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Supporting Information

Facile synthesis of multifunctional manganese(II)-carbon dots hybrid and application as efficient magnetic-fluorescent imaging probes for ovarian cancer cell imaging

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Experimental Section

Chemical and Materials

Ethylenediaminetetraacetic acid (EDTA), manganese chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$), Triethylenetetramine and ethylene glycol were purchased from Sinopharm Chemical Reagent Co., Ltd, (Beijing, China). CellTiter 96 was purchased from Promega (Madison, Wis). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) and N-hydroxysuccinimide (NHS) were purchased from Sigma (Shanghai, China). Anti-HE4 mAb was brought from Abcam (Cambridge, MA USA). All aqueous solutions were prepared with ultrapure water, obtained from a Mill-Q system (Millipore Corp., Bedford, USA).

Characterization

The morphology and microstructure of Mn-CDs were examined with high-

resolution transmission electron microscopy (HTEM) (Tecnai G2 F20 S-TWIN, 200KV, USA). The concentration of manganese content was measured by inductively coupled plasma-mass spectrometry (ICP-MS) (Agilent 7500ce, USA). The surface functional groups of Mn-CDs were recorded by FT-IR spectra from the infrared absorption spectroscopy (Bruker, Germany). The surface oxidation state of the manganese was evaluated by X-ray photoelectron spectroscopy (XPS) (Thermo escalab250Xi, USA). The UV-vis spectra were performed on a T6 series UV-vis spectrophotometer (Puxi Co., Beijing, China). Fluorescence measurements were carried out using a LS-45/55 Fluorescence/Phosphorescence Spestrometer (PerkinElmer, USA). MRI properties were performed on 3.0 T human magnetic resonance scanner (GE Signa, USA). The fluorescent images were taken at a fluorescent inverted microscope (Leica DMI 3000B, Germany) at UV light excitation. Fluorescent images in vivo were performed on a small animals living visible light imaging system (Berthold technologies NightOWL II LB 983 NC100, Germany).

Synthesis of Mn-CDs

Mn-CDs were synthesized through a one-pot solvothermal method. Briefly, EDTA (0.2 mmol) and $MnCl_2 \cdot 4H_2O$ (0.2 mmol) were added to the mixed solution of triethylenetetramine (2 mL) and ethylene glycol (10 mL). Then the solution was transferred to flask (50 mL) and heated at 150 ^oC in air for 10 h with vigorous stirring. After the reaction, the reactors were cooled to room temperature naturally. The product, which was dark brown and transparent, was

transferred to dialysis for 48 h to obtained purified nanoparticles. The solid product was collected after freeze-drying for further applications.

Bioconjugation of Mn-CDs with Anti-HE4 mAb

Anti-HE4 was conjugated with Mn-CDs in the presence of EDC and NHS. Briefly, EDC·HCl (5 mg) was added into the water-dispersed Mn-CDs (2.5 mg/mL) for 15 min to activate carboxyl group. Then, 2 mg NHS was added to the solution for another 15 min. Finally, Anti-HE4 mAb (10 μL, 1.789 mg/mL) was mixed and incubated for 2 h at 37 °C. After the reaction, the supernatant was collected by removing the large cross-linked particles through centrifugation at 10 000 rpm for 10 min. After centrifugal ultrafiltration (5 500 rpm, 20 min) with a Millpore ultrafiltration tube (molecular weight cutoff: 3 000 D), the obtained nanoparticles were redissolved with 2 mL of PBS buffer (pH 7.4) for further study.

