Electronic Supplementary Material (ESI) for Biomaterials Science.

Supporting Information

Rapid and accurate tumor-target bio-imaging through specific *in vivo* biosynthesis of fluorescent Europium complex

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1. Experiment section

Materials

DMEM (high glucose) culture solution and trypsin were purchased from Hyclone. Europium Nitrate Hexahydrate ($Eu(NO_3)_3$•$6H_2O$), fetal calf serum (FBS), penicillin, streptomycin and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma, and stored in the dark. Phosphate buffer solution (PBS) (0.1 M, pH 7.4) was prepared by using double distilled water. Dimethyl sulfoxide (DMSO), hematoxylin and eosin were purchased from Sinopharm Chemical Reagent *Co.* (China). Deionized water was used in all experiments. All of the chemicals used were of analytical grade and were used without further purification.

Cell culture and Cytotoxicity assay

HepG2 cells (human hepatocarcinoma) and Hela (cervical carcinoma) were purchased from Shanghai Institute of Cells, Chinese Academy of Sciences. L02 cells (human embryo liver cell) were provided by Third Military Medical University (Chongqing, China).HepG2, Hela and L02 cells were grown in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycinat 37°C with 5% CO_2 in a 95% humidified atmosphere.

In vitro cytotoxicity was measured by performing methyl thiazolyl tetrazolium (MTT) assays on the cells. Hela cells, HepG2 and L02 cells in log phase were trypsinized and then seeded in 96-well plates at 5×10^3/well. After 24 h resting time, each cell type was rinsed in DMEM and incubated with various concentrations of $Eu(NO_3)_3$ solution additions (0.0001, 0.001, 0.01, 0.1, 1.0 mmol/L) for 48 h. Then 20 μL MTT solution was added to each wells, and the cells were incubated for 4 h. Thereafter, 150 μL DMSO was added to solubilize the formazan crystals using an automated shaker to stir the cells slightly for 10 min. Absorbance of the suspensions was measured by optical density (OD) at a wavelength of 492 nm. Then the cell viability was expressed as follows: cell viability (%) = $[A]_{test}$ / $[A]_{control}$ × 100%, where [A] represents the absorbance value at 492 nm.

***In vitro* biosynthesis and characterization of Eu complex from cells**

Aqueous solution of europium nitrate ($Eu(NO_3)_3$) (Sigma, USA) was freshly prepared and used for the biosynthesis of Eu complex. Eu complex were biosynthesized in situ through incubation of HepG2 cells with 0.01 mmol/L aqueous solution of europium nitrate added to usual cells under culturing conditions. After 24 h or 48 h incubation, the presence of biosynthesized Eu complex was characterized by fluorescence and energy dispersive X-ray spectroscopy (EDS). No formation of Eu complex was observed in normal cells like L02 control cells incubated with the same protocols. Fluorescence spectra were also measured from Eu complex solutions extracted from incubated Hela cells by a repetitive freeze-thaw method, using a UV-Vis-NIR spectrophotometer (Shimadzu, UV3600), and a fluorescence spectrometer (PerkinElmer, LS-55), respectively. EDS of Europium complex immobilized on an indium-tin oxide (ITO) glass substrate were taken on a field-emission scanning electron microscope (Zeiss, Ultra Plus). The valence state of europium atoms in the in situ catalyzed biosynthesis Eu complex was investigated by a PHI 5000 Versa Probe X-ray photoelectron spectrometer.

Confocal fluorescence imaging of cells

Confocal fluorescence microscopy imaging of living cells incubated with the $Eu(NO_3)_3$ solution was performed on laser confocal fluorescence microscope. For the fluorescence microscopy imaging, the continuous wave laser at 488 nm provided the excitation, and fluorescence emission at 500-650 nm was collected as output signal. A 60 × oil-immersion objective lens was used.

