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Supporting Information

for *Small*,DOI: 10.1002/ smll.201100012

Graphite-Coated Magnetic Nanoparticles as Multimodal Imaging Probes and Cooperative Therapeutic Agents for Tumor Cells

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Submitted to small
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Graphite-coated magnetic nanoparticles as multimodal imaging probes and cooperative therapeutic agents for tumor cells**

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[**] We thank Prof. Huixin He for helping us with the hyperthermia studies, Profs. Sang-Wook Cheong and Martha Greenblatt for their support for the SQUID measurements and the IAMDN center for allowing us to use their high resolution TEM facility. We are grateful to the KBLEE group members (Aniruddh Solanki, Shreyas P. Shah and Michael Koucky) for their valuable suggestions for the manuscript. J.K.P acknowledges the research program at KRICT and Drs. Young-Duk Suh & Kee-Suk Jun for the Raman imaging. K.-B.L. acknowledges the NIH Director's Innovator Award (1DP20D006462-01) and is also grateful to the N.J. Commission on Spinal Cord Research grant (09-3085-SCR-E-0).

Synthesis of FeCo/C NPs

7 nm FeCo/C NPs were prepared by dissolving $Fe(NO_3)_3 \cdot 9H_2O$ (2.424 g, 6 mmol, Aldrich, 98%) and $Co(NO_3)_2 \cdot 6H_2O$ (1.164 g, 4 mmol, Aldrich, 98%) in 30 mL of distilled water and then 2.9 mmol of sucrose (Aldrich, 99%) was added. The mixture was stirred vigorously to form a clear solution and then placed in a 45 mL capacity Teflon-lined stainless steel autoclave, which was heated in an oven to 190 ^oC for 9h. For 11 nm FeCo/C NPs, the mixture was heated to 220 ^oC for 9h. The products were washed several times with distilled water, filtered off and finally dried in a drying oven at 80 ^oC for 5h. Subsequently, the dried products were annealed at 1000 ^oC for 3h under Ar atmosphere to allow for the growth of the carbon graphite shell on the surface of FeCo NPs. To remove the remaining carbon graphite, the products were washed several times with distilled water and separated using a bar magnet. The number of graphite shells was controlled by varying the amount of sucrose between 2.9 mmol and 8.8 mmol. To prepare large amounts of FeCo/C magnetic NPs in one step, 13.5744 g of $Fe(NO_3)_3 \cdot 9H_2O$ (33.6 mmol) and 6.5184 g of $Co(NO_3)_2 \cdot 6H_2O$ (22.4 mmol) were added to 30 mL of distilled water, which was followed by the addition of 14.5 mmol of sucrose under the above mentioned reaction conditions. The amount of the FeCo/C NPs was as much as 5 g with a yield greater than 95 %.

Synthesis of Fe_3O_4 Nanoparticles

Synthesis of Iron-Oleate Complex: Monodisperse iron oxide (magnetite; Fe_3O_4) nanoparticles (NPs), 11nm in diameter were synthesized according to previously reported protocol[12a]. Briefly, the iron-oleate complex was prepared by dissolving iron chloride ($FeCl_3.6H_2O$, 4 mmol, Aldrich, 98%) and sodium oleate (12mmol, TCI, 97%) in a mixture solvent composed of 8 ml ethanol, 6 ml distilled water and 14 ml hexane under inert atmosphere. The resulting solution was then heated to 70 °C and maintained at that

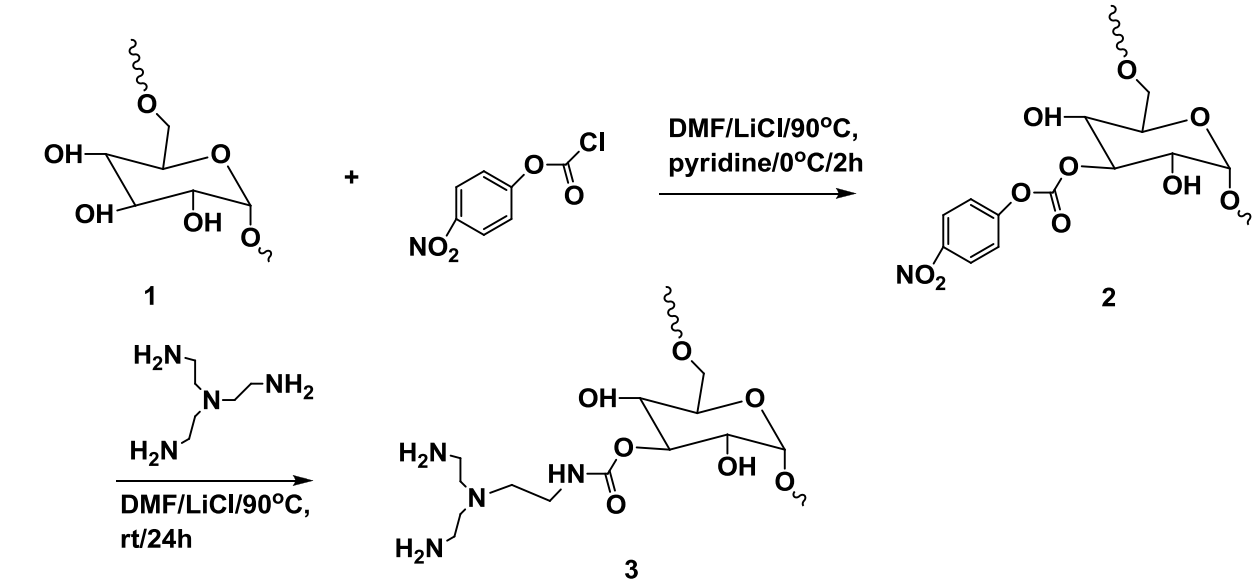
temperature for four hours. Thereafter, the upper organic layer containing the iron oleate complex was washed three times with distilled water in a separatory funnel to remove traces of unreacted starting materials. Following washing, hexane was evaporated off to yield iron-oleate complex in a waxy solid-like form.

Synthesis of Fe_3O_4 Nanoparticles: 3.6 g of the previously synthesized iron-oleate complex and 0.57 g of oleic acid (Aldrich, 90%) were dissolved in 20 g of 1-octadecene (Aldrich, 90%) at room temperature. The resulting mixture was heated upto 320 °C at a constant heating rate of 5 °C min^{-1} and kept at that temperature for 30 min. At 320 °C, the initial transparent reddish orange solution became turbid and brownish black. The resulting solution was then cooled down to room temperature and the nanocrystals were then precipitated out using ethanol and centrifuged to yield monodisperse 11nm Fe_3O_4 nanoparticles. The nanoparticles were then characterized using transmission electron microscopy.

