

Cross-linked magnetic nanoparticles from poly(ethylene glycol) and dodecyl grafted poly(succinimide) as magnetic resonance probes

Hee-Man Yang,^a Chan Woo Park,^a Sujin Lim,^b Sung-Il Park,^a Bong Hyun Chung,^b and Jong-Duk Kim^{*a}

^a Department of Chemical and Biomolecular Engineering (BK21 Graduate Program), Korea Advanced Institute of Science and Technology, Daejeon 305-701, Republic of Korea.

^b BioNanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), P. O. Box 115, Yuseong, Daejeon 305-701, Republic of Korea.

* Corresponding author. Tel.: +82-42-350-3961. Fax: +82-42-350-3910. E-mail: jdkim@kaist.ac.kr.

Experiment

Materials

Iron (III) acetylacetonate, benzyl ether, oleic acid (90%), oleylamine (>70%), 1,2-hexadecanediol (90%), L-aspartic acid, mesitylene, sulfolane, dodecylamine, hexamethylenediamine, hexylamine, Nile red, and tetrahydrofuran were purchased from Sigma-Aldrich and were used as received. Poly(ethylene glycol)-amine ($M_w = 2$ KDa) was purchased from Sunbio (Korea). Phosphoric acid, dimethyl sulfoxide, and N,N-dimethylformamide were purchased from Junsei. Dimethyl sulfoxide- d_6 (DMSO- d_6) used in NMR experiments were Sigma-Aldrich products.

Synthesis of poly(ethylene glycol) and dodecyl grafted poly(succinimide) (PSI-g-PEG-C₁₂)

The precursor polymer, poly(succinimide)(PSI), was synthesized via acid-catalyzed polycondensation of L-aspartic acid using phosphoric acid as the catalyst. Purified PSI (0.97 g, 10 mmol succinimide unit) was dissolved in water free DMF (7ml), followed by aminolysis with 10 mole % of dedecylamine (0.18535 g) at 70 °C for 24 h and aminolysis with 5 mole % of poly(ethylene glycol) at 70 °C for 24 h. The reaction mixture was precipitated twice cold ether and dried in vacuo at 50 °C. The product was dissolved in DMSO, extensively dialyzed against DDI water, and freeze-dried. PSI was aminolyzed with excess 2-aminoethanol for conversion to PHEA to determine the molecular weight (Mn) of PSI calculated from Mn of PHEA by gel permeation chromatography (GPC). The obtained molecular weight (Mn) of PHEA was 19 800 Da (polydispersity index = 1.32).^{10a} Thus, the calculated Mn of PSI was 12 156 Da.

Synthesis of as-synthesized iron oxide nanoparticles

Fe₃O₄ nanocrystals were produced by a seed-mediated growth method using 6 nm sized nanocrystals synthesized using the thermal decomposition method.¹² Briefly, for the synthesis of 6 nm Fe₃O₄, iron (III) acetylacetonate (0.706 g, 2 mmol), 1,2-hexadecanediol (2.5845 g, 10 mmol), oleic acid (1.902 mL, 6 mmol), oleylamine (1.974 mL, 6 mmol), and benzyl ether (20 mL) were mixed under a nitrogen atmosphere. The mixture was heated to 200 °C for 2 h and further heated to 300 °C for 1 h under reflux. After being cooled to room temperature, excess ethanol was used to wash the reactant. Nanocrystals were collected by centrifugation.

Preparation of cross-linked magnetic nanoparticles (CL-MNPs)

20 mg of magnetic nanocrystals and 20 mg of PSI-g-PEG-C₁₂ with or without 1 mg of Nile red were dissolved in 2.5 mL of a mixed solvent (THF/DMF = 4:1, v/v). The solution was constantly stirred for 30 min, and the mixture subsequently added dropwise to 15 mL DDI water. After 3 h of stirring, the mixture was dialyzed against DDI water to remove mixed organic solvent (THF and DMF), and centrifuged (6000 rpm, 10 min) to remove the aggregated nanoparticles. PSI-g-PEG-C₁₂ coated nanoparticles were selectively collected by centrifugation at 15000 rpm. Next, an appropriate amount of HMD was added dropwise to the PSI-g-PEG-C₁₂ coated nanoparticles solution. The mixture was stirred for 12 h to induce the cross-linking reaction. Then, excess hexylamine was added to complete the aminolysis of the unreacted succinimide units in PSI. After further stirring for 12 h, cross-linked nanoparticles solution was dialysed against DDI water and then freeze-dried.

