatic indicators, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bile acid (TBA), and three indicators for kidney functions, creatinine (CRE), blood urea nitrogen (BUN), urea (UA) were measured. These kits were purchased from Nanjing Jiancheng Bioengineering Institute. The liver, and kidney were removed and fixed in 4% paraformaldehyde fixative for hematoxylin (Beyotime Biotechnology Co., Ltd., Shanghai) and eosin (H&E) staining.

11. The extraction of DBR

DBR was extracted twice under reflux with 80% ethanol, 4 h and 3 h respectively. Combining the extract and evaporating it under a rotary vacuum evaporator at 60 °C, the residue was resolved in water and freeze-dried to afford the DBR extract with the yield of 12.5%. The extract was suspended

in 0.5% sodium carboxymethyl cellulose (CMC-Na, Sinopharm) aqueous solution and used subsequently.

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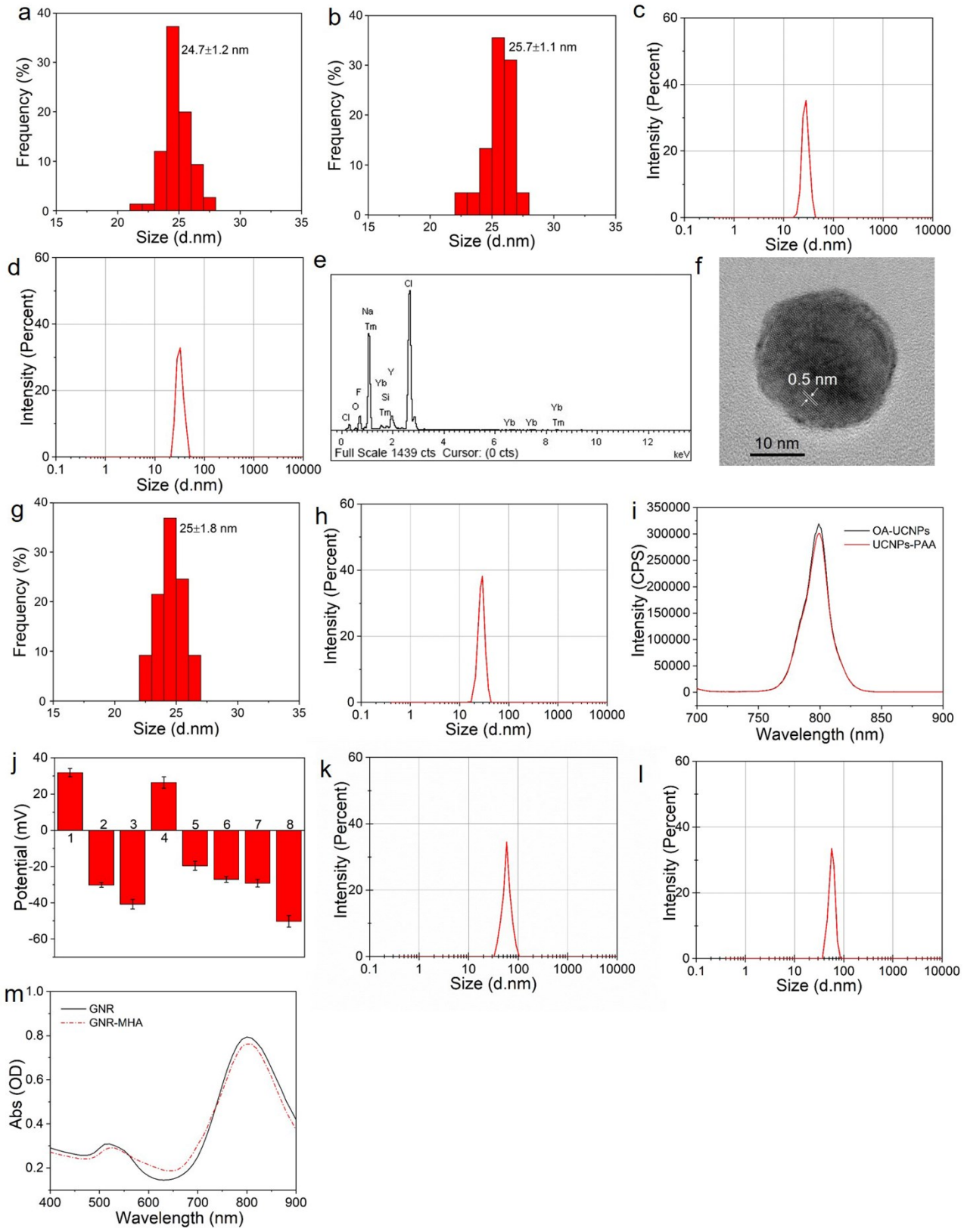

Figure S1. Statistics of the particle size distribution of (a) core: $NaYF_4$:Yb,Tm and (b) OA-UCNPs in Figure 2a,b. Dynamic light scattering (DLS) of (c) $NaYF_4$:Yb,Tm and (d) OA-UCNPs. (e) EDS spectroscopy analyses of OA-UCNPs. (f) The high resolution TEM image of OA-UCNPs. (g)

Statistics of the particle size distribution of PAA-UCNPs in Figure 2c. (h) DLS of UCNPs-PAA. (i) The upconversion luminescence (UCL) spectra of OA-UCNPs and UCNPs-PAA. (j) The zeta potentials of (1) GNR-CTAB, (2) GNR-MHA, (3) GNR-H1, (4) OA-UCNPs, (5) UCNPs-PAA, (6) UCNPs-H1, (7) UCNPs-H2, (8) UCNPs-H1/H2-GNR. DLS of (k) GNR-CTAB and (l) GNR-MHA. (m) The UV/Vis absorption spectra of GNR-CTAB and GNR-MHA. (data are medians ±quartiles, n = 3).