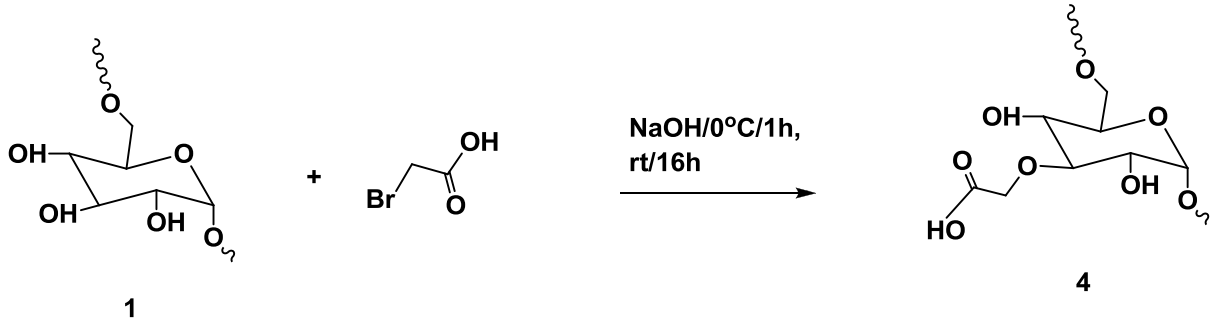
Physical Characterization of the FeCo/C NPs

The NPs were characterized using XRD (Bruker D8 X-ray diffractometer with CuKα radiation (Ni filter)), TEM (Tecnai G2 and JEOL JEM-2010F high-resolution transmission electron microscope operated at an accelerating voltage of 200 kV), Raman spectroscopy (Renishaw Micro-Raman 2000 with He-Ne laser excitation of 632.8 nm) and SQUID magnetometry (Quantum design magnetometer). The stoichiometry of Fe and Co and metal concentration in the NPs were determined using Inductively Coupled Plasma-Atomic Emission Spectrometer (ICP-AES, data not shown).

Synthetic Routes to Functionalized Dextrans:

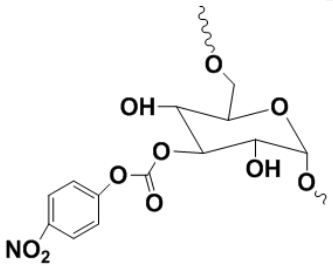


Scheme 1. Synthesis of dextran functionalized with 4-nitrophenyl chloroformate groups and amine groups.



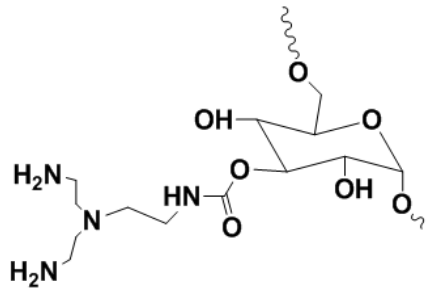
Scheme 2. Synthesis of dextran functionalized with carboxyl groups.

Synthesis of Functionalized Dextrans:



Dextran functionalized with 4-nitrophenyl chloroformate groups (4-NC dextran) (2)

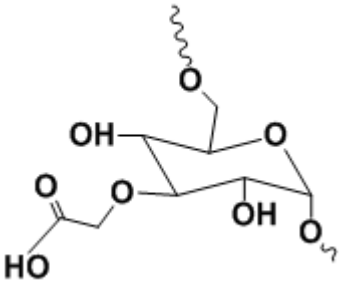
Scheme 1 shows the synthesis of 4-NC dextran[25]. For a complete dissolution of dextran (**1**) in DMF, 15 g (92 mmol) dextran (from *Leuconostoc spp*, Aldrich, M_r = 6,000) and 5.0 g (118 mmol, 2 w/v %) water-free lithium chloride (LiCl, Acros Organics, 98%) were suspended in 250 ml DMF and stirred at 90 °C until a clear solution appears. The reaction mixture was cooled to 0 °C, and pyridine (4.2 mL, 52 mmol, Alfa Aesar, 99%) was added to the dextran solution followed by 4-NC (10.5g, 52 mmol, Acros Organics, 97%). The reaction mixture was stirred for 4 h at 0 °C, and finally the 4-nitrophenyl carbonated dextran was isolated by precipitation in cold ethanol, washed with diethyl ether, and dried under vacuum. Yield: 14.5 g, 97%. ^{1}H NMR (D_2O): δ 3.27-4.13 (m, dextran glucosidic protons), 4.68 (s, dextran anomeric proton), 7.83 and 8.67 (dd, aromatic protons).



Dextran functionalized withaminegroups (3)

For a complete dissolution of 4-NC dextran in DMF, 7 g (37 mmol) 4-NC dextran and 1.13 g (26.7 mmol, 1 w/v %) water-free LiCl were suspended in 56 mL DMF and stirred at 90 °C until a clear solution appeared. The solution was then cooled again to 0 °C. Tris(aminoethyl)amine (7 mL, 45 mmol, Alfa Aesar, 96%) was dissolved in DMF (56 mL) and added drop wise under stirring to the 4-NC dextran solution. The reaction was stirred for 24 h at room temperature. Subsequently, the product was isolated by precipitation in cold ethanol and washed several times with ethanol to remove *p*-nitrophenol. The product was filtrated and dried under vacuum. Finally the completion of the reaction was determined by a Ninhydrin color test and NMR spectroscopy. Yield: 4.6g, 66%. ^{1}H NMR (DMSO-*d*6): δ 1.0

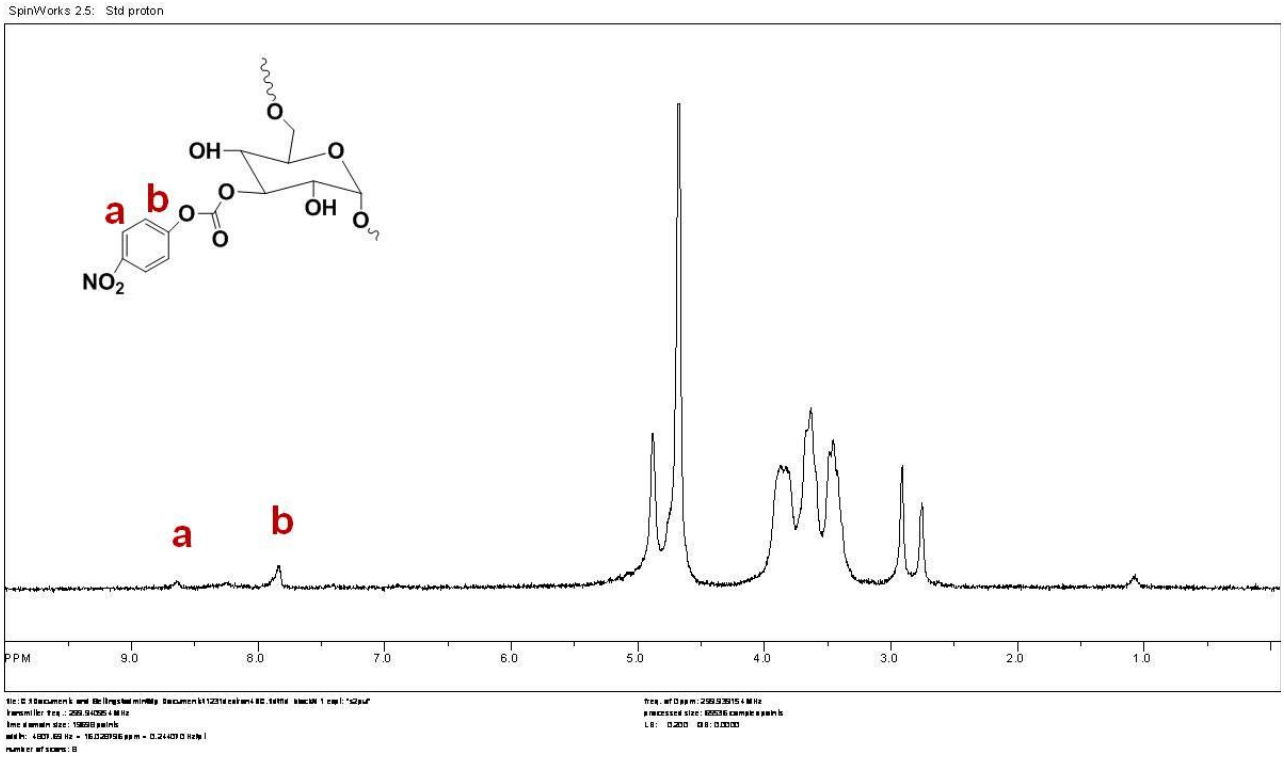
(t, amine protons), δ 2.42-2.57 (m, amine protons), δ 3.1-3.8 (m, dextran glucosidic protons), 4.08 (s, dextran glucosidic protons), 4.69 (s, dextran anomeric proton), 8.0 (s, amide protons).