Characterization of cross-linked magnetic nanoparticles

X-ray diffraction was performed to explore crystal structure of magnetic nanoparticles using a Rigaku D/max-RB apparatus and a Cu K α source ($\lambda = 0.154$ nm). Transmission electron microscopy (TEM) was performed using a Philips CM-200 instrument operating at 200 kV. For TEM imaging, cross-linked magnetic nanoparticle solution containing 0.1% (w/v) phosphotungstic acid (PTA; a negative stain) was placed on a copper grid covered with a formvar carbon membrane. The grid was exposed to air, to evaporate solvent. Hydrodynamic diameter of nanoparticles were measured by dynamic light scattering employing the particle size option in the Malvern instrument (Zetasizer nano zs). Fourier transform infrared (FTIR) spectra were recorded using a Bruker IFS66V/S & HYPERION Model 3000 instrument at room temperature. Spectra were recorded in the range 4000-650 cm⁻¹ in the attenuated total reflection (ATR) objective with ZnSe crystal. The saturation of magnetization was evaluated using a vibrating-

sample magnetometer (Lakeshore, model 955287(A)). Thermogravimetric analysis (TGA) was carried out on a setsys 16/18 (Setaram, France). The atomic weight percentage of Fe or Mn in each nanoparticles was measured by inductively-coupled plasma atomic emission spectrometer (ICP-AES, model : Jarrell Ash IRIS-AP, Thermo) for further in vitro study.

Release of hydrophobic Nile red from CL-MNPs

The amount of loaded Nile red was determined by UV-vis spectroscopy (Jasco V-530, Japan) at 550 nm after dissolving Nile red loaded CL-MNPs in DMSO. For a release experiment, a Nile Red loaded CL-MNPs solution (4 mg in 1 mL of PBS) was sealed in a dialysis membrane (MWCO = 6~8 kDa), and incubated in PBS buffer (pH 7.4) To monitor the release of Nile red, absorbance (at 575nm) of the Nile red loaded CL-MNPs solution was measured at appropriate time intervals.

Cell viability: MTT assay

5×10^3 HeLa cells were seeded on each well of a 96-well plated and cultured for 24 h at 37°C. Various concentrations of cross-linked Fe₃O₄ nanoparticles were then treated to the cells. After 24h incubation, the medium containing nanoparticles were exchanged with a fresh medium, and 10 µL of MTT reagent (5 mg/mL of stock solution) were added to each well. After culturing for 2 h, The 100 µL of DMSO were added to each well at room temperature. The absorbance of sample was measured by using a microplate reader (Bio-rad model 680) at 570 nm. The viability of cells was determined as the percentage of viable cells of the untreated control and analyzed in triplicate.

Confocal microscopy

2×10^3 cells were seeded on each well of a u-slide 8-well microscopy chamber slides (ibidi Integrated Biodiagnostics, Munich, Germany) and incubated at 37 °C for 12 hr. The cell medium was replaced with appropriate amount of cross-linked Fe₃O₄ nanoparticles solution and discarded after 24 h incubation at 37 °C. The cells were

washed twice with PBS and submerged in a fixation and permeabilization solution (BD biosciences). After storage for 20 min, the cells were washed twice with PBS and treated with Hoechst 33342 to stain the nuclei of the cells for 20 min. The cells were washed twice with PBS and visualized by a Deltavision RT (Applied Precision Technologies, Issaquah, WA).

Prussian blue staining

To demonstrate ferric iron on HeLa cells, 2.5×10^4 cells were seeded on each well of 8-chamber slide (Nunc, Roskilde, Danmark) and grown for 24 h. Then the cells were incubated with cross-linked Fe_3O_4 nanoparticles containing medium. After 24 h, the medium was removed then cells were washed with PBS solution. The cells were generally fixed with 4% paraformaldehyde (Sigma-Aldrich, MO, USA) for 20 min at room temperature. After removing the fixing agent, the cells were washed and stained according to manufacturer's protocol (Prussian Blue Iron Stain Kit, Polyscience, PA, USA). Briefly, nanoparticles-labelled and control cells were incubated with 1:1 mixture of 4% potassium ferrocyanide and 4% hydrochloric acid for 20 min, and washed with distilled water several times. To stain nuclei of the cells, Nuclear Fast Red solution (Polyscience, PA, USA) was treated to the cells for 5 min and then rinsed in running tap water for 1 min. After drying cells, cover slip was mounted by using mounting medium (DAKO, CA, USA) and then the cells were observed using light microscopy.

T₂-weighted MR Imaging

A total of 1×10^6 cells were seeded on each well of a six-well plate and grown for 24 h. The cells were then incubated with a medium containing cross-linked Fe_3O_4 nanoparticles. After 24 h, the medium was removed and then the cells were washed with a PBS solution. Nanoparticles labeled and control cells were, respectively, detached from the well using a trypsin/EDTA solution and harvested by centrifugation at 9000

rpm for 3 min. The cells were resuspended in a PBS solution containing 1% paraformaldehyde, followed by incubation at 4 °C for 2 h. The cells were subsequently washed with PBS solution and again harvested at 9000 rpm for 3 min. Cell pellets were finally suspended with 2% solution of low melting agarose, solidified at room temperature, and kept at 4 °C. All MR imaging experiments were performed using a 4.7 T clinical MRI instrument (Bruker BioSpec 47/40). The parameters were as follows: TE = 7.4 ms, TR = 8000 ms, FOV = 5.0 × 5.0 cm, matrix = 128 × 128, slice thickness = 2 mm.