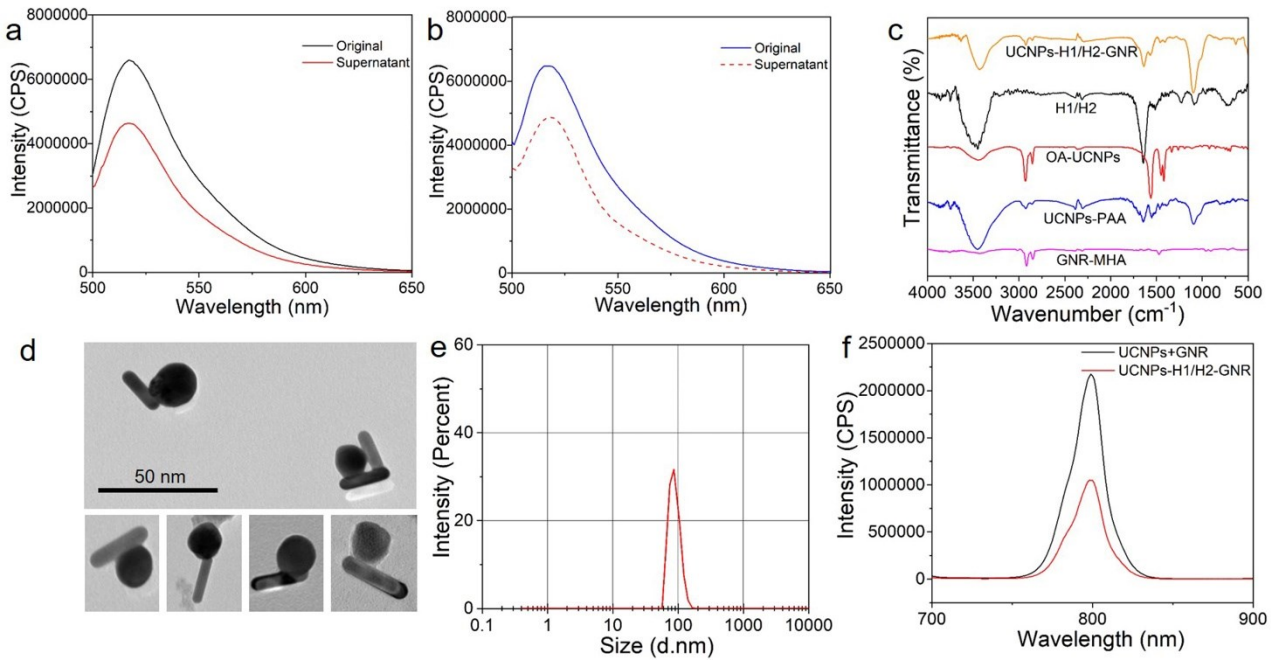

Figure S2. (a) The fluorescence spectra of H1F in original (5×10^{-8} M) and supernatant solutions when it was modified to GNR. (b) The fluorescence spectra of H2F in original (5×10^{-8} M) and supernatant solutions when it was modified to UCNPs. (c) FT-IR spectra of H1/H2, GNR-MHA, OA-UCNPs, UCNPs-PAA and UCNPs-H1/H2-GNR. (d) TEM image of UCNPs-H1/H2-GNR. (e) DLS of UCNPs-H1/H2-GNR. (f) The UCL spectra of UCNPs + GNR and UCNPs-H1/H2-GNR under 980 nm (1 W) irradiation.

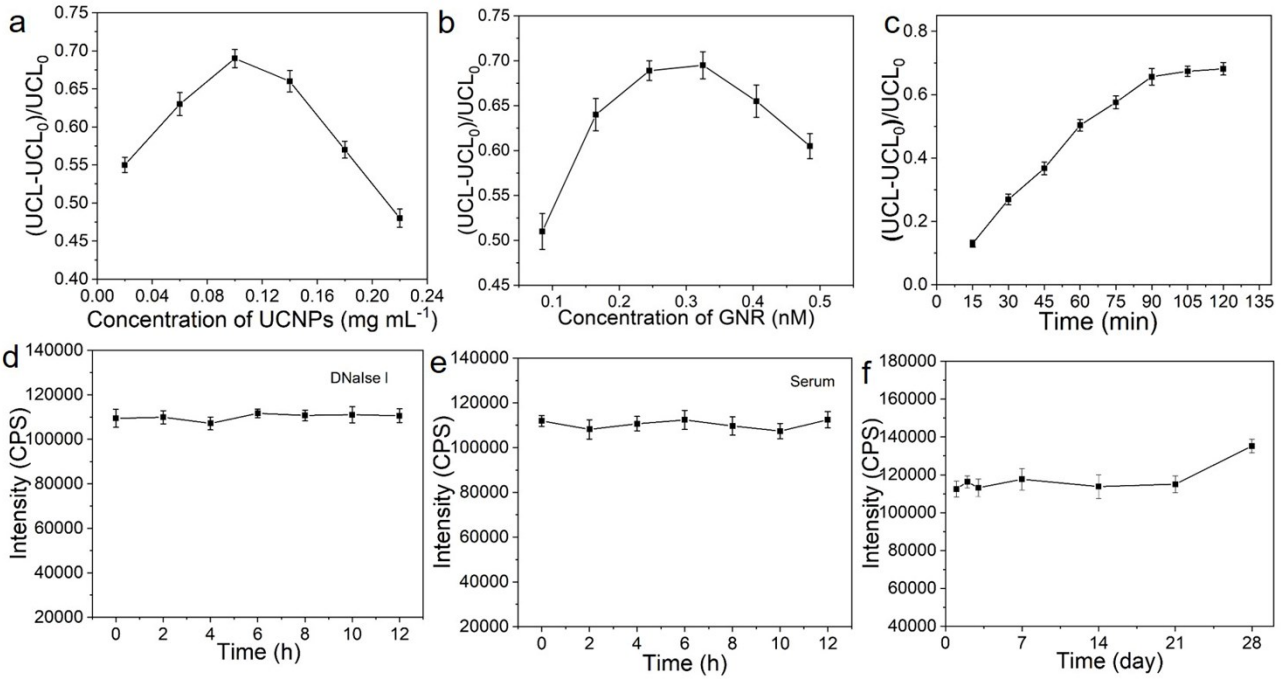

Figure S3. (a) Optimization of UCNPs concentration. UCNPs were ranged from 0.02 mg mL^{-1} to 0.22 mg mL^{-1}, GNR was 3.25×10^{-2} nM, GSH was 5 mM, miR122 was 1×10^{-9} M. (b) Optimization of GNR concentration. GNR was ranged from 8.5×10^{-3} nM to 4.85×10^{-2} nM, UCNPs was 0.1 mg mL^{-1}, GSH was 5 mM, miR122 was 1×10^{-9} M (c) Optimization of the incubation time of UCNPs-H1/H2-GNR and miR122. UCNPs was 0.1 mg mL^{-1}, GNR was 3.25×10^{-2} nM GSH was 5mM and miR122 was 1×10^{-9} M. The stability of UCNPs-H1/H2-GNR in (d) DNase Ⅰ (0.1 U μL^{-1}) and (e) serum who was diluted to 8-fold by 1 × PBS, as well as (f) the long-term stability of UCNPs-H1/H2-GNR in 4 ℃. UCNPs was 0.1 mg m L^{-1}. 980 nm laser was 1 W. (data are medians ±quartiles, n = 3).