Construction of the xenografted tumor mouse model

Four weeks of age and weight-matched (18-22 g) of BALB/c female mice were purchased from Peking University Health Science Center. All animals were maintained in a specific pathogen free (SPF) house at 24 ± 2 °C with a standard 12-hour light/12-hour dark cycle and received water and food. The mice were randomly assigned to groups for experimental purposes. Tumor models were

generated by the subcutaneous inoculation (0.10 mL volume containing 5×10^7 cells/ml media) of Hela cells or HepG2 cells into left forelimb (posterior limb) oxter-endermic using a 1 ml syringe with a 25 G needle. Tumor growth was monitored until a palpable size of about 0.2 - 1.0 cm was reached in all directions.

***In vivo* bio-imaging study of the tumors**

For *in vivo* bio-imaging of Eu complex in the tumor location, some of the xenograft (Hela cells and HepG2 cells) tumor mice received a subcutaneous injection of 1 mmol/L $Eu(NO_3)_3$ solution prepared in normal saline (0.1 mL) near the tumor location and the others received the relevant solution through tail vein injection of 10 mmol/L $Eu(NO_3)_3$ solution. The animals were fully anesthetized by inhalation of a mixture of oxygen with 5% isoflurane 4 h, 8 h, 24 h, 48 h and 72 h after the injection. The in vivo bio-images were acquired by using an in vivo fluorescence imaging system (Cambridge Research & Instrumentation, *Inc.*, Maestro EX). The ROI (regions of interest) analysis was measured under the assistance of *CRi* Maestro Image software. The studies were approved by the National Institute of Biological Science and Animal Care Research Advisory Committee of Southeast University, while experiments conducted by following the guidelines of the Animal Research Ethics Board of Southeast University.

Histological and Hematology Examinations

The 10 mmol/L $Eu(NO_3)_3$ solution prepared in normal saline (0.1 mL) were injected into xenograft tumor mice (n=3) through the tail vein and this group of mice was test as the experimental group. Normal athymic nude mice (n=3) with injection of the same dose $Eu(NO_3)_3$ solution were selected as the control group in the histological study. Xenograft tumor mice (n=3) with injection of the same dose normal saline were selected as the control group in hematology study. After 48 h, blood and tissues samples were harvested from mice post-injection. Blood was collected from the orbital sinus. Upon completion of the blood collection, mice were sacrificed. The liver, spleen, and kidney were removed, and fixed in paraformaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The histological sections were observed under an optical microscope. The images were obtained by using a BX53 microscopy system (Olympus, Tokyo, Japan) that was equipped with a color CCD (DMK 41BU02, *Sony Co.*, Tokyo, Japan). All experiments involving mice were approved by the National Institute of

Biological Science and Animal Care Research Advisory Committee of Southeast University, and experiments were conducted by following the guidelines of the Animal Research Ethics Board of Southeast University.

Europium species uptake by tumor and Bio-distribution by ICP-MS

After injection the 10 mmol/L $Eu(NO_3)_3$ solution for 48 h, We have collected tumor and the vital organs (liver, kidney and spleen) separately and submitted them for inductively coupled plasma mass spectrometry (ICP-MS) analysis for the determination of europium elements in order to evaluate the tumor uptakes and the bio-distribution of europium in different vital organs. Both tumor and organs (liver, kidney and spleen) were washed with phosphate buffered saline (PBS) and blotted dry to minimize the contribution of any contaminants. Then they were weighed and then eroded in HNO_3 and H_2O_2.

Proteomics research

Hela cells were seeded at a density of 2.5×10^4 cells/cm^2 with 0.01 mmol/L $Eu(NO_3)_3$ solution for 24 h at 37 °C. Cells cultured with DMEM/high glucose medium were used as a control. In order to remove contamination from bovine serum proteins in the medium, cells were washed with cold phosphate-buffered saline solution (PBS) thrice before cell total protein extraction. The proteomics analysis was performed by Shanghai Weijunsheng Biological Technology *Co. Ltd* (Shanghai, China). The prepared samples were labeled with 8-plex iTRAQTM(Applied Biosystems, USA) and analyzed by using EASY-nLC 1000 liquid chromatography (Proxeon Biosystems) /Q-Exactive mass spectrometry (Thermo Scientific, SCX/HR-HPLC-ESI-MS/MS). The iTRAQ data were searched with MASCOT engine (Matrix Science, London, UK; version 2.2) embedded into Proteome Discoverer 1.3 (Thermo Electron, San Jose, CA.) against Uniprot Human database (133549 sequences, download at March 3rd, 2013) and the decoy database for protein identification and quantification. The experiment was performed in two independent runs. The proteins with fold changes >1.2 or rate <0.667were considered differentially expressed.

