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Supporting Information

"Green" Functionalization of Magnetic Nanoparticles via Tea Polyphenol for Magnetic Resonance/Fluorescent Dual-imaging

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Contents

1 Experimental Section

1.1 Materials and Methods

Synthesis of metal-doped SPIONs

Spinel MFe_2O_4 (M=Fe, Mn, or Zn) nanocrystals were prepared by thermal decomposition method with some improvement.[1] In brief, Iron (Ⅲ) acetylacetonate ($Fe(acac)_3$, 2.0 mmol), 1,2-hexadecanediol (10.0 mmol), oleylamine (6.0 mmol) and oleic acid (6.0 mmol) were dissolved in 20 mL benzyl ether. The solution was heated to 200 °C for 2 h, refluxed at 300 °C for another 1 h, and finally cooled to room temperature. For Mn doped superparamagnetic iron oxide nanoparticles (SPIONs), $Mn(acac)_2$ were mixed with $Fe(acac)_3$ in the molar ratio of $n_{Fe}/n_{Mn}=2/1$. For Mn/Zn doped SPIONs, $Mn(acac)_2$, $Zn(acac)_2$ and $Fe(acac)_3$ were mixed in the molar ratio of $n_{Fe}/n_{Mn}/n_{Zn}=2/0.7/0.3$. Both mixture reacted with the similar amount of reducing agent (1,2-hexadecanediol) and surfactants (oleylamine and oleic acid) as mentioned above. However, the mixture were heated to 200 °C for 1 h and 250 °C for another 1 h in order to achieve sufficient doping of Mn and Zn in iron oxide. At last, the mixture was refluxed at 300 °C for another 1 h. The obtained black particles were precipitated via appropriate ethanol and collected by centrifugation at 8000 rpm for 10 min. Particles were reprecipitated and washed with ethanol for three times, and were finally redispersed in *n*-hexane and stored at 4 °C. The products were abbreviated by OA-IO, OA-MnIO and OA-ZnIO for oleic acid-coated SPIO, Mn doped SPIO, Mn/Zn doped SPIO, separately. In addition, unmodified hydrophilic SPIONs were synthesized followed Massart method by chemical co-precipitation,[2, 3] in order to make a comparison with TP modified SPIONs in cellular uptake.

Surface functionalization of metal-doped SPIONs by ligand-exchange method

This process was similar with our previous report related to functionalize SPIONs with peptide dendrimer as shown in **Fig. S1**.[4] For oleic acid-coated SPIONs (OA-IO), different amount of TP was dissolved in the mixture solvent of hexane/chloroform/methanol (1/1/2 v/v/v) with the mass ratio of m_{TP}: m_{NPs}=1:9, 1:3, 1:1, 3:1, separately, to give sample 0.1TP-IO, 0.3TP-IO, TP-IO and 3TP-IO. For Mn, Mn/Zn doped SPIONs (OA-MnIO and OA-ZnIO), the addition quantity of TP retained as m_{TP}: m_{NPs}=1:1, the obtained products were abbreviated as TP-MnIO and TP-ZnIO. Each mixture was shaken overnight to facilitate ligand-exchange and the solvent was removed under reduced pressure. The particles were precipitated in *n*-hexane and collected by centrifugation at 3500 rpm. After washing with *n*-hexane and magnetic separation for three times, the products were re-dispersed in distilled water. The extra solvents

and *non*-chelated TP were removed by dialysis using a dialysis bag (M_W=8000~14000) for 24 h in Milli-Q water.

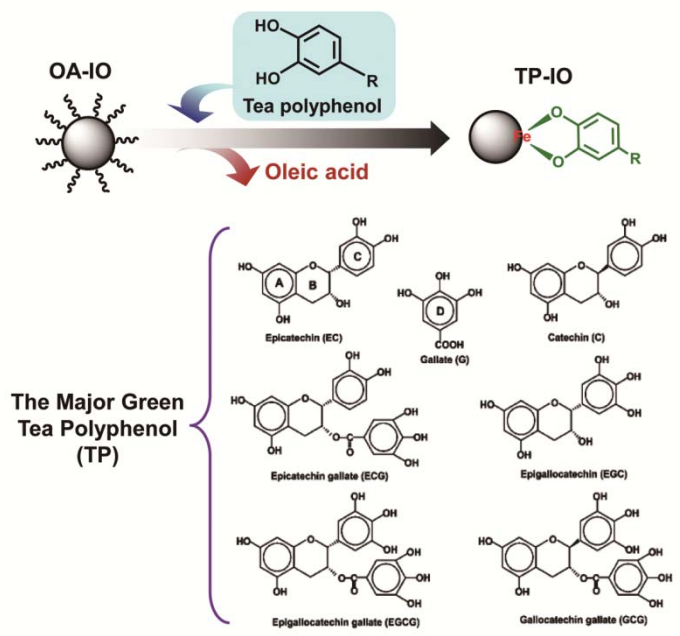

Fig. S1. Ligand-exchange process of metal-doped SPIONs by TP.