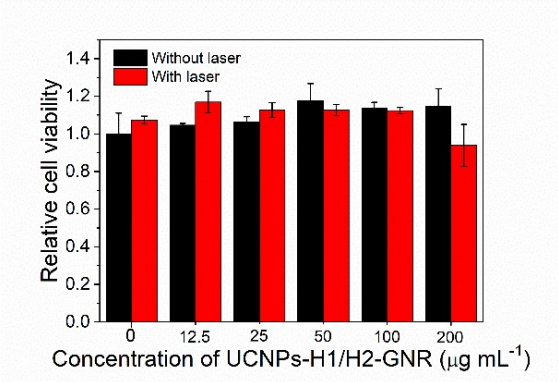

Figure S4. Cytotoxicity of UCNPS-H1 /H2-GNR at different concentrations with or without 980 nm laser (1 w) irradiation.

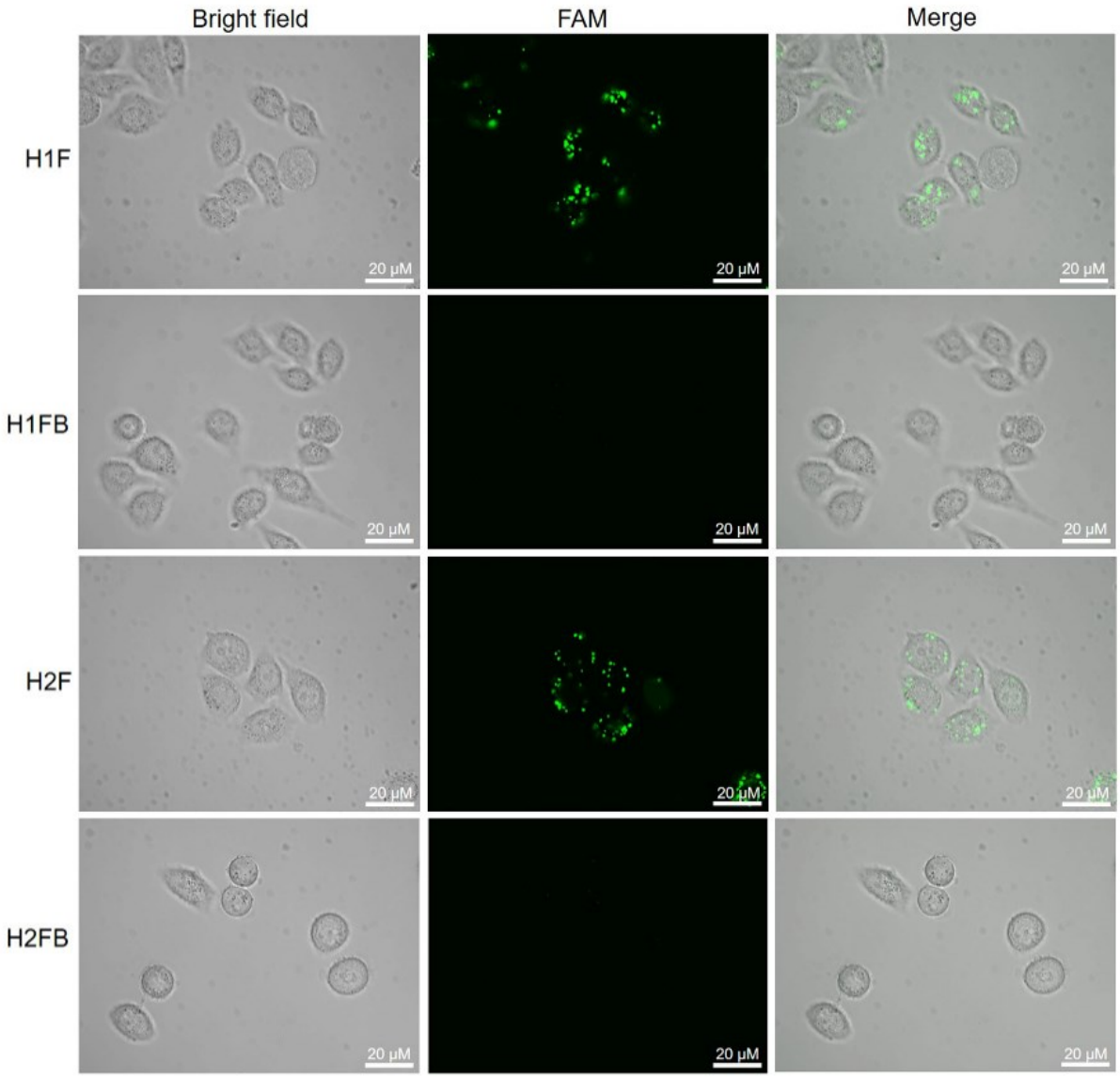

Figure S5. Confocal images of 7702 cells cultured with H1F, H1FB, H2F and H2FB respectively. Incubation time was 4 h. The excitation wavelength of FAM was 488 nm and the laser power was 20 mW× 4 %.

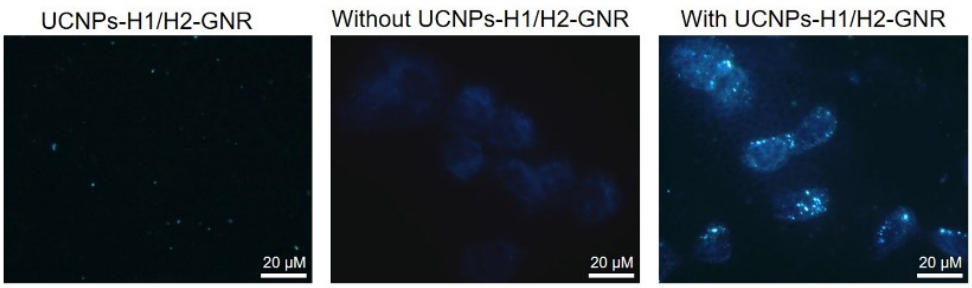

Figure S6. The darkfield images of UCNPs-H1/H2-GNR and HL7702 cells cultured without or with UCNPs-H1. Incubation time was 2 h.