Bioinformatics analysis

The third level of Gene Ontology (GO) analysis and KEGG pathway analysis of differentially expressed proteins were performed by DAVID software (http://david.abcc.ncifcrf.gov/).

Data analysis

Data was initially stored in MS excel and statistical program SPSS version 18 was used for analysis of variance (ANOVA), where data were expressed as the means ± SD (standard deviation) from at least three independent experiments. One-tailed unpaired Student's t-test was used for significance testing, and $p < 0.05$ was considered significant.

2. Supporting Figures

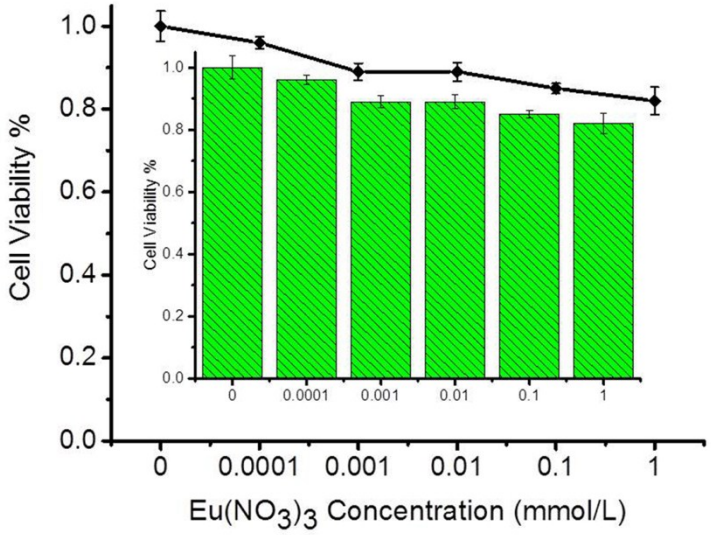

Fig. S1 MTT assay assessment of dose-dependent cytotoxicity towards L02 cells 48 h after incubation with $Eu(NO_3)_3$ solutions. The data is represents three individual experiments done in triplicates.