Cell Culture

Human hepatocellular liver carcinoma cell line (HepG2), Human cervical adenocarcinoma cell line (Hela), human breast adenocarcinoma cell line (MCF-7) and human embryo liver cell line (L02) were purchased from ATCC (American Type Culture Collection). All the cells were cultured in DMEM (Invitrogen, Germany) culture medium supplemented with 10% FBS, 100 units penicillin and 100 g/mL streptomycin in a humidified incubator (37 °C, 5% CO_2). Cells suspensions were seeded at a density of 30,000 cells/cm^2 in each well and allowed a sufficient amount of time to adhere to cell culture plates before protein adsorption, cell viability evaluation and cells labeling analyzed by magnetic resonance imaging (MRI) and confocal laser scanning microscopy (CLSM).

Protein corona on the surface of TP-IO

The protein adsorption and separation procedure were modified according to the method reported by Lundqvist.[5] In brief, the culture medium in 24 well plates with HepG2 or L02 cells attaching were removed. TP-IO were diluted by DMEM (10% FBS) into 0.08 mg(Fe)/mL and added into each well for incubating 1, 4 and 24 h, respectively. The TP-IO suspension in DMEM (10% FBS) without cells was used as control. All the samples were cultured in a humidified incubator (37 °C, 5% CO_2). After specific

time, the suspension were pipetted out off the culture plates, centrifuged at 13,000 rcf for 15 min to pellet the SPIO-protein complexes, which was washed by PBS (500 μL/time) and separated by magnetic field for three times to remove unbound proteins. The complexes were suspended in 2% SDS in PBS buffer at a final concentration of 0.08 mg(Fe)/mL for 2 h to elucidate the adsorbed proteins for sodium dodecyl sulfate polyacrylamide (SDS/PAGE) analysis.

1.2 Characterization of the products and cell experiments

Characterization of metal-doped SPIONs

The magnetization of SPIONs was analyzed by vibrating sample magnetometer (VSM, Model BHV-525, Riken Japanese Electronics Company) with field up from 0 to 15,000 Oe at 300 K. Fourier transform infrared (FTIR, PE spectrometer) spectra were measured with wave number range 4000~400 cm^{-1} and step length 4 cm^{-1}. Thermo gravimetric analysis (TGA) was performed using simultaneous thermal analysis (STA 449 C Jupiter, NETZSCH) by heating each sample in an alumina crucible in presence of inert N_2 atmosphere at a heating rate of 10 °C/min from 35 to 1100 °C. The crystal structure of SPIONs was studied by powder X-ray diffraction (XRD, X' Pert Pro MPD, Philips, Netherlands) using Cu Kα (40 KV) radiation with angle range from 25° to 90° at 0.1°/sec. The concentrations of Fe, Mn and Zn in different metal-doped SPIONs and the iron content in the solvent were analyzed by atomic absorption spectrometry (AAS, Aanalyst 800, Perkin Elmer). Hydrodynamic diameter and size distribution of SPIONs were analyzed by dynamic light scattering (DLS, Malvern Nano-ZS, l = 632.8 nm) with samples dispersed in Milli-Q water, hexane or ethanol and tested at 25 °C. Simultaneously, the surface charge alteration of particles was probed by zeta potential (Malvern Nano-ZS). Each measurement was performed for at least five times. The size and morphology of SPIONs were determined by transmission electron microscopy (TEM, Japan electronic) with samples prepared by evaporating dilute suspensions on carbon-coated copper grids in air at room temperature.