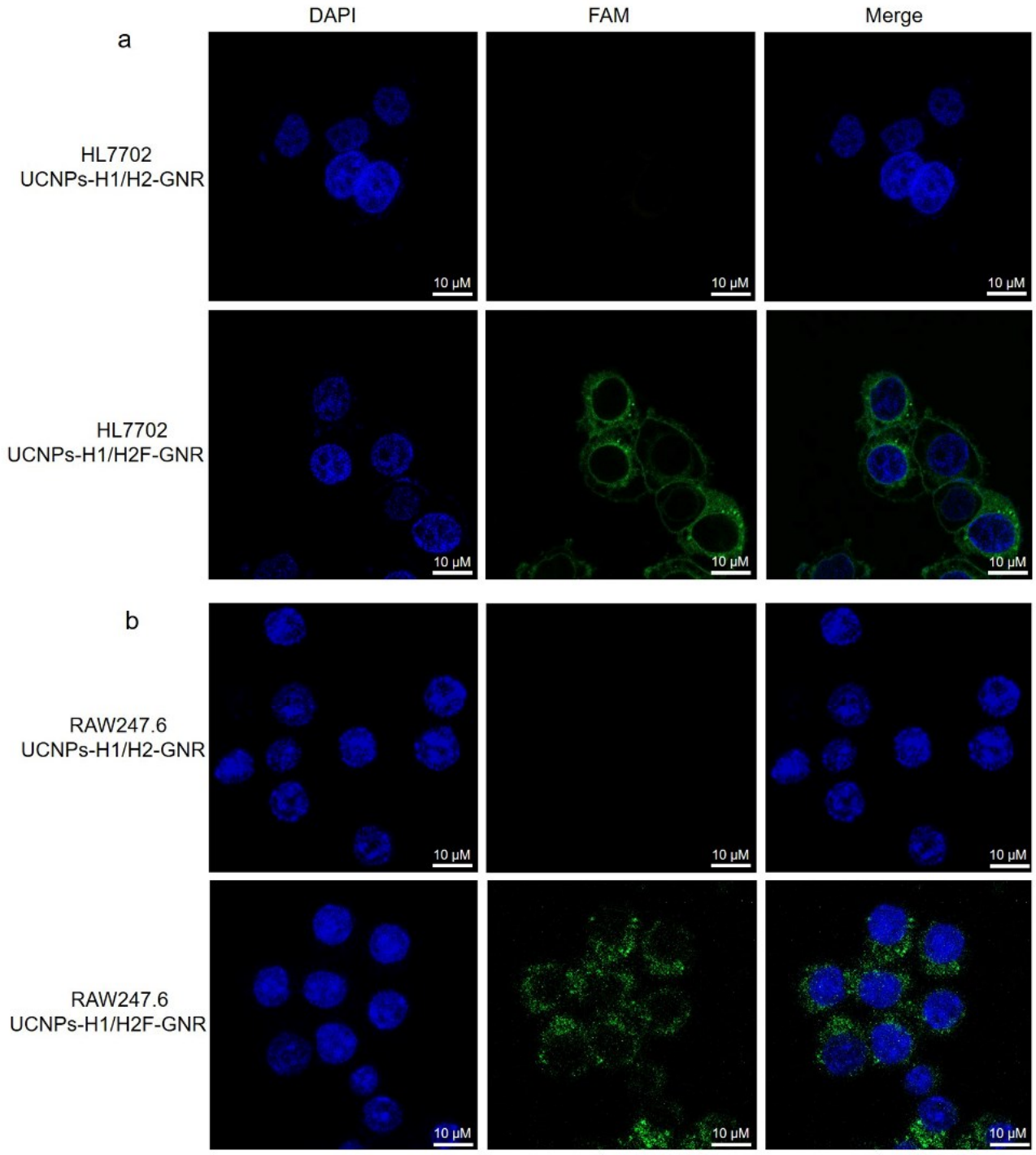

Figure S7. Confocal images of HL7702 and RAW247.6 cells who were treated with UCNPs-H1/H2-GNR and UCNPs-H1/H2F-GNR for 2h respectively. The excitation wavelength of DAPI was 405 nm and the laser power was 50 mW× 2 %. The excitation wavelength of FAM was 488 nm and the laser power was 20 mW× 4 %.