MRI relaxation properties of Mn-CDs

The as-purified 0.491 mM Mn-CDs measured by ICP-MS were dissolved in double distilled water to dilute a series of manganese concentration (0.491, 0.45, 0.40, 0.35, 0.30, 0.25, 0.20, 0.15, 0.10 mM). Then, MR images were acquired by 3.0T human magnetic resonance scanner. The parameters were described as follows: i) T1 weighted images: echo time (TE)=16.5 ms, repetition time (TR)=425 ms, field of view (FOV)=14 cm×14 cm, matrix=384×256, slice thickness=2.0 mm, spacing=1.5 mm; ii) T1-map images: TE=7.4 ms, TR=200-800 ms, FOV=14 cm×14 cm, matrix=384×256, slice thickness=2.0 mm,

spacing=1.5 mm. The T1- map images were reconstructed by function software at a workstation (AW 4.2). The signal intensities of each ROIs in the T1-map (70 pixels) were measured for each concentration. Accordingly, the specific relaxivities (r1), which means the contrasting effect, was measured.

Quantum yield determination

Fluorescence quantum yields (QY) were calculated using quinine sulfate as a standard material. The photoluminescence intensity and the absorbance value were measured at a wavelength of 360 nm. QY values were obtained according to the following equation (1):

$$QY_x = QY_s * [A_s/A_x] * [I_x/I_s] \quad (1)$$

Where A is the absorbance value and I is the integrated photoluminescence intensity. The subscript "s" refers to the standards and "x" refers to the Mn-CDs in present experiment.

Cell cytotoxicity test

Cell cytotoxicity was assessed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay[22] with HO-8910 cell (a human EOC cell line) and EA.hy926 (a human umbilical vein cell line) as the test cell lines. HO-8910 and EA.hy926 cells were incubated in RPMI 1640 medium and Dulbecco's modified Eagle medium (DMEM), respectively, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. These cells were seeded into 96 well flat-bottom microplates at densities of 1×10^4 cells per well, which were grown as

monolayer cultures in the 5% CO_2 incubator at 37 °C for 12 h to nearly fill up the well. Then, after washed twice with PBS, the complete culture medium containing different concentrations of nanoparticles (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/mL) was added to the well and co-cultured for 12 h. Later, the mediums were discarded and the cells were rinsed thrice with PBS, which was cultured with fresh medium 100 μL and CellTiter 96 agent 20 μL per well for 4 h. The absorbance for each sample was detected by the microplate reader (Multiskon MK3, USA) at 490 nm. The absorbance of each concentration was calculated by the average of 6 parallel samples. The relative cell viability (%) compared to control cells was calculated by (sample/control)×100%. All experiments were repeated in triplicate.

In vitro MR imaging and fluorescence imaging

The HO-8910 cell and EA.hy926 cells were cultured in 6-well plates at a density of 2×10^5 cells/well in the 5% CO_2 incubator at 37 °C for about two days to nearly fill up the well. Afterward, the culture media was removed, and the cells were washed with PBS. Then 1 mL Mn-CDs and 1 mL Mn-CDs@Anti-HE4 were respectively added into two kinds of cells to culture for 30 min in the incubator. After washed away the nanoparticles with PBS twice and lysed by typsin, cells were collected by centrifugation at 2000 rpm for 10 min and distributed in 300 μL 4% paraformaldehyde solution and kept at 4 °C for MRI scanning. In vitro MR imaging study was finished with 3.0 T human magnetic resonance scanner. The parameters were same as previous. Similarly, the two

kinds of cells were cultured in 6-well plates about 24 h to grow about 60%-80% for in vitro fluorescence microscopy image. After washing with PBS, 1 mL Mn-CDs (2.5 mg/mL) and 1 mL Mn-CDs@Anti-HE4 were added into two kinds of cells to culture for 30 min in the incubator, consistently. After washed away the nanoparticles with PBS twice, fluorescence images and light microscope images at the same field were obtained using microscope with emission filters.