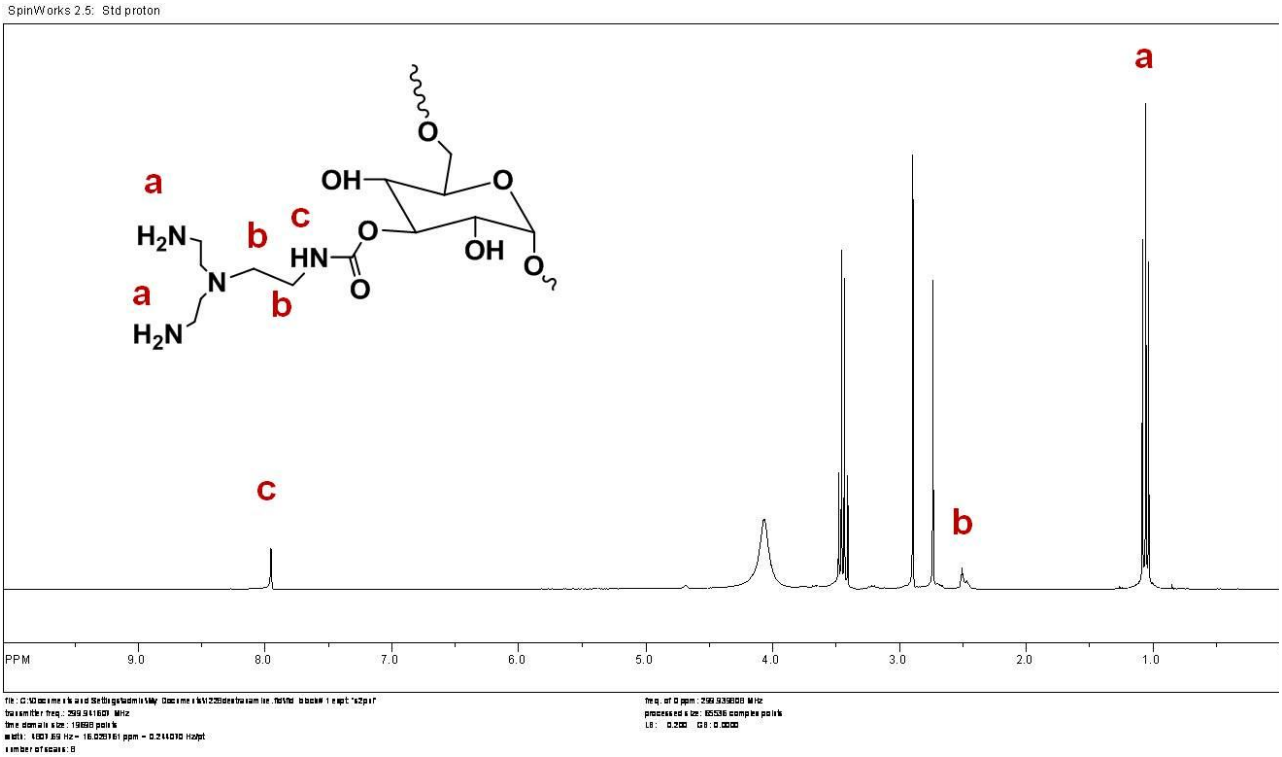


Dextran functionalized with carboxyl groups (4)

Scheme 2shows the representative synthesis for obtainingdextran functionalized by carboxyl groups (**4**). Carboxydextran was synthesized using the methods reported previously[26]. 6 g dextran was dissolved in 20 mL 0.1 M NaOH. Then 1M bromoacetic acid (1.4 g, Aldrich, 98%) and 10 mL 2 M NaOH (0.8 g) were added to 10 mL of the dextran solution under stirring. The solution was stirred for 24 h at room temperature. The carboxydextran was then isolated by precipitation in acetone and subsequently washed with acetone.The product was dried under vacuum. Yield: 5.8g, 96%. ^{1}H NMR (D_2O): δ 3.26-4.18 (m, dextran glucosidic protons), 4.52-4.92 (m, dextran anomeric proton), 5.05-5.10 (s, carboxylic acid protons).

Chemical Charaterization using NMR spectroscopy:

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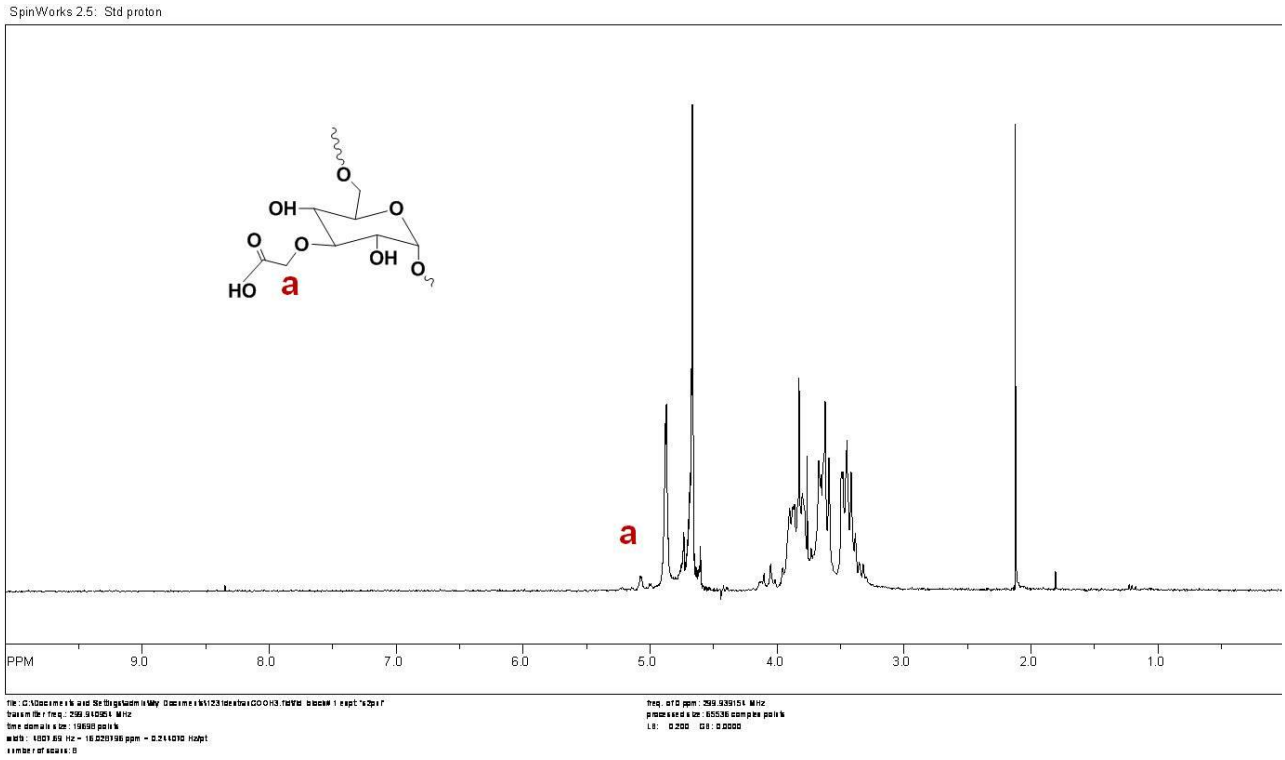
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Surface Modification of the FeCo/C NPs with the Functionalized Dextrans

200 mg of FeCo/C magnetic NPs were dispersed in 10 ml of NaOH (0.5 M) and this suspension was mixed with a solution of 400 mg of functionalized dextran in NaOH (0.5 M) prepared previously. The mixture was sonicated for 24 h at 30 °C and the coated NPs were separated magnetically and washed thrice with water to remove the unreacted functionalized dextran.

T_2 Measurements: Aqueous solutions of varying concentrations of FeCo/C NPs were used for T_2 measurements using a 4.7 T MRI instrument with a 72 mm volume coil (Bruker, Germany). The T_2 values of various phantom solutions were obtained from the Carr-Purcell-Meiboom-Gill (CPMG) sequence at room temperature (TR = 10 s, 128 echoes with 7.4 ms even echo space, number of acquisition = 1, spatial resolution = 391 μm × 391 μm, section thickness =1 mm).

Conjugation of Targeting Molecules to FeCo/C NPs: Amine reactive FeCo/C-Np-Dex (Nitrophenyl ester conjugated dextran; Np-Dex) magnetic NPs were dispersed in degassed PBS. To 50 μl of the above FeCo/C solution (9 mg/ml), 20 μl of EGFR (Epidermal Growth Factor Receptor) antibody (0.5 mg/ml, BD Pharmingen, San Diego, CA) or 25 μl of cRGD solution (5mM, American Peptide Company, Sunnyvale, CA) was added. The reaction solution was stirred for 3-4 hours at 100 rpm. After conjugation, unreacted biomolecules were removed by three cycles of ultracentrifugation at 14,000 rpm and subsequent washing with PBS.

Attachment of siRNA to the FeCo/C NPs: For the siRNA to silence EGFP/EGFRvIII gene in U87-EGFP /EGFRvIII cells, we utilized the layer-by-layer (LbL) method with slight modifications to the reported procedure (**Reference**). 1 ml of 1.0 mg/ml polyethyleneimine (PEI, MW 10,000) in distilled water was added to 0.5 –1.0 mg carboxydextran FeCo/C NPs.

After sonication for 2-4 hours under neutral pH, the coated particles were purified using ultracentrifugation and subsequent washing with distilled water. After characterization of the surface charge using zeta potential measurement (see Fig. S13), 10 μl of 0.1mM siRNA solution in degassed PBS was added to 100 μl of PEI coated FeCo/C solution (5 mg/ml). After mild stirring for 1-2 hours, the NPs were purified by centrifugation and washing. Following this, another layer of PEI was deposited on the siRNA layer of FeCo/C-PEI with the same concentration and reaction volume as first PEI layer by stirring mildly for 1-2 hours. Sonication was avoided to protect the siRNA from heating and degradation. Final products was purified by ultracentrifugation (14,000 rpm) and washing with 1.0 mM NaCl solution. Surface charge was characterized using zeta potential measurement (Zetasizer Nano ZS, Malvern Instruments, Westborough, MA) to perform the siRNA transfection experiment.

Cell Culture of U87-EGFP, U87-EGFRvIII and other Control cells: For the hyperthermia, MR imaging, Raman study and siRNA transfection experiments, EGFP or EGFRvIII overexpressed U87 cells (U87-EGFP or U87-EGFRvIII) and several controls cells (PC-12 and Astrocytes) were cultured using previously reported methods, albeit with minor modifications. For U87-EGFP and U87-EGFRvIII cells, DMEM with high glucose, 10% FBS, 1% Streptomycin-penicillin and 1% Glutamax (Invitrogen, Carlsbad, CA) were used as basic components of growth media including Geneticin G418 (100 μg/ml, Invitrogen) as a selection marker for U87-EGFP and Hygromycin (100 μg/ml, Invitrogen) for U87-EGFRvIII. Astrocytes were cultured in the above mentioned growth media without any selection markers, while PC-12 cells were cultured in DMEM with 10% horse serum, 5% FBS and 1% Streptomycin-penicillin. All cells were cultured at 37 ^{o}C in humidified 5% CO_2 atmosphere.

In Vitro Imaging

MRI Imaging: For the MR imaging of the single layer of the cells with nanoparticles, U87-EGFP cells were grown on 15mm diameter plastic cover slips (EMS, Hatfield, PA) in 12-well

plates. After treatment with two different nanoparticles - FeCo/C MNPs and Fe_3O_4 MNPs (90-120 μg/ml) for 2 hours, cells were fixed with 4% paraformaldehyde solution. MRI imaging was performed at 4.7 T using the following parameters: point resolution: 156 × 156 μm, section thickness of 0.6 mm, TE = 60 ms, TR = 4000 ms, number of acquisitions = 1.

Raman Imaging: Internalization of nanoparticles into U87-EGFP cells was performed in 48-well plates using the above mentioned conditions. To each well, FeCo/C MNPs with different number of carbon graphite layers were added using the previously mentioned conditions. The cells were then imaged using a Renishaw Micro-Raman 2000 instrument with He-Ne laser excitation of 632.8 nm.