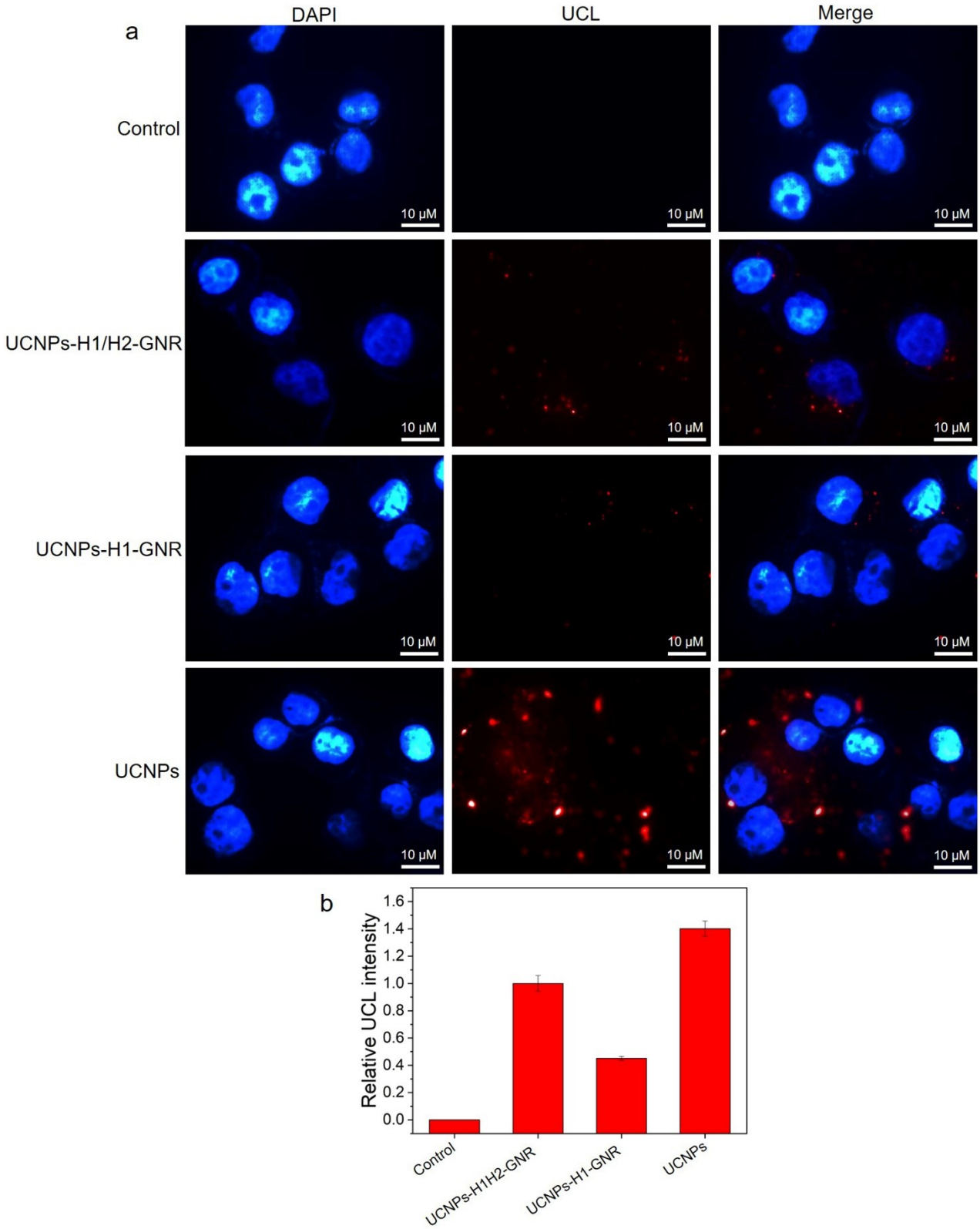

Figure S8. (a) UCL images of HL7702 cells pre-treated with UCNPs-H1/H2-GNR, UCNPs-H1-GNR and UCNPs, respectively, wild cells were use as control. Incubation time was 2 h. (b) Quantification of the relative UCL intensity of each cells in (a). The power of 980 nm laser was 0.5 W. (data are medians ±quartiles, n = 3).

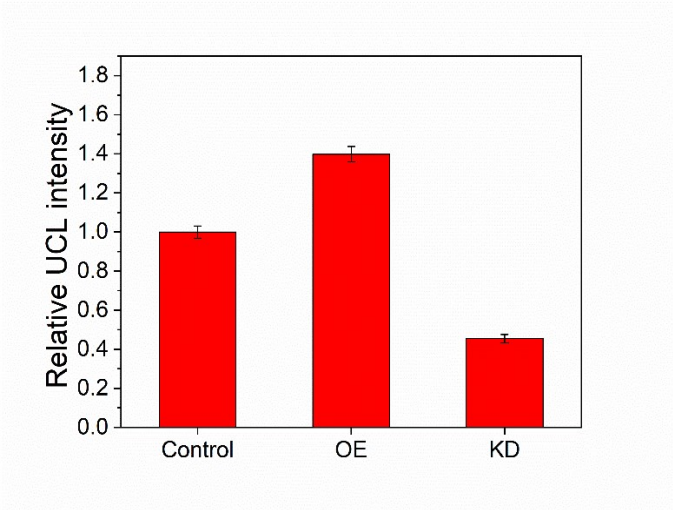

Figure S9. Quantification of the UCL intensity of cells with miR122 overexpressed (OE) or knockdown (KD). The power of 980 nm laser was 0.5 W. (data are medians ±quartiles, n = 3)

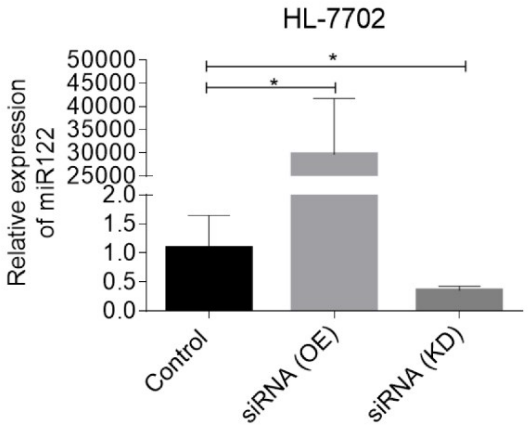

Figure S10. RT-qPCR analysis of the relative expression levels of miR122 in HL7702 cells whose miR122 were OE and KD respectively. (data are medians ±quartiles, n = 3).

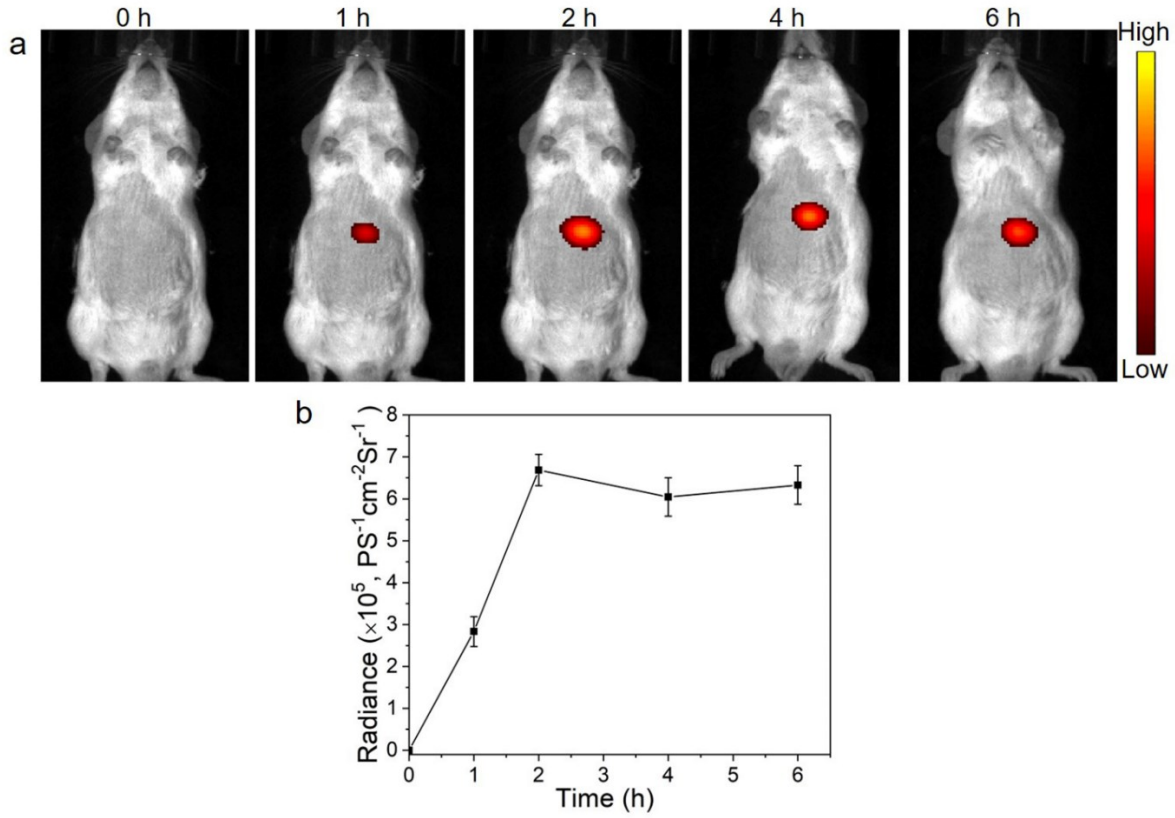

Figure S11. (a) Representative whole-body UCL images of WT ICR mice after intravenously (i.v) administrated with UCNPs-H1/H2-GNR at different time. The dosage of UCNPs-H1/H2-GNR was 20 mg kg^{-1}. (b) Quantification of the UCL intensity of mice after treatment with UCNPs-H1/H2-GNR at different time (data are medians ±quartiles, n = 3).