In vivo toxicity assessment of Mn-CDs

All animal experiments were conducted under protocols approved by the Animal of the Ministry of Health of the People's Republic of China and the Guidelines for the Care and Use of Laboratory Animals of Xuzhou Medical College. Kunming female mice aged 4-6 weeks were purchased from the Animal Research Center of Xuzhou Medical College. Mn-CDs (100 mg/kg) was injected via the tail vein into three healthy Kunming female mice in each group, other three mice were used as the untreated controls. The body weight of animals and their behavior were carefully recorded daily during the course of the experiment. Three weeks after injection, three mice in a group were respectively sacrificed to collect various organs and tissues for histological examination. Tissues were stained with hematoxilin/eosin as indicated to assess for potential effects of Mn-CDs treatment on the organ morphology and cellular damage.

In vivo imaging

Female BALB/c mice aged 4-6 weeks were purchased from the Animal Research Center of Xuzhou Medical College. Tumor-bearing mice model were established by injection of 2×10^7 HO-8910 cells into each mouse. Three weeks later, HO-8910 tumor-bearing mouse was anesthetized by 2% pentobarbital and put in a small animal RF coil, and the Mn-CDs contrast agent solution was injected into the tail vein; the dose was 100 mg/kg. T1WI, T2WI and T1WI enhanced images were made before and after the injection of the contrast agent solution. All the in vivo MRI experiments were performed on the Discovery MR 750w 3.0 T human magnetic resonance scanner (GE Discovery, USA). The parameters as follows: i) Coronal T1-weighted spin-echo sequence: echo time (TE)=12 ms, repetition time (TR)=400 ms, field of view (FOV)=8 cm×8 cm, slice thickness=2.0 mm, spacing=0.2 mm; ii) Coronal T2-weighted fast-spin-echo sequences: TE=58 ms, TR=3000 ms, FOV=8 cm×8 cm, slice thickness=2.0 mm, spacing=0.2 mm iii) Axial T1-weighted spin-echo sequence: TE=Min Full, TR=400-850 ms, FOV=6 cm×6 cm, slice thickness=1.5 mm, spacing=0.2 mm. The in vivo fluorescence experiments were performed on small animals living visible light imaging system. After anesthetized, the Mn-CDs solution (100 μL, 15 mg/mL) was injected into the tumor directly.

Fig. S1 Dynamic light scattering size distribution graphs of a) Mn-CD and b) Mn-CDs@Anti-HE4.

Table S1 The XPS data from Mn-CDs sample.

Name	Start BE	Peak BE	End BE	Height CPS	FWHM eV	Area (P) CPS.eV	Atomic %
C1s	291.53	284.81	280.73	16214.32	2.42	48004.47	65.56
N1s	403.83	399.01	395.43	7894.27	1.76	16341.31	13.69
O1s	536.18	530.79	527.78	16476.9	2.08	38659.35	19.58
Mn2p	661.08	640.14	635.43	1705.92	2.97	9514.99	1.17

Table S2 The details on the measurement and quantum yield value of Mn-CDs obtained from application of equation (1).

	Absorbance	PL intensity	Integrated PL intensity	QY
Quinine sulfate	0.149	687.62	62115.51	54%
Mn-CDs	0.082	602.84	57475.09	90.79%