Protein corona analysis by 1D SDS/PAGE

Hard protein corona on the surface of TP-IO was studied by SDS/PAGE gel electrophoresis (4% stacking gel, 10% resolving gel). The SPIO-protein complexes in 2% SDS were 5-fold diluted with Sample Loading Buffer (5×) (0.5M DTT, 10% SDS, 250 mM Tris/HCl pH 6.8 and 0.02% bromophenol blue in 30% Glycerol) and heated to 100 °C for 5 min. The mixture was centrifuged at 13,000 rcf for 10 min and the supernatant was loaded into the stacking gel with 10 μL per lane. Gels were run at 120 mV for ~80 minutes until bromophenol blue penetrating the gel. The samples were stained by Coomassie Brilliant

Blue and destained in acetic acid solution diluted by Milli-Q water. The gel images were acquired using BIO-RAD (Universal Hood Ⅱ). Each sample was prepared repeatedly for three times and separated by independent gels. The semiquantitative results for protein composition and content could be obtained by the software (Image Lab, version 2.0.1) according to the bend intensity and area in different gels. The error bars are expressed as the SD of the values obtained from three independent measurements and results were presented as mean±SD.

Cell viability

Viability of HepG2 and L02 cells labeled with TP-IO for 24 and 48 h was evaluated by CCK-8 assay in 96 well plates. Both attached cells were treated with TP-IO at the concentration ranged from 0.005 to 0.32 mg(Fe)/mL in DMEM (10% FBS) at 37 °C in 5% CO_2 atmosphere. The labeled cells were washed with PBS to remove unbound particles and cellular debris, while the cells without uptake treatment were used as control. 0.1 mL Cell Counting Kit-8 solution (CCK-8, Dojindo) (1 mg/mL in cell complete medium) was added to each well and incubated with cells at 37 °C for 4 h. Optical density at 470 nm was measured by microplate reader (BIO-RAD 550). The spectrophotometer was calibrated to zero absorbance, using CCK-8 solution without cells. The relative cell viability (%) was calculated as follows: % Cell Viability = ($OD_{470\,nm}$ of test sample)/($OD_{470\,nm}$ of control) ×100%.

Cells labeling and cellular uptake of TP-IO (CLSM and iron content quantification)

TP-IO was diluted by the cell culture media to achieve TP-IO dispersion at final iron concentrations of 0.02 and 0.08 mg(Fe)/mL. To evaluate the potential of TP-IO in fluorescent imaging, CLSM was introduced to observe the cellular uptake process of TP-IO in L02, HepG2 and Hela cells in the early time from 1 h to 24 h. All cell lines were seeded into 35 mm glass bottom cell culture dishes at a density of 5000 cells per well. After cell attachment for 24 h, the medium for each sample was changed into 250 μL freshly mixed TP-IO dispersion (0.08 mg(Fe)/mL) for cells labeling and internalization. Then all the samples were washed by PBS to remove free TP-IO and stained by Hoechst 33258 (200 μL, Dojindo) at 37 °C and 5% CO_2 for cell nuclei observation. Green florescent property of TP facilitated successful cell labeling and imaging by CLSM (Leica TSC SP5), which were excited at the wavelength of 488 nm.

In addition, the attached cells, HepG2 and Hela, were incubated separately with different concentrations of TP-IO dispersion (0.02 and 0.08 mg(Fe)/mL) in 96-well cell culture plate for 24 and 48 h. Cellular uptake quantification of iron content in both cells were analyzed by ferrozine assay.[6] Briefly, both labeled and unlabeled cells were mixed with iron-releasing reagent (a freshly mixed solution of equal

volume of 1.4 M HCl and 4.5% (w/v) $KMnO_4$ in H_2O) and incubated for 2 h at 60 °C. After the mixture had cooled to room temperature, the iron-detection reagent (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 M ammonium acetate, and 1 M ascorbic acid dissolved in water) was added to each sample. 30 min later, the absorbance of the sample solution was measured at 550 nm using a microplate reader (Model 680, Bio-RAD). A standard curve for the iron was made under identical conditions using known amount of $FeCl_3 \cdot 6H_2O$ solution. The iron content of the sample was calculated by comparing its absorbance to that of a range of standard concentrations in equal volume. All experiments were carried out in triplicate with the results expressed as mean standard deviation.