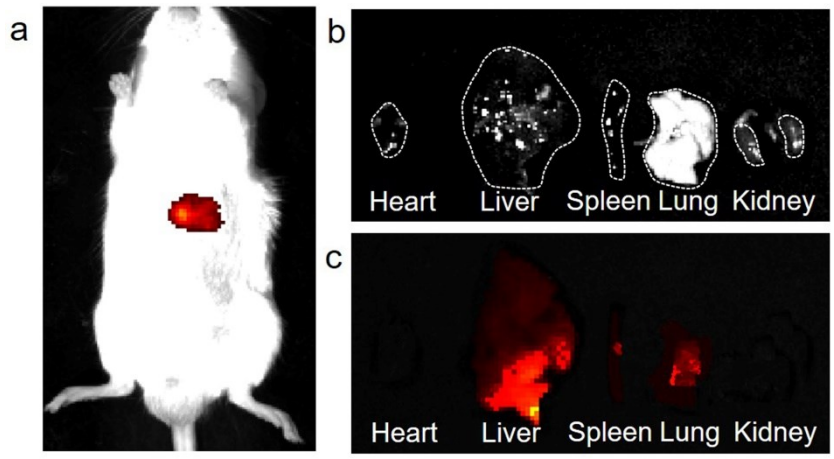

Figure S12. (a) Representative UCL image of mice after i.v injection with UCNPs-H1/H2-GNR for 2 h. The dosage of UCNPs-H1/H2-GNR was 20 mg kg^{-1}. (b) White field of heart, liver, spleen, lung, kidney. (c) UCL images of each organ obtained from the organs image after background subtracted.

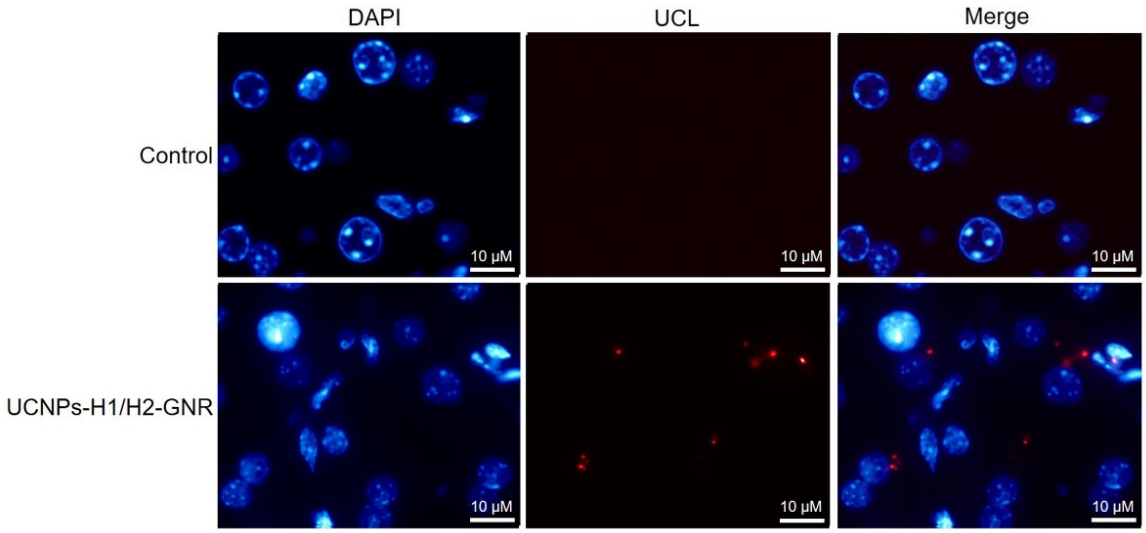

Figure S13**.** UCL images of the liver of mice after injected with nanoparticles for 2 h. WT mice was control. The dosage of UCNPs-H1/H2-GNR was 20 mg kg^{-1}. The power of 980 nm laser was 0.5 W.

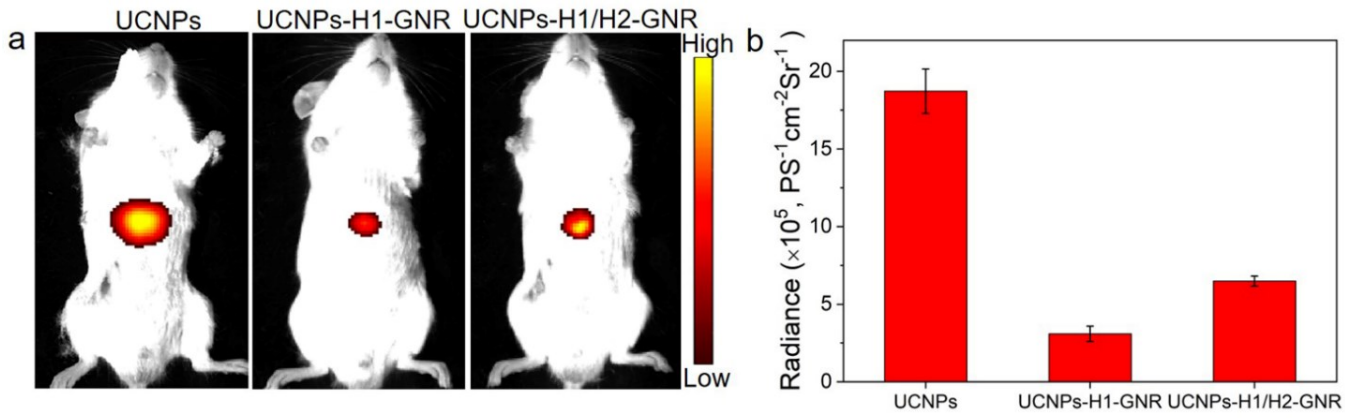

Figure S14 (a) Representative images of mice after *i.v* injection with UCNPs, UCNPs-H1-GNR and UCNPs-H1/H2-GNR (20 mg kg^{-1}) for 2 h. (b) Corresponding UCL intensity of mice after *i.v* injection with UCNPs, UCNPs-H1-GNR and UCNPs-H1/H2-GNR. (n=3 mice per group).