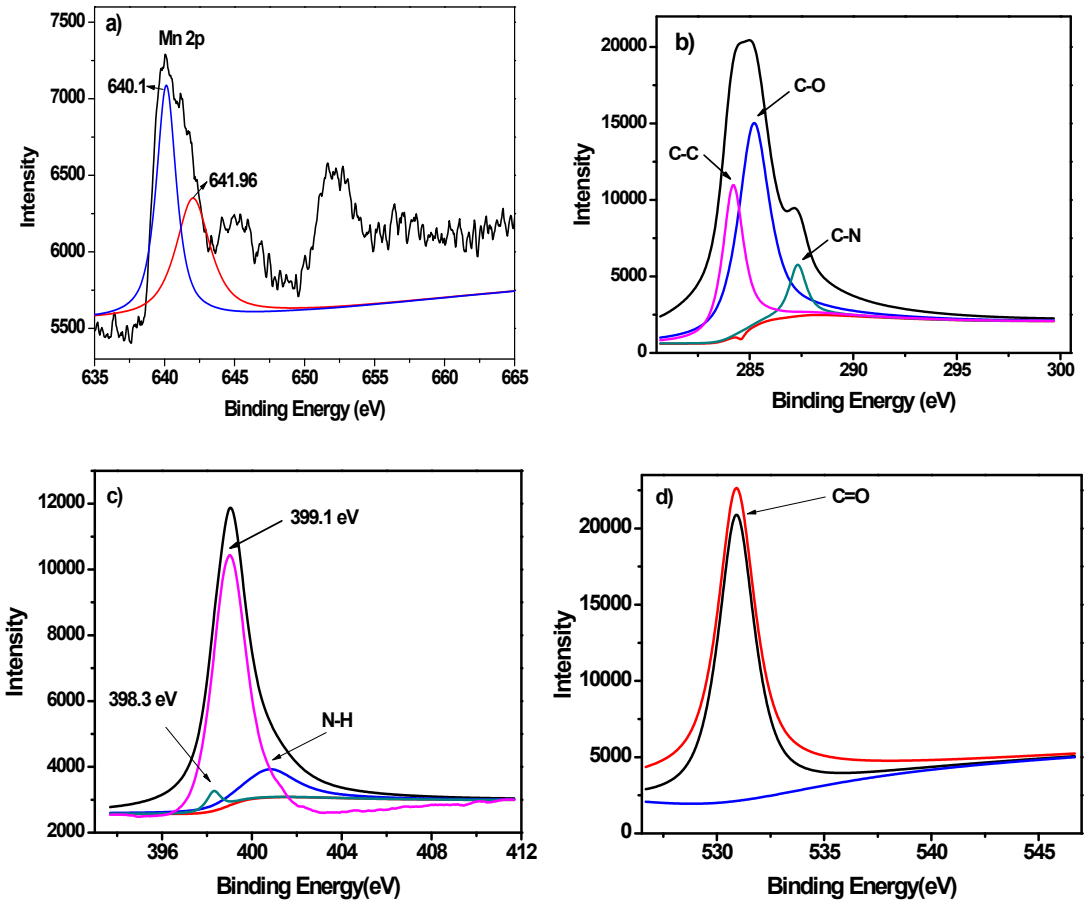

Fig. S2 XPS spectra of the Mn-CDs. High-resolution Mn2p a), C1s b), N1s c), O1s d) peaks for the Mn-CDs. The peak observed at a binding energy of 284.6 eV in the high-resolution C1s spectrum is mainly attributed to graphitic (C-C/C=C), and the peak at 285.2 eV and 287.3 eV suggest the presence of C-O and C-N. The high-resolution N1s spectrum reveals the presence of pyridinic groups (398.3 and 399.1 eV), and the peak at 400.9 eV is associated with N-H groups. The high-resolution O1s spectrum reveals the presence of C=O-containing groups (531.1eV).

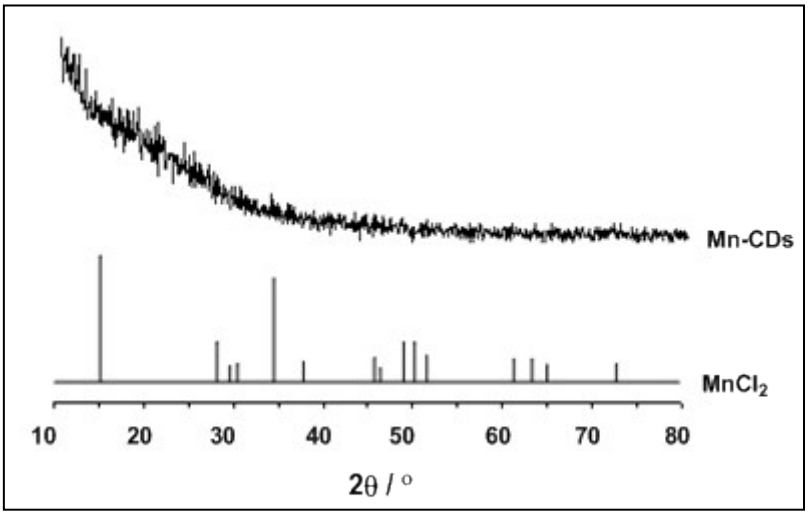

Fig. S3 The XRD pattern of Mn-CDs and $MnCl_2$ (JCPDS Card No. 07-0230).