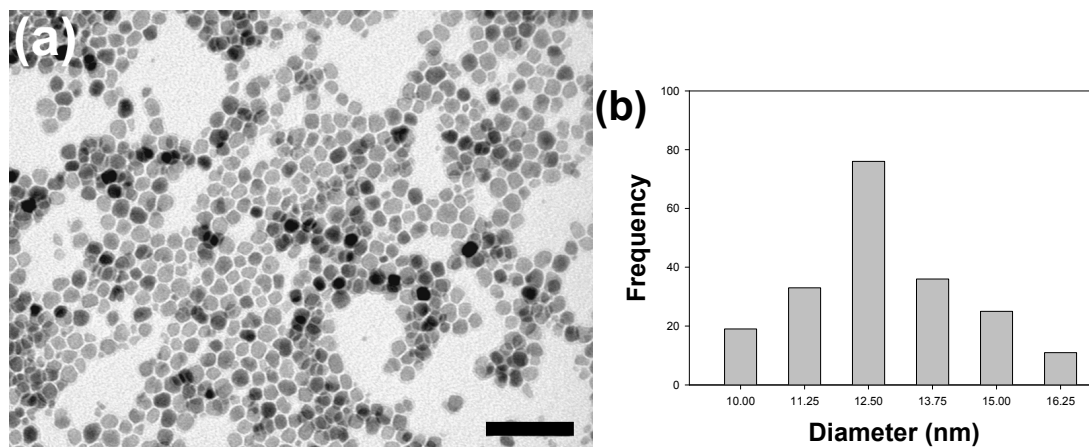


Fig. S1. (a) TEM images of and (b) size histogram of as-synthesized Fe_3O_4 nanocrystals in hexane and. All scale bars are 80 nm.

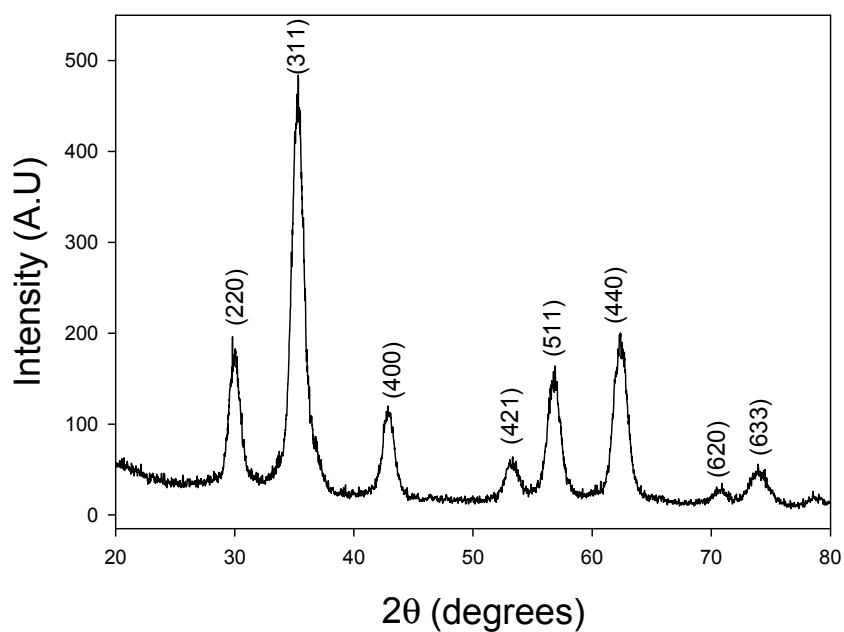


Fig. S2. X-ray diffraction patterns of as-synthesized Fe_3O_4 nanocrystals obtained using a Rigaku D/max-RB apparatus and a Cu $K\alpha$ source ($\lambda = 0.154$ nm); powder samples were analyzed.