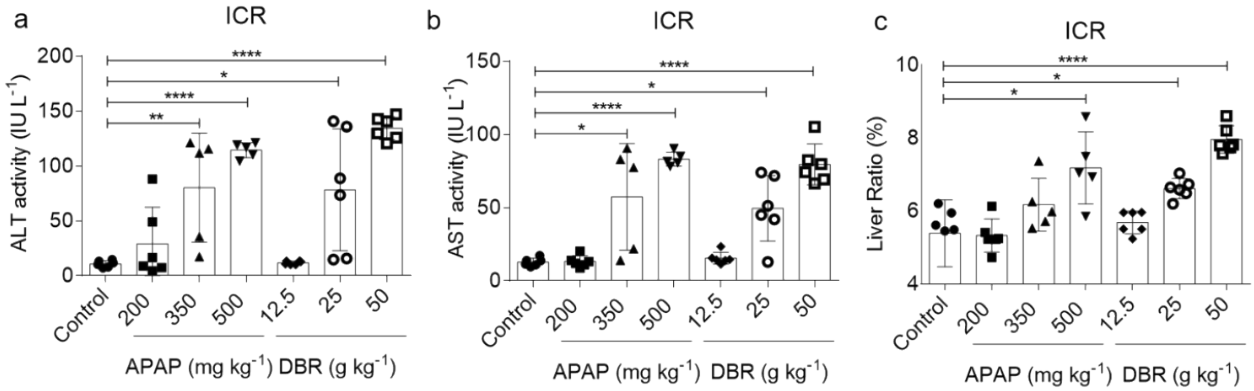

Figure S15. Dose-dependent changes of serum (a) ALT activities, (b) AST activities and (c) liver ratio of mice after i.g administration of APAP and i.p administration of DBR.

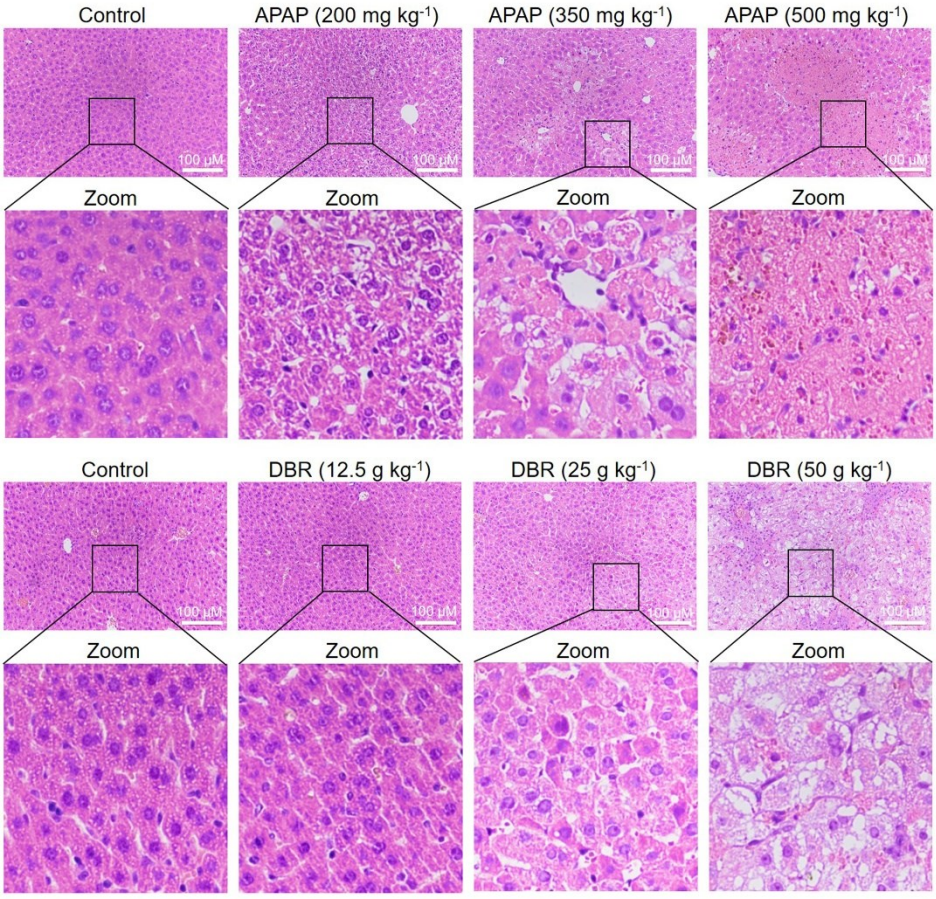

Figure S16. Histopathologic evaluation (H&E staining) of liver tissues obtained from mice treated with APAP or DBR at different dosage.

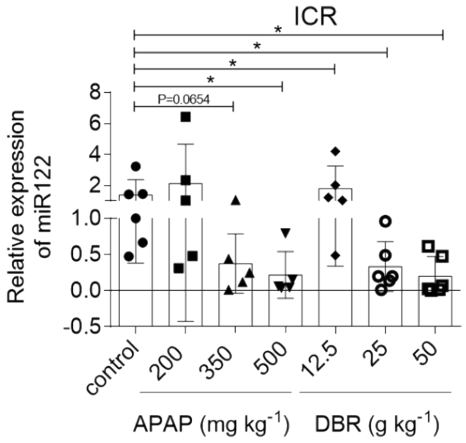

Figure S17. RT-qPCR analysis of the relative expression levels of miR122 in liver of mice administrated with APAP of 200 mg kg^{-1}, 350 mg kg^{-1} and 500 mg kg^{-1} and DBR of 12.5 g kg^{-1}, 25 g kg^{-1} and 50 g kg^{-1}. WT mice were control.