Magnetic resonance imaging

To validate the potential of TP-IO as a MRI contrast agent, we acquired the T_2-weighted MR images and measured the T_2 relaxation rates as a function of iron concentration and number of labeled cells using a 7 T MRI scanner (Bruker Biospec 70/30 USR). T_2 relaxation rates of materials, TP-IO and 3TP-IO, were determined with particles suspended in water at different concentration (0~0.4 mM(Fe)). For *in vitro* iron oxide enhanced-MRI, L02, HepG2, Hela and MCF-7 cells were incubated with TP-IO for 24 h at 37 °C and 5% CO_2, washed twice by PBS and subsequently trypsinized using trypsin/EDTA (0.25%/0.5%). The obtained different cell suspensions in fresh culture medium were centrifuged at 1200 rpm for 5 min. The cell pellets were re-suspended in PBS for washing and fixed by 4 % paraformaldehyde in PBS for 30 min before counting the total cell numbers in the suspension. Finally, the cell pellets were dissolved in glutaraldehyde in PBS (1/300 v/v) to have 4×10^5 cells in 0.5 mL as the initial cell concentration, which was diluted to obtain a reduced cell number from 4×10^5 to 0.1×10^4 in 100 μL glutaraldehyde solution. All the suspensions were redispersed in 100 μL 1% agarosein Eppendorf tubes. In addition, unlabeled cells and distilled water in 1% agarose in the same volume were used as comparisons. All the samples were stored at 4 °C before analysis. T_2-weighted spin-echo scans were used with the following parameters: TR = 5000 ms, TE from 11 to 704 ms, number of averages = 1, FOV = 140×140 mm, slice thickness = 2.0 mm. Signal intensities of different TE time were used to calculate T_2 value of each sample.

2 Physicochemical properties of metal-doped SPIONs

Table S1. Physicochemical properties of metal-doped SPIONs before and after ligand-exchange by TP.

Referred to as	Magnetic nanoparticles	Ratio of m_{TP}: m_{NPs}	Size (nm) by DLS			TGA (w%)	M_s(emu/g)	
			Hexane /ethanol	water	PDI		VSM	TGA
OA-IO	Fe_3O_4	-	9.46	-	-	34.15	48.0	-

0.1TP -IO	Fe_3O_4	1:9	-	-	-	35.21	44.7	47.2
0.3TP-IO	Fe_3O_4	1:3	9.80	32.55	0.170	48.58	32.4	37.4
TP-IO	Fe_3O_4	1:1	10.55	19.56	0.169	45.64	33.4	39.6
3TP-IO	Fe_3O_4	3:1	10.18	41.20	0.137	46.84	31.1	38.7
OA-MnIO	$Fe_{2.42}Mn_{0.58}O_4$	-	10.62	-	-	32.08	55.0	-
TP- MnIO	$Fe_{2.42}Mn_{0.58}O_4$	1:1	-	17.13	0.156	43.68	48.1	47.0
OA-ZnIO	$Fe_{2.42}Mn_{0.35}Zn_{0.23}O_4$	-	10.05	-	-	34.21	55.0	-
TP- ZnIO	$Fe_{2.42}Mn_{0.35}Zn_{0.23}O_4$	1:1	-	20.90	0.233	46.65	33.4	40.5

In **Fig. S2**, hydrophobic SPIONs (OA-IO) synthesized by thermal decomposition method were only dispersed in *non*-polar solvent, such as hexane. After the ligand-exchange by different content of TP, for m_{TP}:m_{NPs}=1:9, the obtained particles (0.1TP-IO) could not realize adequate phase transfer. Some particles floated in the interface between hexane and water, while others deposited on the bottom of the bottle. For the increased amount of TP at ratio of m_{TP}:m_{NPs}≥1:3 (w/w), all the modified particles (0.3TP-IO, TP-IO and 3TP-IO) were dispersed excellently in water with similar black color.

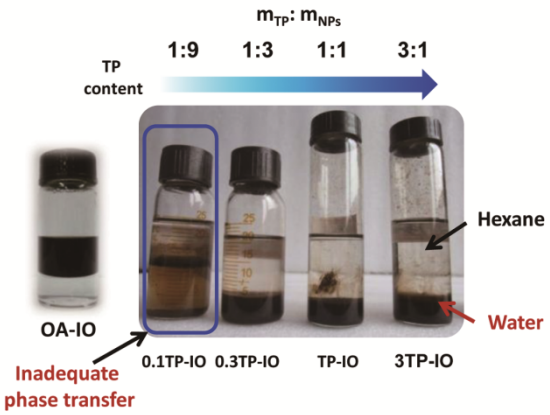

Fig. S2. Stability and dispersion of SPIONs in hexane or water before and after ligand-exchange by different amount of TP.