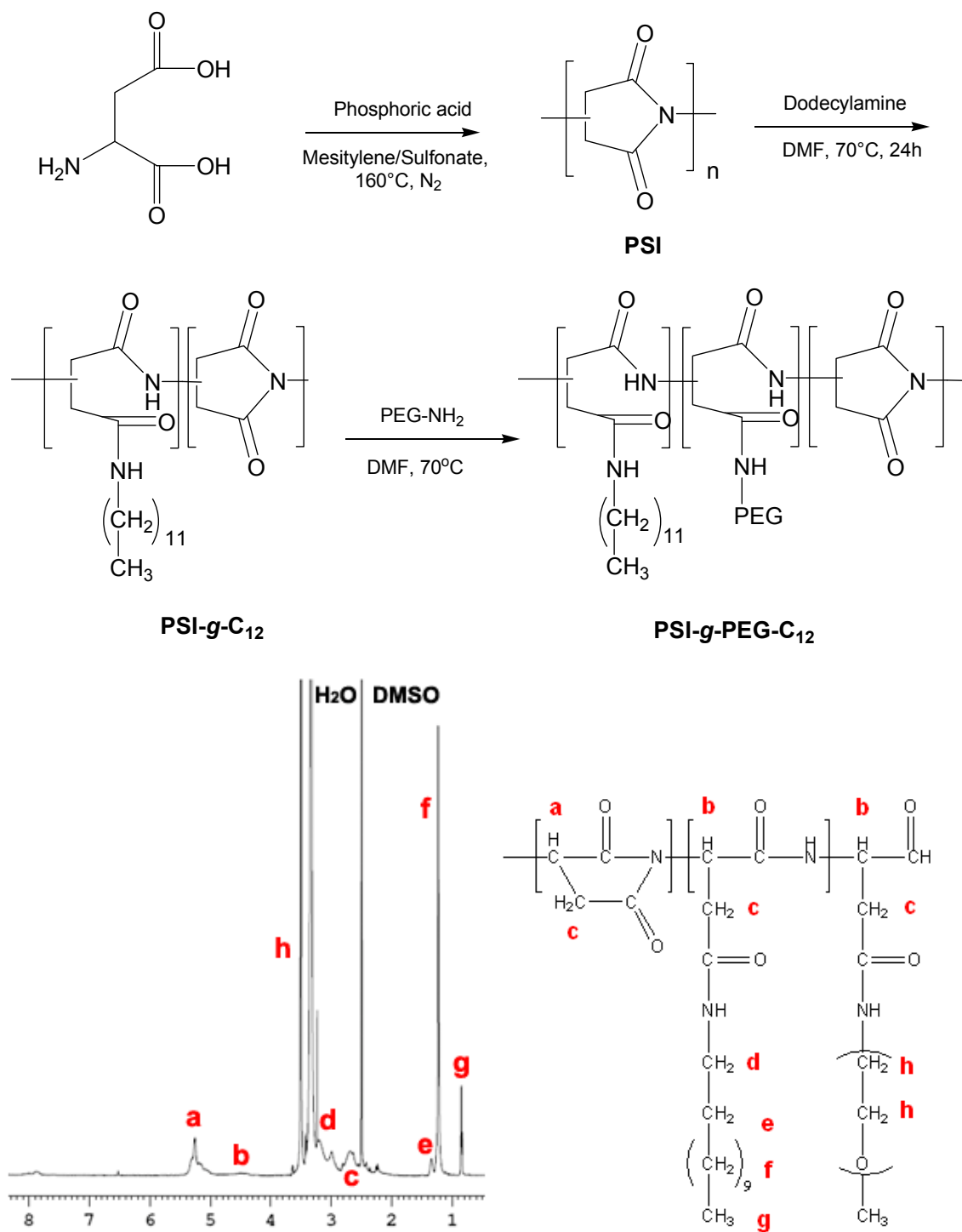


Fig. S3 (a) Synthetic scheme of PSI-g-PEG-C₁₂ (b) ¹H NMR spectra of PSI-g-PEG-C₁₂ in DMSO-d₆.