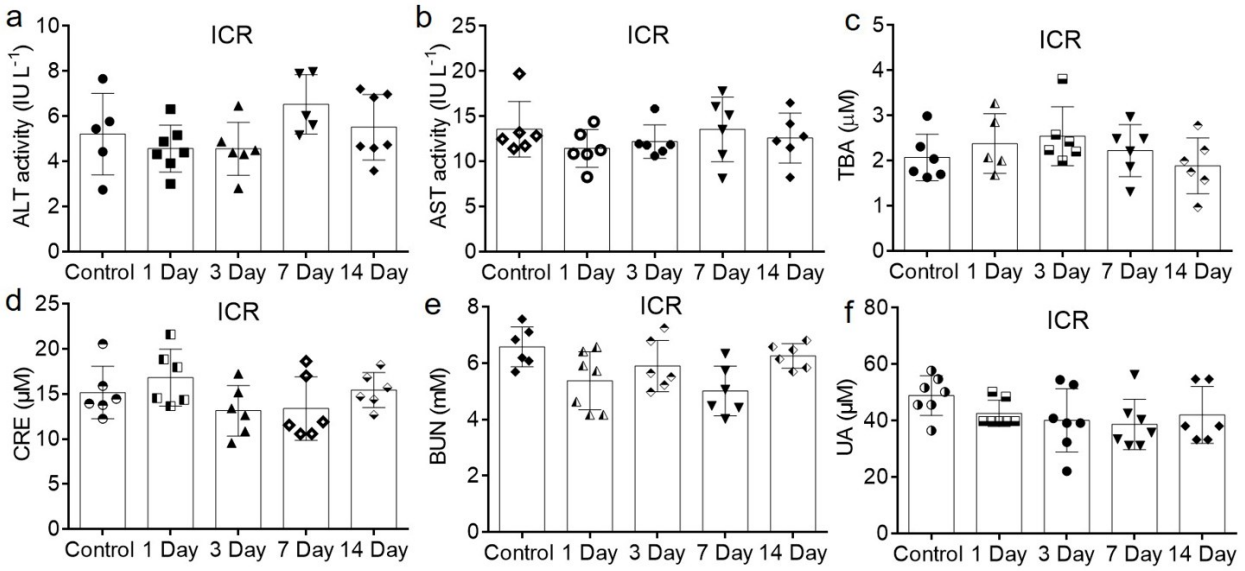

Figure S18. Serum biochemistry results obtained from mice injected with UCNPs-H1/H2-GNR (20 mg kg^{-1}) for different time and mice receiving no injection (Control). (a) alanine aminotransferase (ALT), (b) aspartate aminotransferase (AST), (c) total bile acid (TBA), (d) creatinine (CRE), (e) blood urea nitrogen (BUN), (f) urea (UA).

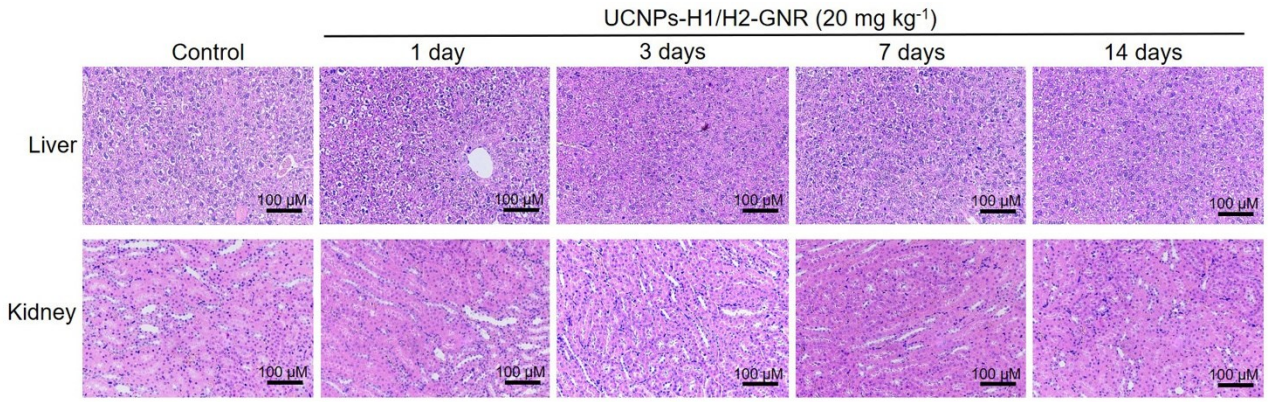

Figure S19. Histopathologic evaluation (H&E staining) of (a) liver and (b) kidney tissues obtained from mice i.v. with UCNPs-H1/H2-GNR (20 mg kg^{-1}) continuously for different time and mice receiving no injection (Control)

Table S1 The recovery of this nanoprobe (n=3)

Sample	Added concentration (M)	Recovery (%)	RSD (%)
Serum	10^{-12}	104.50±2.20	2.1
	10^{-9}	107.83±3.92	3.6
	10^{-6}	96.67±2.03	2.1

Table S2 Sequences of nucleic acids

Names	From 5’ to 3’
miR122	UGGAGUGUGACAAUGGUGUUUG
H1	SH-CAAACACCATTGTCACACTCCACCATGTGTAGATGGAGTGTGACA ATGGAATTAA-NH_2
H2	NH_2-AAAA-S-S-ACACTCCATCTACACATGGTGGAGTGTGACA ATG GCCATGTGTAGA
H1FB	FAM-CAAACACCATTGTCACACTCCACCATGTGTAGATGGAGTGTGACA ATGGAATTAA-BHQ
H1F	SH-CAAACACCATTGTCACACTCCACCATGTGTAGATGGAGTGTGACA ATGGAATTAA-FAM
H2F	NH_2- AAAA-S-S-ACACTCCATCTACACATGGTGGAGTGTGACA ATG GCCATGTGTAGAATT-FAM
mis-miR-122	AGCAGAGUGACAAUGGUGUUUG
miR155	UUAAUGCUAAUUGUGAUAGGGGU
miR192	CUGGCCUAUGAAUUGACAGCC
Stem-loop Primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAAAC A
mQ Primer R	AGTGCAGGGTCCGAGGTATT
Specific Primer	CGCGTGGAGTGTGACAATGG
U6 Primer (Forward)	CTCGCTTCGGCAGCACA
U6 Primer (Reverse)	AACGCTTCACGAATTTGCGT

Table S3 Comparison of the performances of the nanoprobe with the reported upconversion-based nanoprobes

Energy acceptors	Detection targets	Linear range	Detection limit	Detection site	References
Au NPs	miR21	10 pM-0.1nM	0.74 pM	cell	29
Cy3	miR21	200 pM-1.4 nM	0.095 nM	miRNA buffer	30
AF555	miR21	10 nM-200 nM	1.02 nM	cell	31
Au NPs	gIgG	5 nM-400 nM	3.4 nM	whole blood	33
TAMRA	miR-21	25 pM-2.5 μM	11.2 pM	cell	34