Table S2. TGA analysis of metal-doped SPIONs in different decomposition steps.

Referred to as	Magnetic nanoparticles	Ratio of m_{TP}: m_{NPs}	TGA (w%)		
			Total	Step 1	Step 2
OA-IO	Fe_3O_4	-	34.15	18.08	16.07
0.1TP -IO	Fe_3O_4	1:9	35.21	16.06	19.15
0.3TP-IO	Fe_3O_4	1:3	48.58	24.90	23.68

TP-IO	Fe_3O_4	1:1	45.64	22.10	23.54
3TP-IO	Fe_3O_4	3:1	46.84	22.72	24.12
OA-MnIO	$Fe_{2.42}Mn_{0.58}O_4$	-	32.08	17.41	14.67
TP- MnIO	$Fe_{2.42}Mn_{0.58}O_4$	1:1	43.68	20.22	23.46
OA-ZnIO	$Fe_{2.42}Mn_{0.35}Zn_{0.23}O_4$	-	34.21	18.68	15.53
TP- ZnIO	$Fe_{2.42}Mn_{0.35}Zn_{0.23}O_4$	1:1	46.65	25.91	20.74
Pure TP	-	-	62.49	42.77	19.72

In **Fig. S3**, XRD patterns of TP modified SPIONs revealed that the characteristic diffraction peaks of all the samples corresponded to cubic spinel-type of magnetite crystals (JDPCS card 19-0629), which indicated that different contents of TP on the surface of SPIONs did not affect their crystallinity.

For size distribution analysis in **Fig. S4**, hydrophilic TP ligands on the surface of SPIO facilitated particles with excellent dispersibility in both ethanol and water. 0.3TP-IO, TP-IO and 3TP-IO showed the similar particle size as OA-IO did in ethanol, which were close to 10 nm. However, the hydrodynamic diameters of TP modified particles in water were significantly larger than that in ethanol. This could be attributed to different hydration properties of phenolic hydroxyl groups on the surface of the particles between ethanol and water. The hydrodynamic diameter was increased to 32.55 and 41.20 nm for 0.3TP-IO and 3TP-IO, separately, while TP-IO processed the smallest hydrodynamic diameter of about 19 nm in water as shown in **Table S1**.

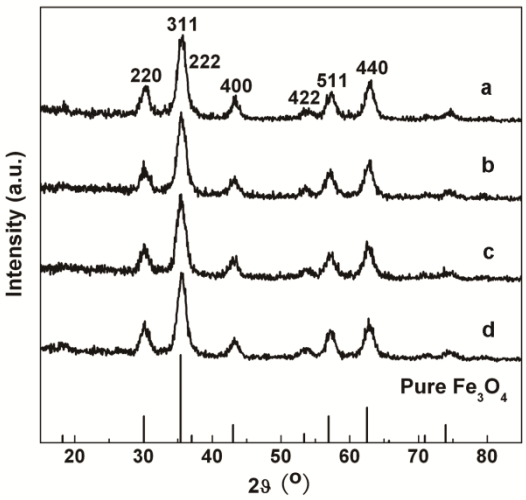

Fig. S3. XRD patterns of SPIONs under different reaction amount of TP: (a) OA-IO, (b) 0.3TP-IO, (c) TP-IO and (d) 3TP-IO. (JDPCS card 19-0629)

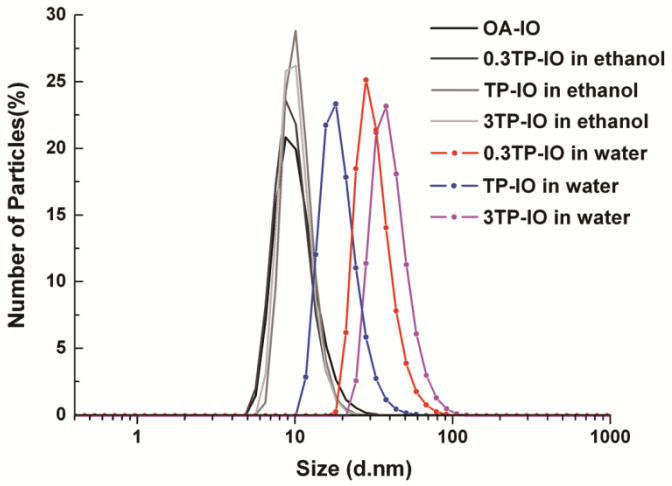

Fig. S4. Hydrodynamic diameters of metal-doped SPIONs in hexane, ethanol or water, before and after surface ligand-exchange by different contents of TP.