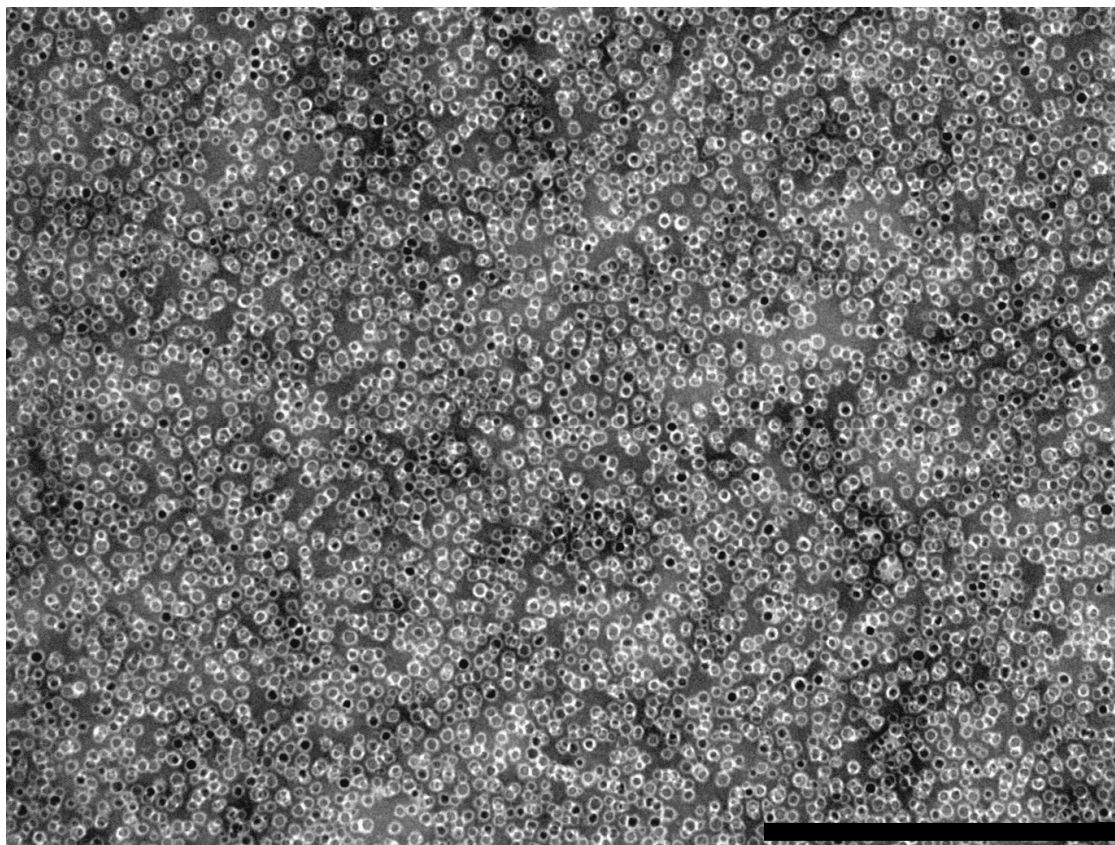


Fig. S4. TEM image of CL-MNPs at lower magnification of figure 2(a). All scale bars are 500 nm.

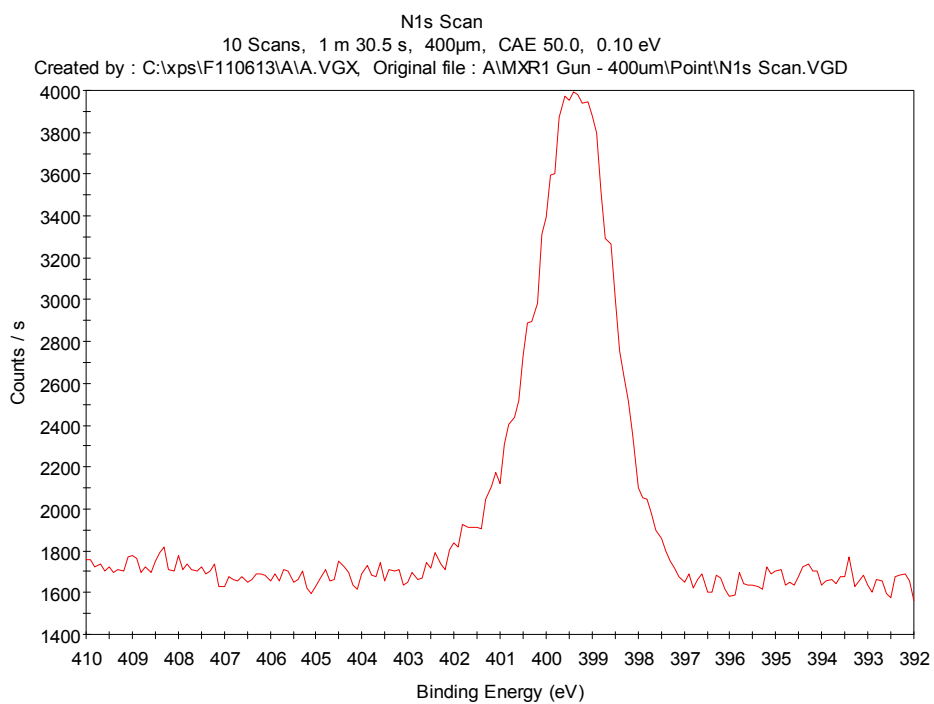


Fig. S5. XPS spectra of the N1s region obtained from CL-MNPs

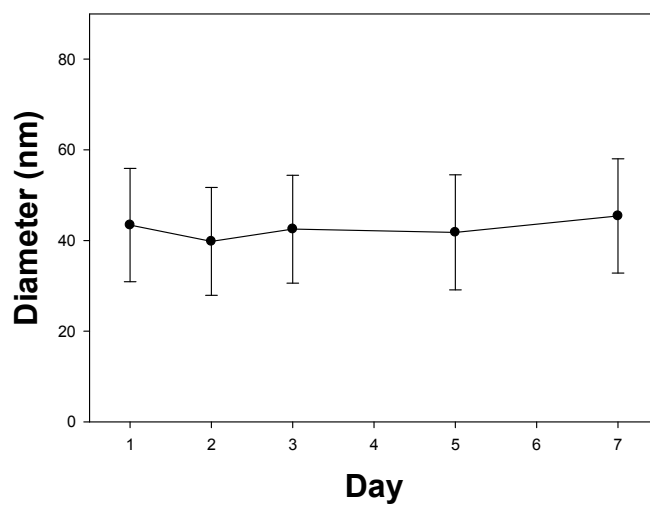


Fig. S6. Size distributions of CL-MNPs in PBS buffer solution determined using DLS for 1 week.