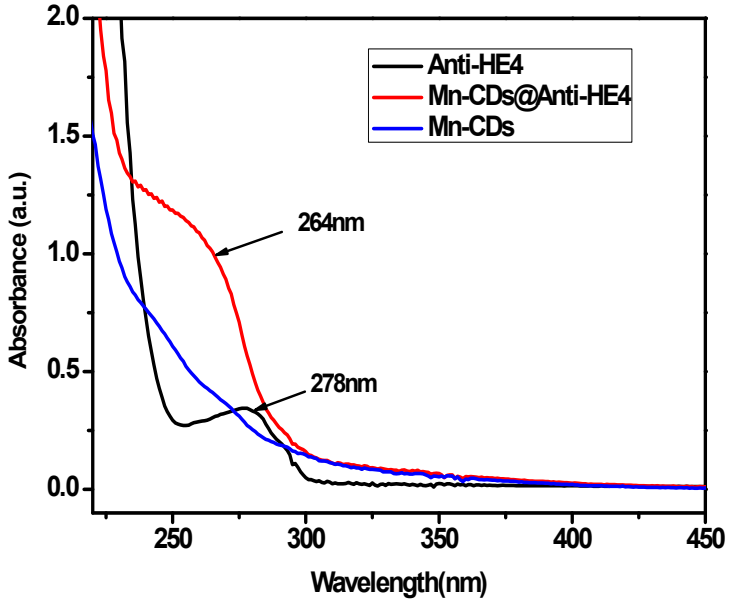

Fig. S4 UV-vis absorption spectra of Mn-CD@Anti-HE4 (red line), Anti-HE4-mAb (black line) and Mn-CDs (blue line).

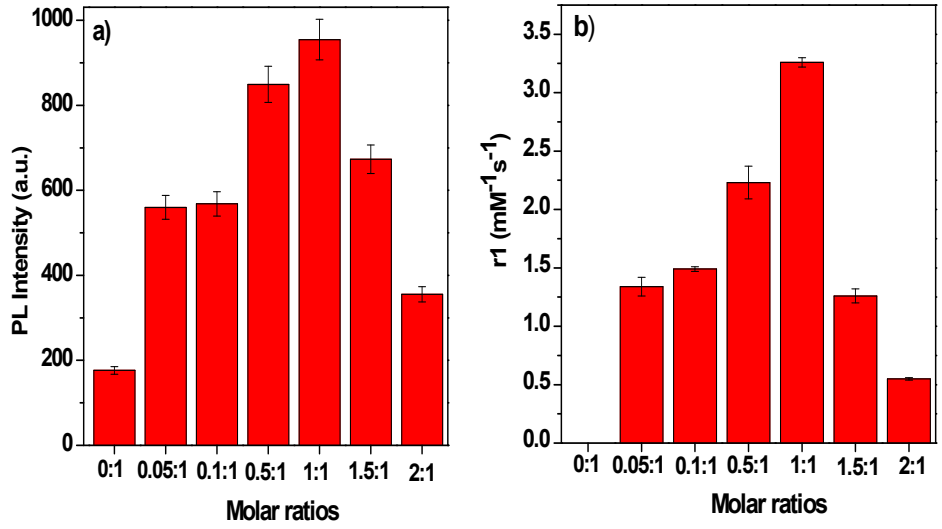

Fig. S5 The effect of different molar dosage of $MnCl_2 \cdot 4H_2O$ on a) the fluorescent capability and b)MRI relaxation properties of Mn-CDs.