3 Protein corona analysis

Table S3. Quantities of distinguishable proteins in the hard corona of TP-IO in various systems with increasing the incubation time.

Incubation time (h)	DMEM (10% FBS)	HepG2 in DMEM (10% FBS)	L02 in DMEM (10% FBS)
1	23	19	19
4	19	20	20
24	18	17	18

In **Table S3**, the quantities of distinguishable proteins were acquired by Image Lab (version 2.0.1) according to the number of countable bends in SDS/PAGE gel (Fig. 3a in the manuscript). The number of countable proteins slightly decreased with the incubation time increasing in DMEM (10% FBS), while for the particles in the presence of cells, the number of countable proteins was smaller than that in DMEM (10% FBS), which was merely 19 at 1 h. For a longer incubation time up to 24 h, the number of proteins were all decreased and kept similar among different medium, indicating the excellent protein adsorption resistance of TP layer on the surface of particles.

4 Cell labeling and cellular uptake of TP modified SPIONs

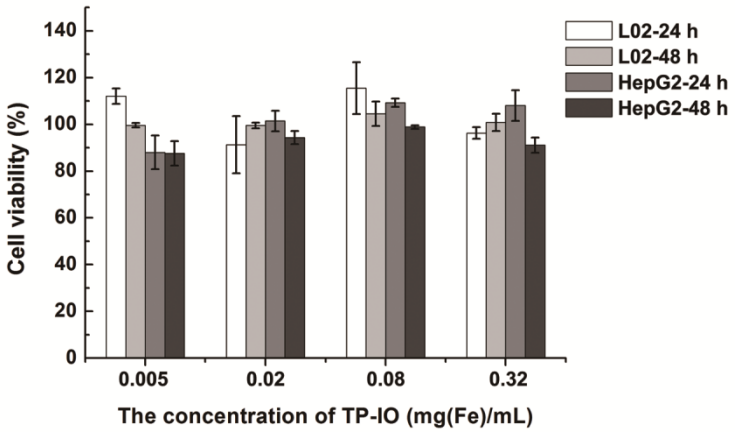

Fig. S5. Viability of L02 and HepG2 cells labeled with TP-IO of iron concentration at 0.005, 0.02, 0.08 and 0.32 mg(Fe)/mL.

In **Fig. S5**, after incubated with TP-IO for 24 and 48 h, both normal and cancer cells (L02 and HepG2) showed high viability larger than 90%, indicating the excellent biocompatibility and low cytotoxicity of TP modified particles for the concentrations lower than 0.32 mg(Fe)/mL. From CLSM images in **Fig. S6**, we can observe that the cell membrane and cytoplasm emitted green light due to autofluorescence of TP, suggesting that TP was successfully chelated onto the surface of SPIONs. The particles exhibited distinct distributions among different cells. TP-IO mainly existed in the cytoplasm of L02 cells in small aggregates, but dispersed homogenously in the cell cytoplasm of Hela cells. For HepG2 cells, particles tended to accumulate on the cell membrane at first and dispersed in cytoplasm later. Additionally, the cellular uptake was time-dependent due to the stronger intensity of green fluorescent for a longer incubation time up to 4 h, which was more obvious in HepG2 and Hela cells.

In **Fig. S7**, the cellular uptake quantification of TP-IO in cancer cells (HepG2 and Hela) was compared with the unmodified SPIONs in the same diameter. Hydrophilic SPIONs with hydroxyl groups on the surface were synthesized by co-precipitation method.[2, 3] TP-IO always showed a higher cell labeling efficiency among different incubation time (0~24 h), because the cellular uptake of TP-IO by both cancer cells was significantly larger than naked SPIONs. Moreover, Hela cells showed the largest cellular uptake of TP-IO up to 48 pg(Fe)/cell at 4 h. The sharply decreased iron content of cellular uptake at 24 h was mainly ascribed to the fast cell division of cancer cells.