***In vivo* MR Imaging:** We injected 11 nm FeCo/C NPs (10 μL, 0.25 mg of Fe) and Resovist (100 μL, 2.5 mg of Fe) into a rat's tail vein. T_2-weighted MR images before injection and at 30 minute intervals post-injection were obtained using a 4.7 T MRI instrument (Bruker, Germany). The T_2-weighted MR signal intensity was also measured in the various organs over an extended period of 7 days. We used the following parameters: resolution of 234 × 256 μm, section thickness of 2.0 mm, TR = 400 ms, TE = 15 ms, number of acquisitions = 8. Flip angle = 30^o.

Hyperthermia Measurements: All the AC magnetic field experiments were conducted using a Comdel CLF-5000 RF generator in a magnetic field with a frequency of 334 kHz and at amplitude of 150 Oe. To measure the temperature variation of the magnetic NPs suspensions, 2 mL of each suspension was taken in a double- walled test tube where the space between the outer and inner walls was evacuated to minimize any heat loss. The tube was placed at the center of the induction heater coil and an alcohol thermometer was used to measure the temperature increase in the suspension, thereby negating the electrical and magnetic effects of the generator on the thermometer. For hyperthermia measurements in cells, 15 mm diameter cover glass (Fisher scientific, Pittsburg, PA) was autoclaved and sterilized under UV light.

U87-EGFP or U87-EGFRvIII cells were cultured to 40-60 % confluency on the cover glasses in 24-well plates. FeCo/C NPs or Fe_3O_4 NPs in Opti-MEM (Invitrogen, Carlsbad, CA) were added at various concentrations (10, 30, 60, 90 μg/ml). After 2-4 hrs of incubation, cells were washed with DMEM to remove non-specific attachment of NPs and the media was changed to growth media. After overnight incubation, hyperthermia study was carried out. For target-specific hyperthermia experiments, control cells such as PC-12 and astrocytes were co-cultured with U87-EGFP (about 1:1 ratio). EGFR antibody or cRGD conjugated FeCo/C NPs were used as a hyperthermia agents at different concentrations (1 μg/ml and 5 μg/ml). Detailed procedure was same as the one used for FeCo/C and Fe_3O_4 in U87-EGFP cells.

siRNA delivery and EGFP knockdown using FeCo/C NPs: PEI/siRNA/PEI-FeCo/C NPs were dispersed in the transfection media (Opti-MEM) at a final concentration of 30-120 μg/ml. 100 μl of the nanoparticle solution was added to the U87-EGFP cells at 50-60% confluency in 96-well plates. After 6-8 hours of incubation, the solution in each well was exchanged with the growth medium, followed by washing with DMEM. Fluorescence images were obtained at 48, 72 and 96 hours after transfection.

siRNA delivery against EGFRvIII and combined hyperthermia treatment: U87-EGFRvIII cells were grown on 15 mm diameter cover glass in 24-well plates at 40-50% confluency. 30-120 μg/ml of PEI/siRNA/PEI-FeCo/C in the transfection media was added to the cells. After 2-4 hours of incubation, the wells were washed with DMEM and media replaced with growth medium. Hyperthermia treatment (334 kHz, 5min) was performed at 72, 96 and 120h post-siRNA treatment. MTS assay using the CellTiter 96 Aqueous One Solution (Promega, Madison, WI) was performed within 4 hours of incubation after hyperthermia (protocol as recommended by manufacturer) in order to quantify the synergistic cell death.

References:

[1] J. Park, K. J. An, Y. S. Hwang, J. G. Park, H. J. Noh, J. Y. Kim, J. H. Park, N. M. Hwang, T. Hyeon, *Nature Materials* **2004**, *3*, 891.

[2] J. C. Ramirez, M. Sanchezchaves, F. Arranz, *Angewandte Makromolekulare Chemie* **1995**, *225*, 123.

[3] S. Lee, V. H. Perez-Luna, *Analytical Chemistry* **2005**, *77*, 7204.

Supporting Figures

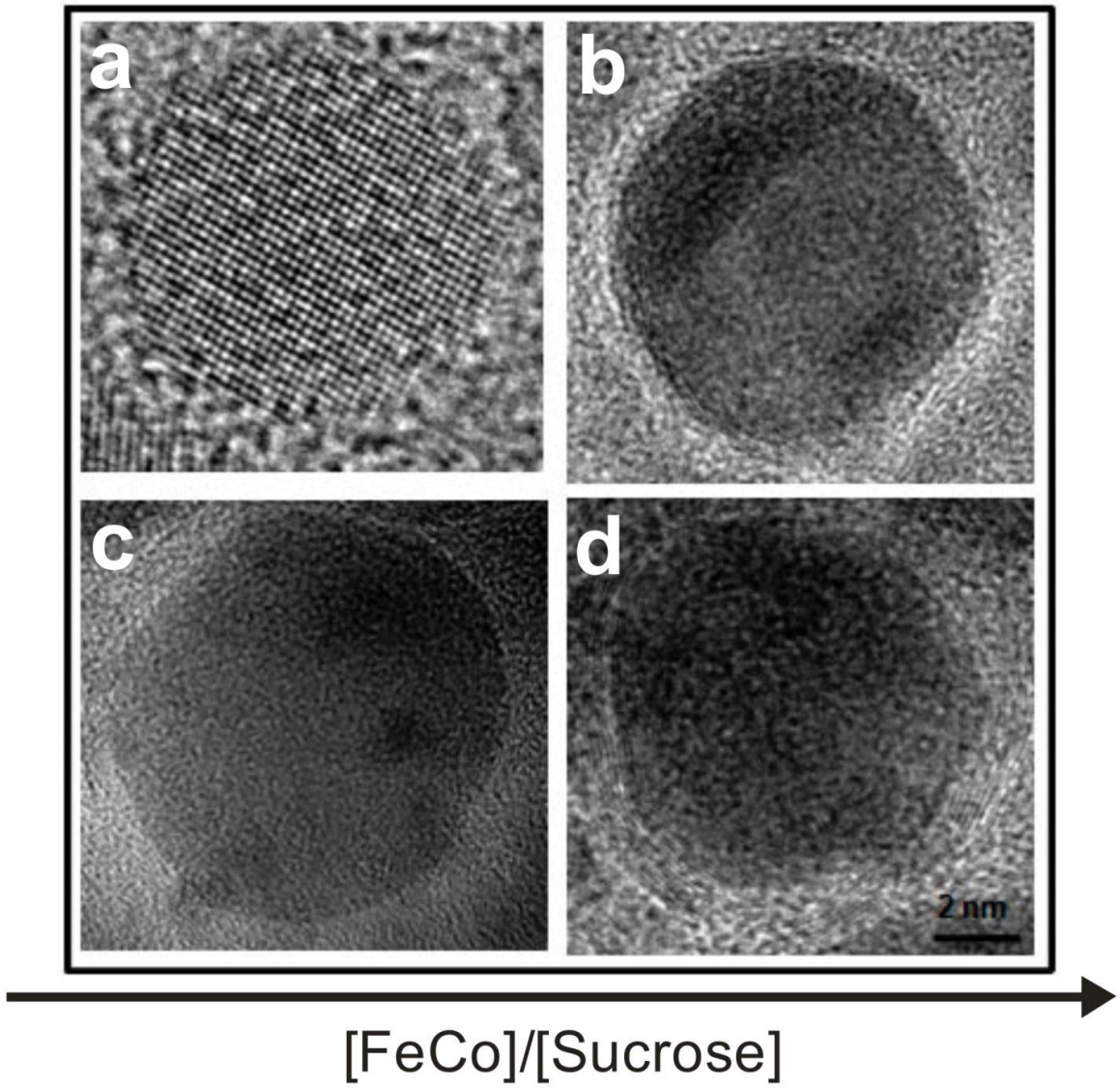

Figure S1. HR-TEM image of FeCo/C MNPs as a function of [FeCo]/Sucrose ratio. The shell thickness can be easily controlled by varying the [FeCo]/[Sucrose] ratio between **a)** 2.9 **b)** 4.4 **c)** 5.8 and **d)** 8.8.