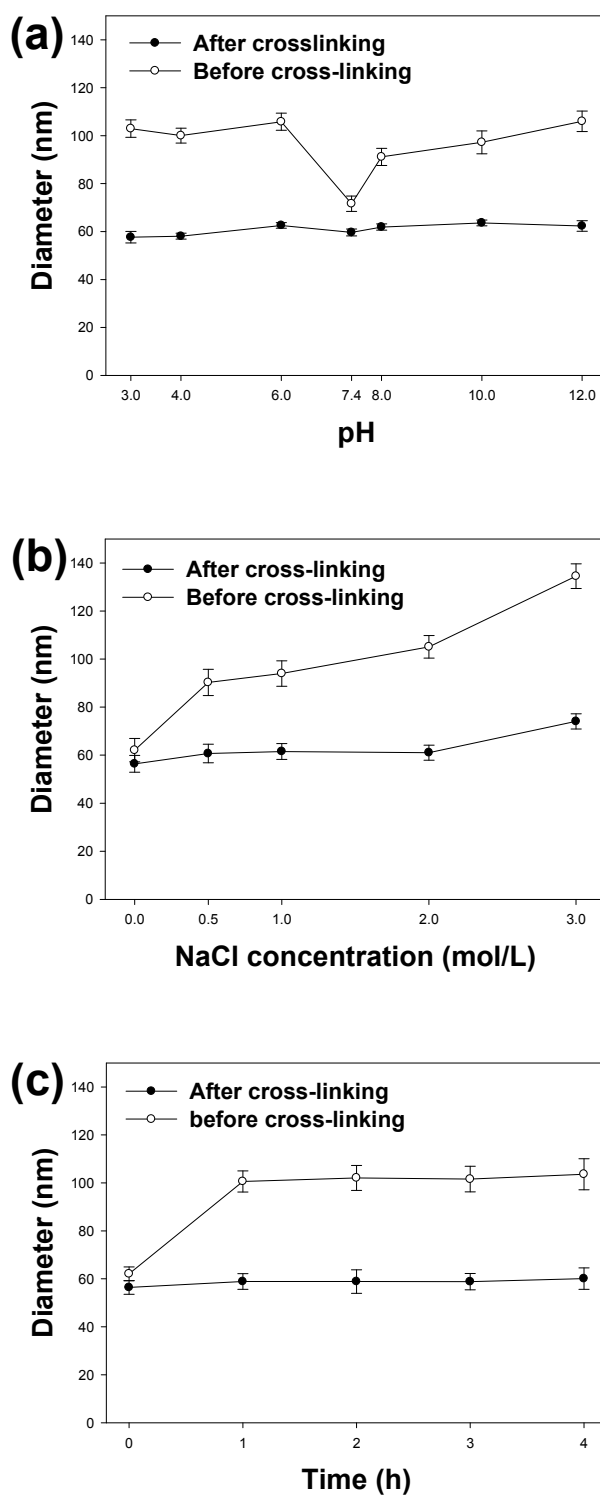


Fig. S7. Colloidal stability test of CL-MNPs monitored with DLS (a) pH stability test, (b) stability in various NaCl solution, and (c) thermal stability test at 60°C. The diameter of CL-MNPs were

measured after 1 day and the mean effective hydrodynamic diameters and calculated from five measurements by DLS.