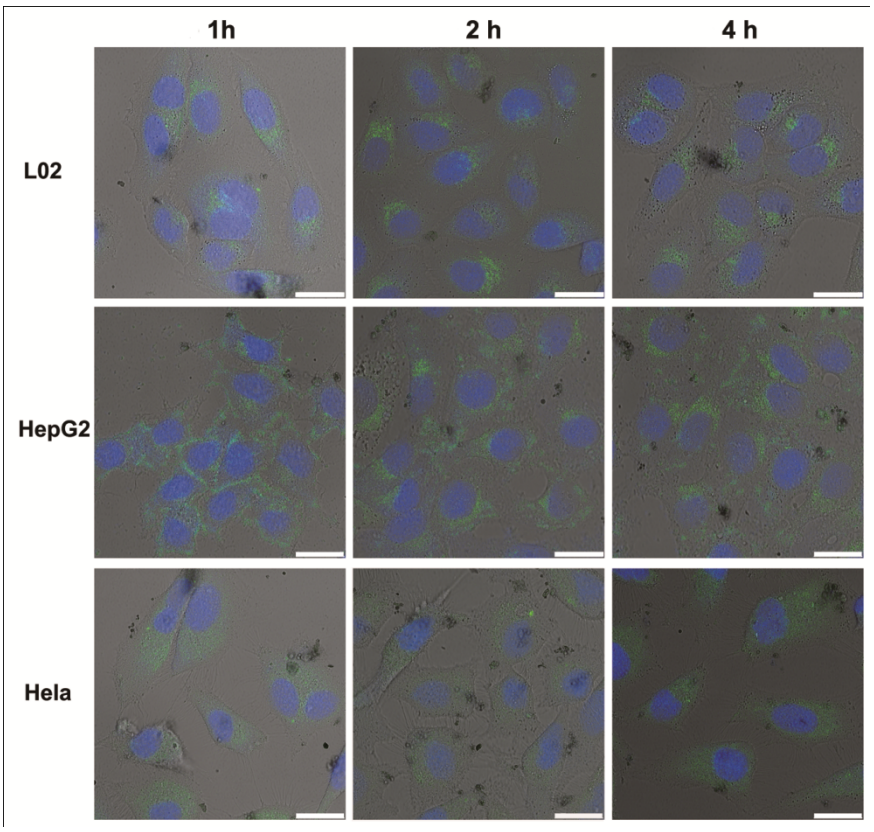

Fig. S6. CLSM images of L02, HepG2 and Hela cells incubated with 0.08 mg(Fe)/mL TP-IO for 1, 2 and 4 h, separately. Images from left to right: overlaid micrograph of the bright field image of the cells, fluorescent image of the cell nuclei stained with Hoechst 33258 (blue channel) and TP-IO (green channel). (Scale bar: 25 μm)

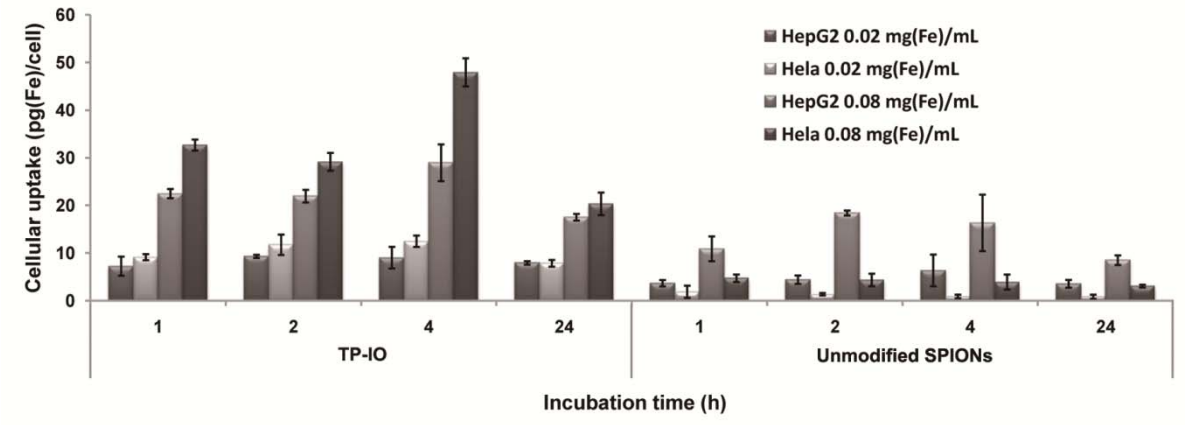

Fig. S7. Cellular uptake quantification of TP-IO and unmodified SPIONs by HepG2 and Hela cell lines at the concentration of 0.02 and 0.08 mg(Fe)/mL for 1, 2, 4 and 24 h, respectively.

5 Reference

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