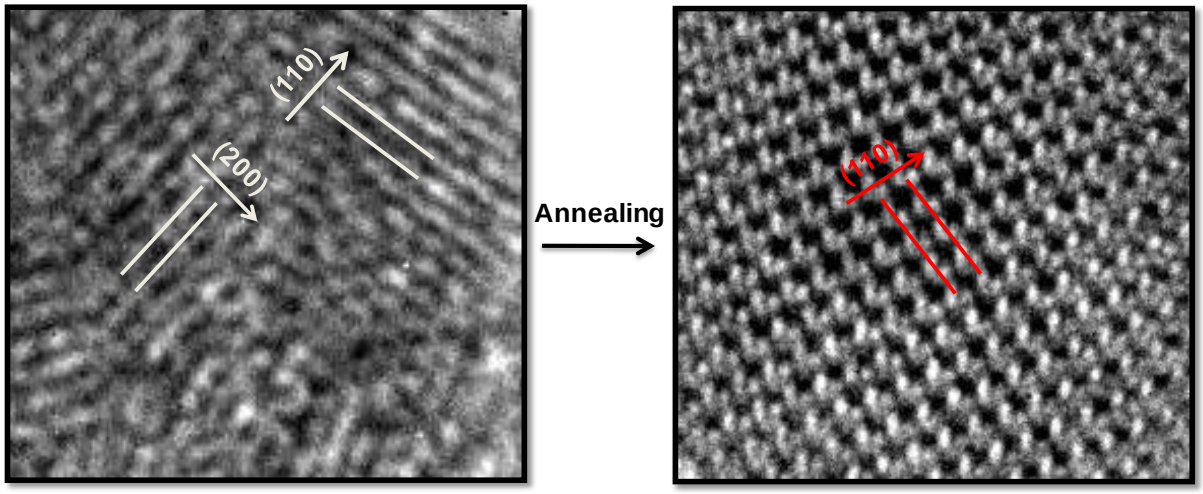

Figure S2. HR-TEM images of FeCo/C MNPs: as prepared (left) and after annealing at 1000 °C (right). The identification of lattice fringes for as-prepared FeCo/C nanoparticles indicated the presence of chemically disordered phase, whereas the annealed FeCo/C nanoparticles show that the particles have uniform lattice fringes across the particles, which is attributed to good crystallinity.

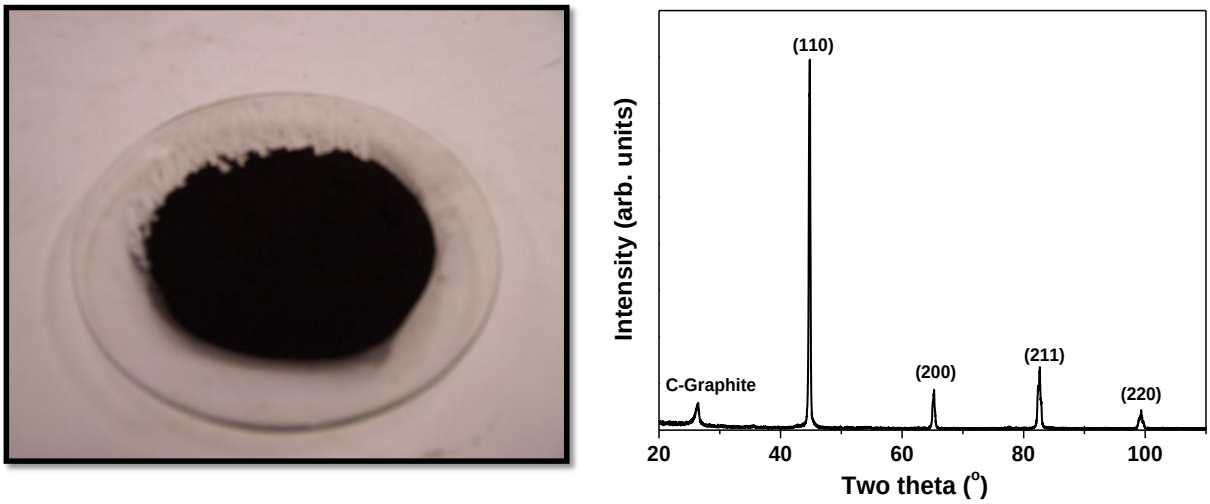

Figure S3. Photograph of the air-stable carbon graphite encapsulated FeCo nanoparticles obtained via a large-scale synthesis and the corresponding powder-XRD spectrum.