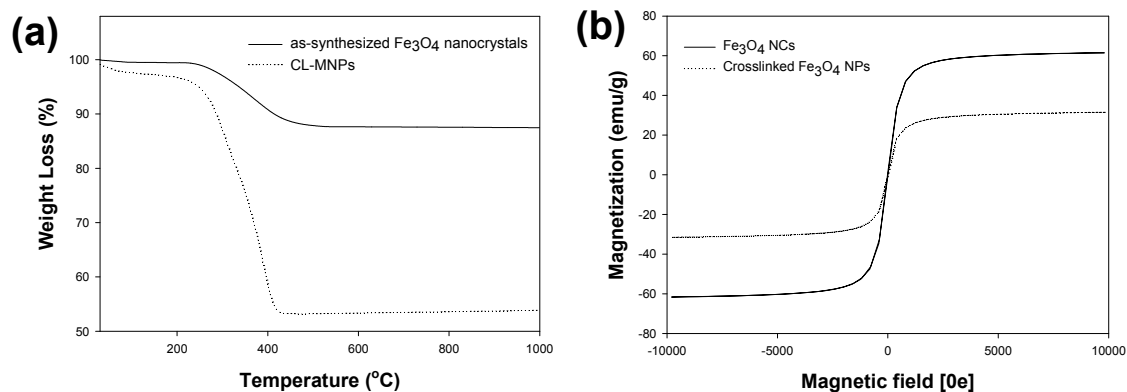


Fig. S8. Thermogravimetry analysis of as-synthesized iron oxide nanocrystals and CL-MNPs. (b) Magnetization curve of as-synthesized iron oxide nanocrystals and CL-MNPs at room temperature.

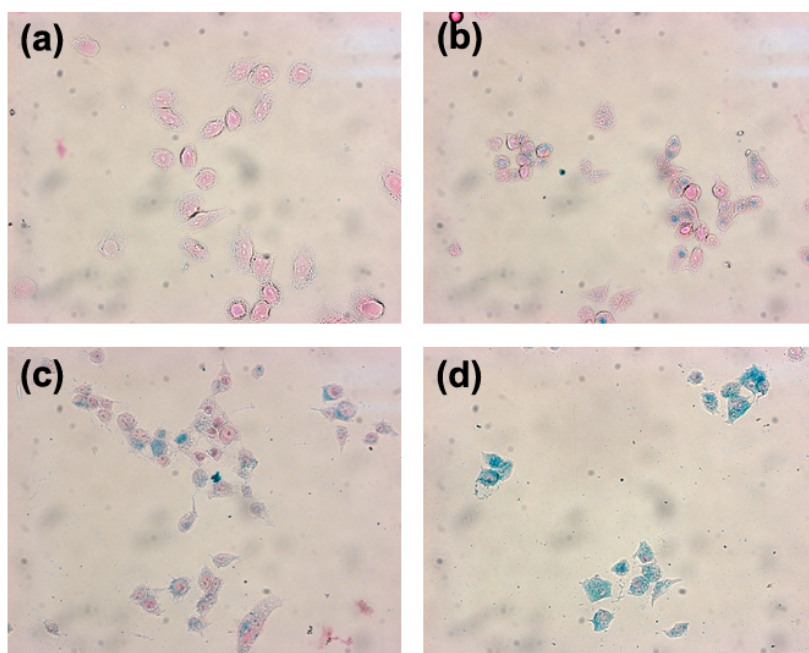


Fig. S9. Prussian blue staining images of (a) control HeLa cells and HeLa cells treated with (b) 0.0625, (c) 0.125, (d) 0.25 mg/ml of Nile red loaded CL-MNPs for 24 h.

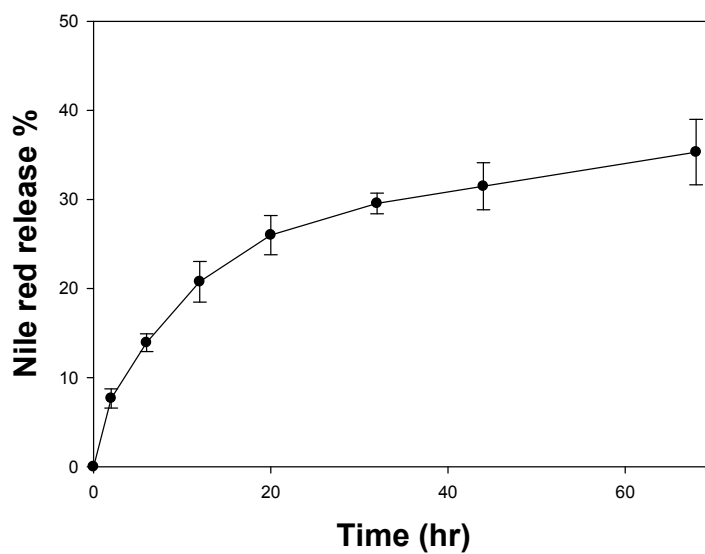


Fig. S10. Nile red release profile of Nile red loaded CL-MNPs at pH 7.4.