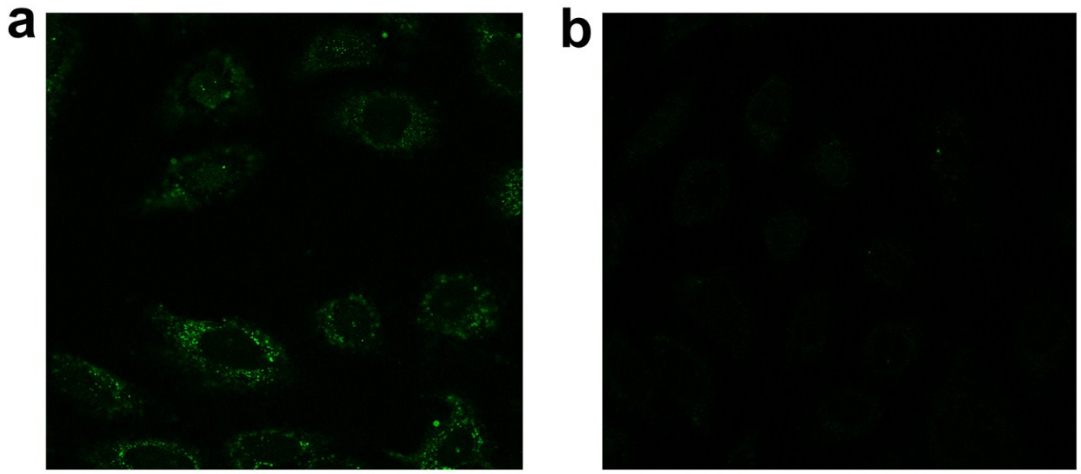

Fig. S2 Laser confocal fluorescence imaging of Hela cells (a and b). (a) incubated with 0.01 mmol/L $Eu(NO_3)_3$ solution for 24 h at 37°C; (b) incubated with DMEM (high glucose) medium as control. Images were acquired at 400-fold magnification.

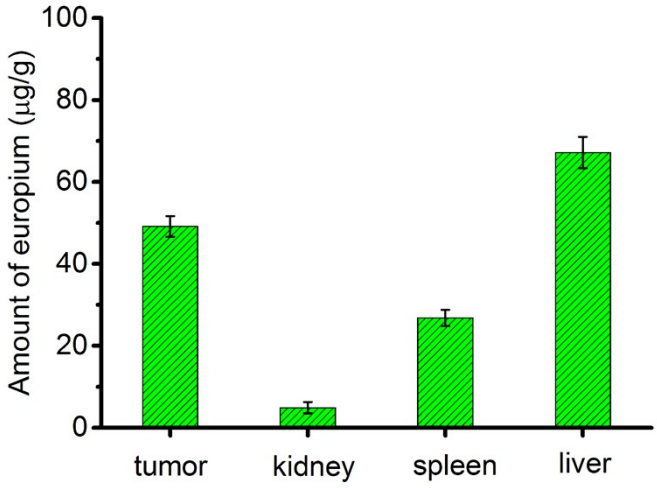

Fig. S3 ICP-MS data shows the bio-distribution of europium element in various organs or tissues of Hela tumor bearing mice with 0.1 ml 10 mmol/L $Eu(NO_3)_3$ solution at 48 h post-injection.

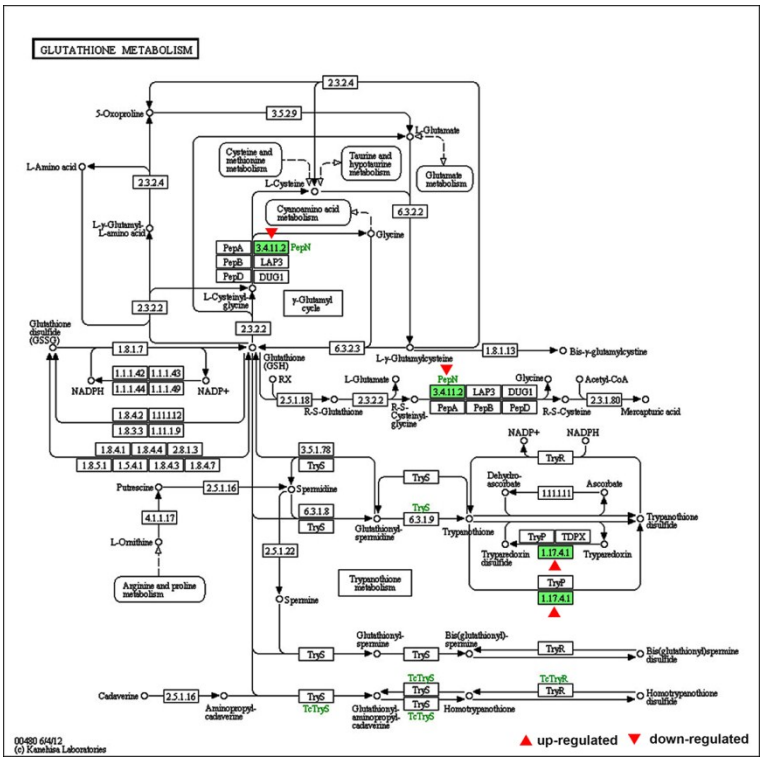

Fig. S4 KEGG biological pathways of glutathione metabolism.