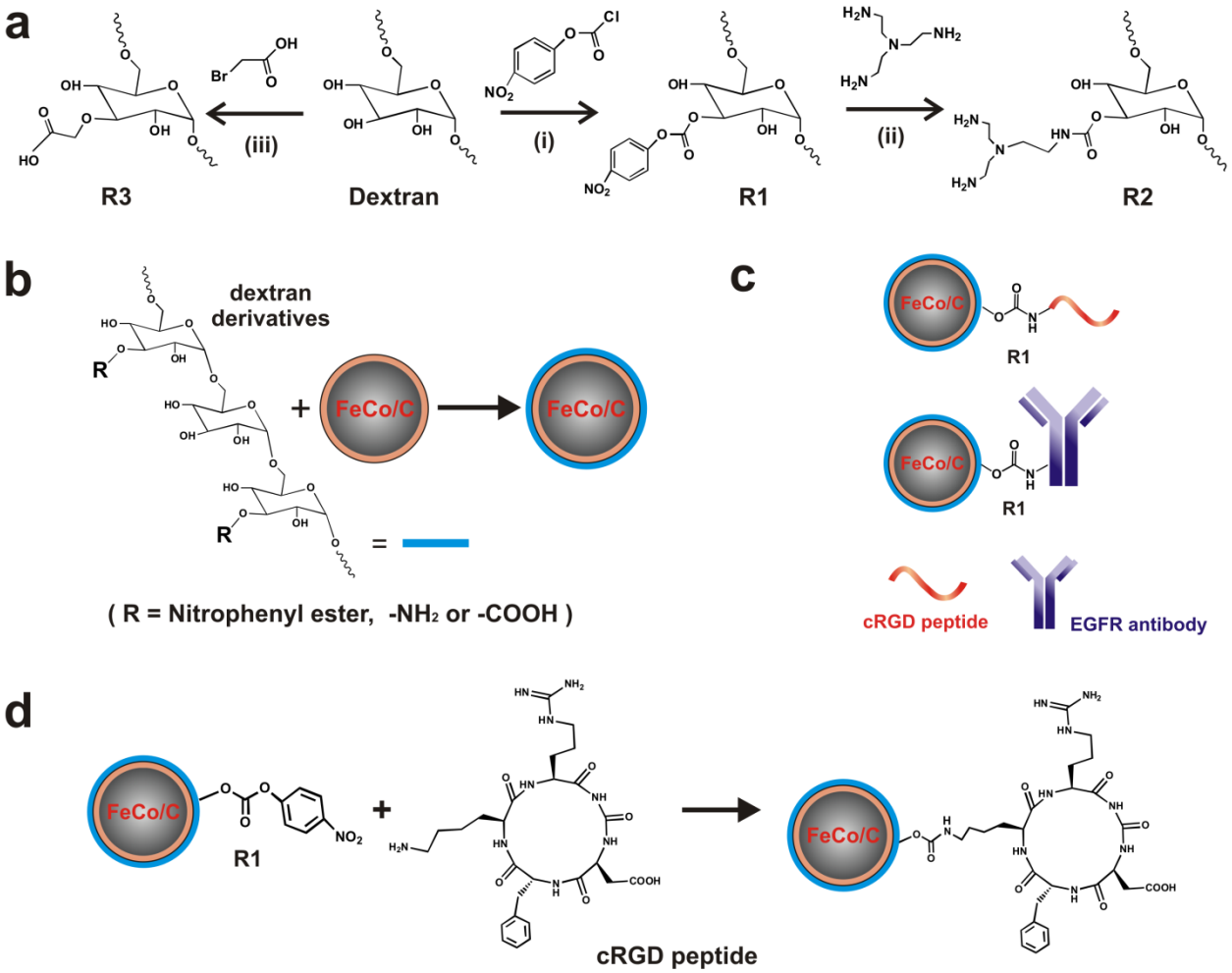

Figure S4. Schematic diagram depicting the conjugation strategies for attaching biomolecules to the surface of FeCo/C nanoparticles. a) Synthesis of dextran derivatives terminated with 4-nitrophenyl chloroformate group (R1), -NH_2 group (R2) and -COOH group (R3). **b)** Coating of the FeCo/C NPs with dextran derivatives to render them biocompatible and enable conjugation of biomolecules onto their surface. **c)** and **d)** Conjugation of EGFR antibody and cRGD peptide to the reactive functional groups present on the dextran coated FeCo/C NPs.

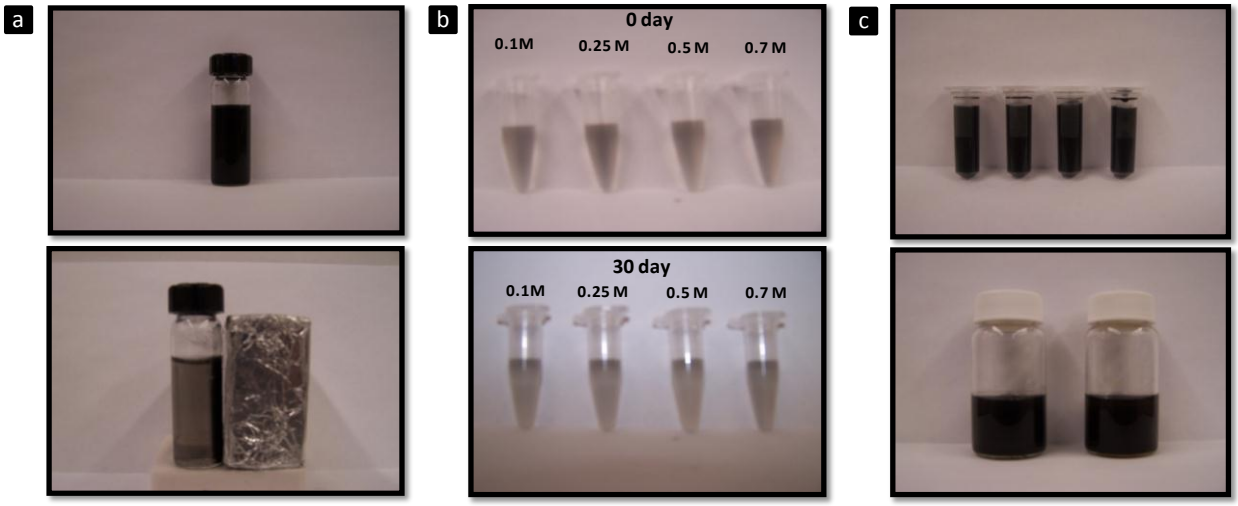

Figure S5. Photographs of colloidal solutions of 11 nm FeCo/C NPs. a) Separation of FeCo/C NPs from a suspension in HNO_3 (53 wt%) by a commercial neodymium magnet. **b)** Colloidal stability in NaCl solution, and **c)** Dispersion of carboxydextran-coated FeCo/C NPs in water.

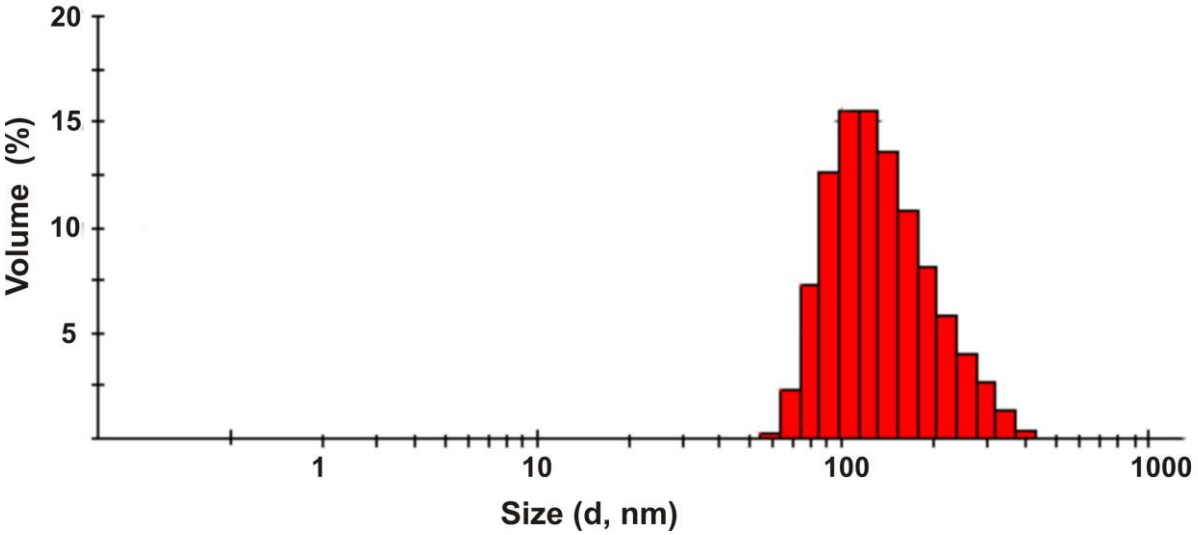

Figure S6. Hydrodynamic diameter of the dextran-coated FeCo/C NPs as determined by DLS measurement. The average size of the NPs was found to be 120-140 nm.