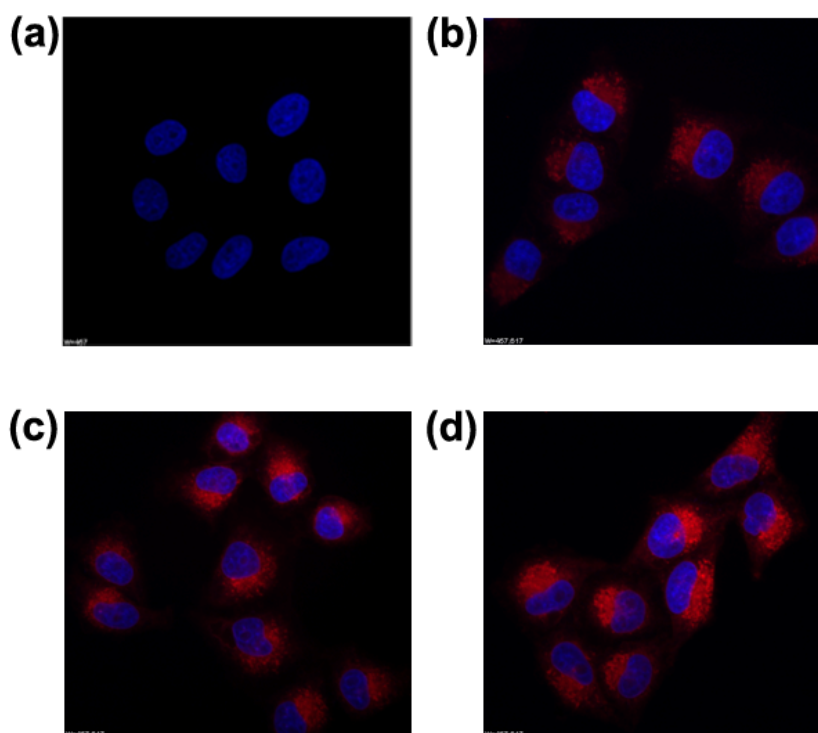


Fig. S11. Confocal laser scanning microscopy images of HeLa cells treated (a) without or with (b) 0.03125, (c) 0.0625, (d) 0.125 mg/ml of Nile red loaded CL-MNPs for 24h of HeLa cells

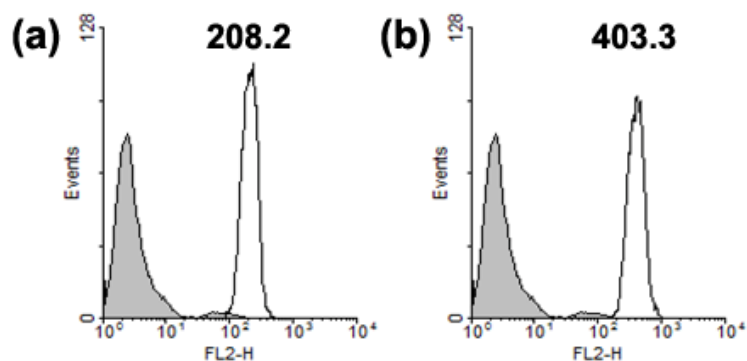


Fig. S12. Cell viability Flow cytometry results of HeLa cells treated with (a) 0.0625, (b) 0.125 mg/ml of Nile red loaded CL-MNPs for 24 h. Gray histograms represent control cells, value in the top middle part of each panel represents the mean fluorescence intensity (MFI) of nanoparticle treated cells.

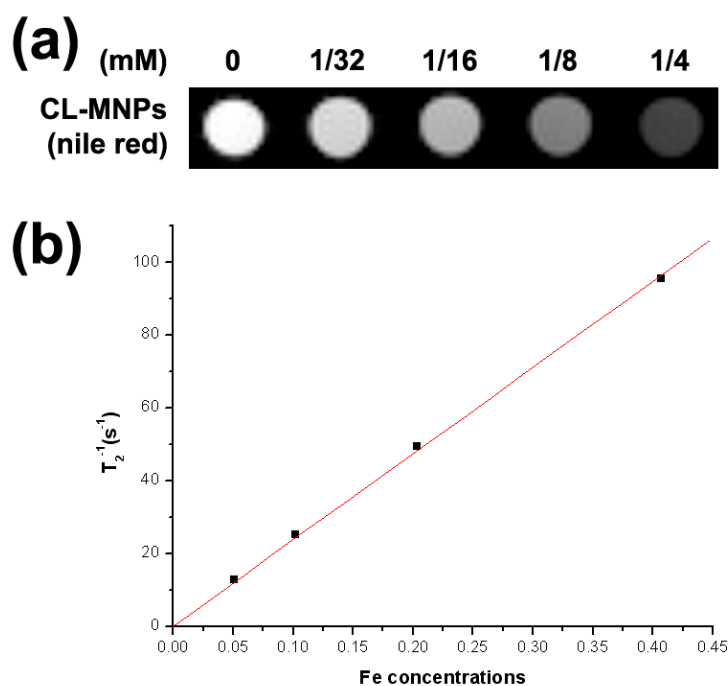


Fig. S13. T₂-weighted MR images of Nile red loaded CL-MNPs in aqueous solution with various concentrations at 4.7 T. (b) Graphs of R₂ against the iron concentration in Nile red loaded CL-MNPs.