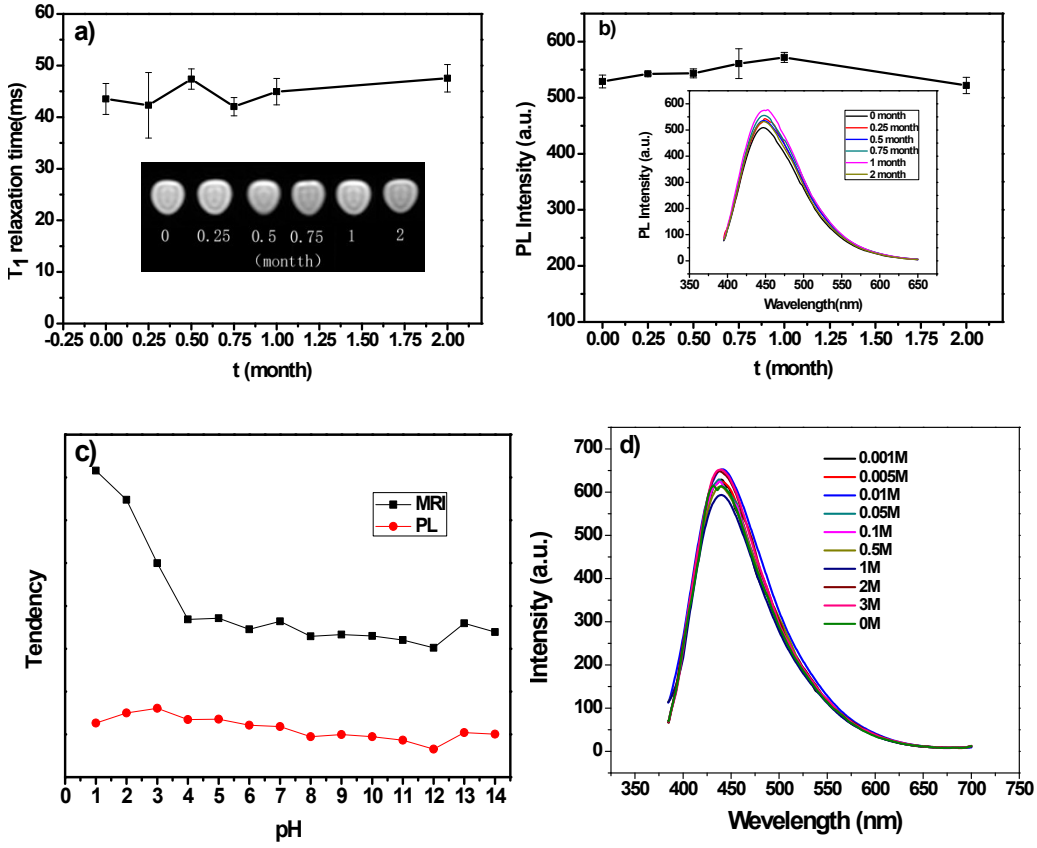

Fig. S6 The stability of Mn-CDs. Effect of storage time on a) fluorescence intensity and b) T_1 relaxation time of Mn-CDs. c) Effect of pH on fluorescence intensity and T_1

relaxation time of Mn-CDs. d) Effect of ionic strengths on the fluorescence intensity of Mn-CDs.

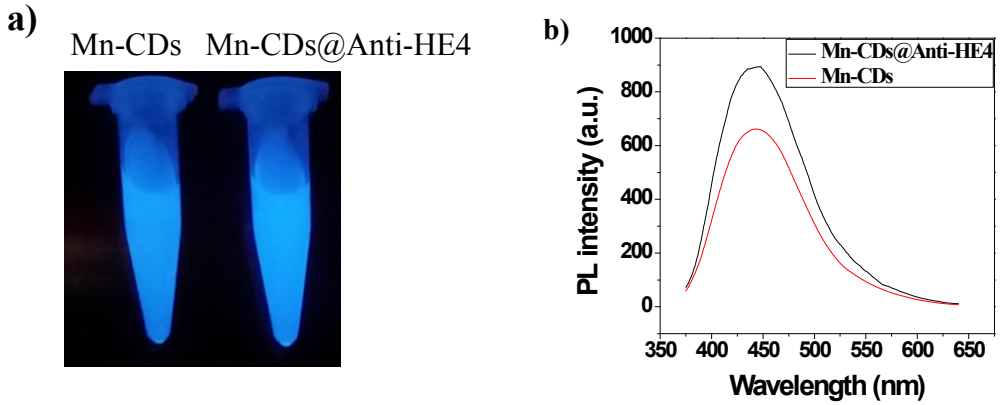

Fig. S7 a) Photographs of Mn-CDs in aqueous solution under UV light (Ex; 360 nm). b) PL spectra of Mn-CDs and Mn-CDs@Anti-HE4.

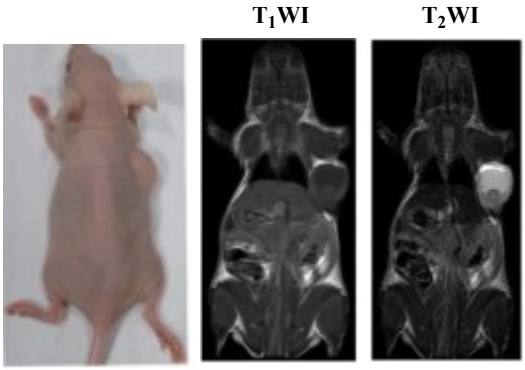

Fig. S8 T_1WI and T_2WI of HO-8910 tumor-bearing nude mice. The tumor bore at right chest wall near frontal leg was a mixed mass with cystic and solid content, which was heterogeneously hypointense on T_1WI and hyperintense on T_2WI.

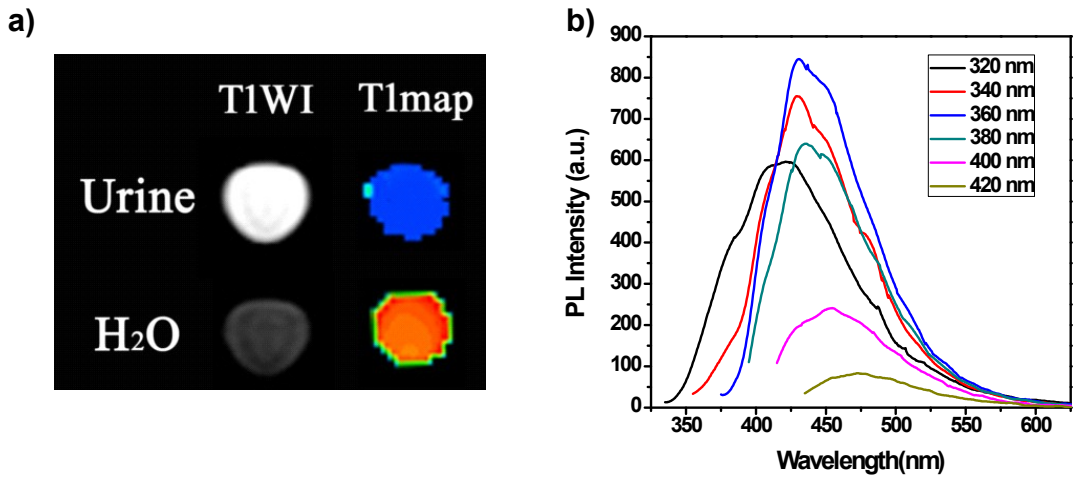

Fig. S9 a) T1-weighted images and b) PL analysis on urine samples, taken ~1 h after the injection of Mn-CDs.