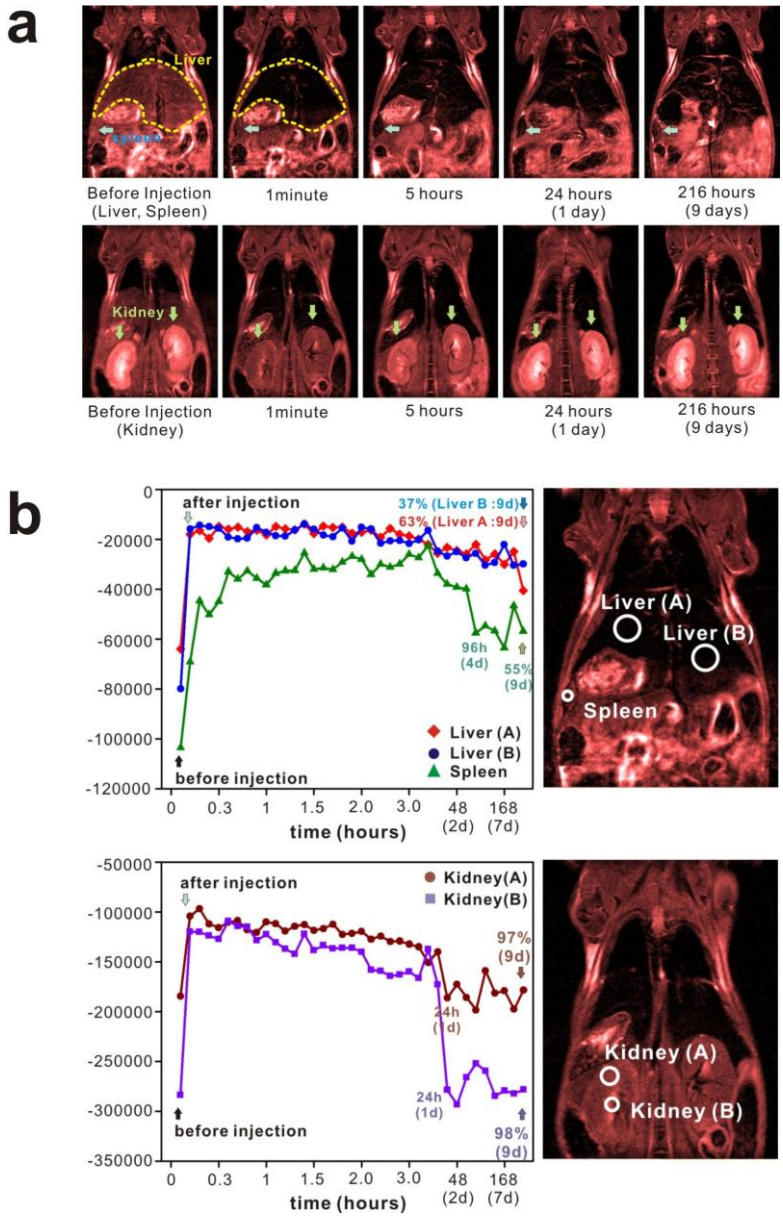

Figure S7. a) The *in vivo* biodistribution and MR imaging contrast of dextran-coated FeCo/C NPs in rats was monitored over 10 days. The FeCo/C NPs predominantly localized to the liver and spleen with a small percentage being found in the kidneys. **b)** Plots of T_2-weighted MR imaging intensity of FeCo/C NPs in the various organs (marked as white circles on the right) as a function of time over a period of 7 days. The FeCo/C NPs were effectively retained in the organs with no significant loss of the MR signal over the period tested.

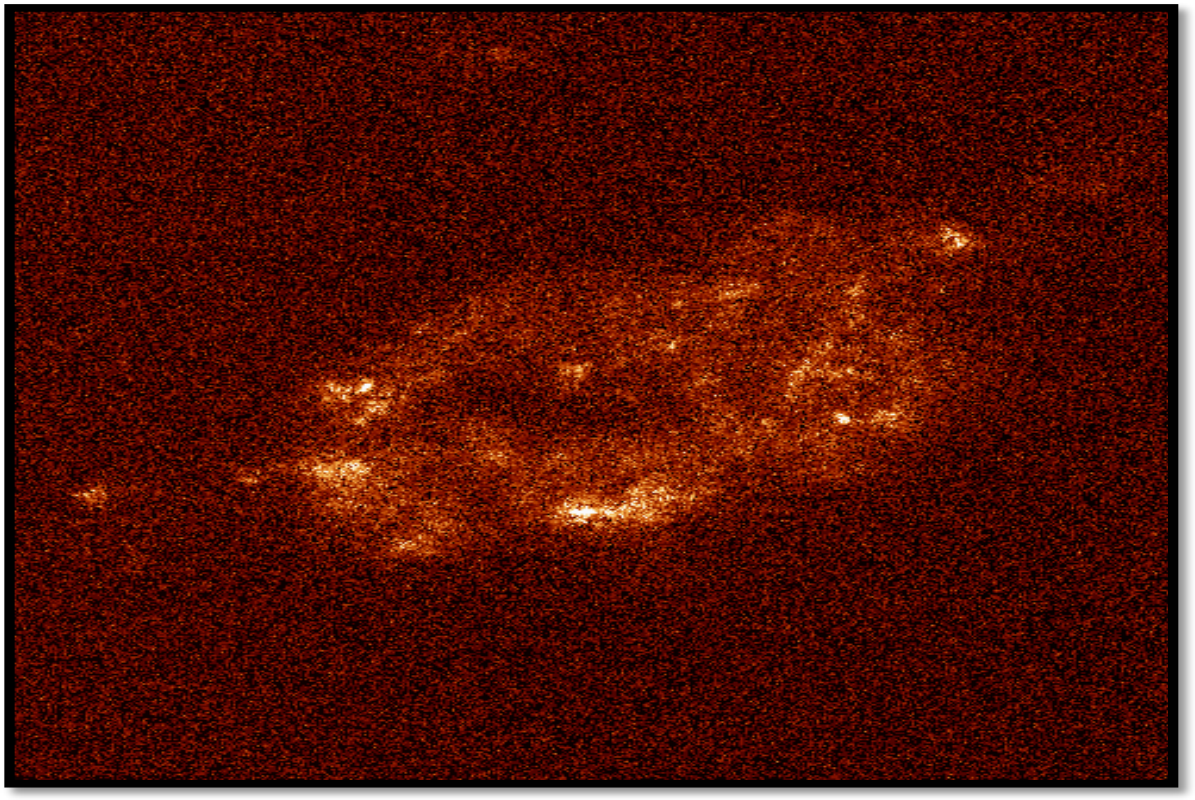

Figure S8. Raman image of single layer carbon graphite encapsulated FeCo NPs internalized into U-87 cell in a field of 60 μm x 60 μm.

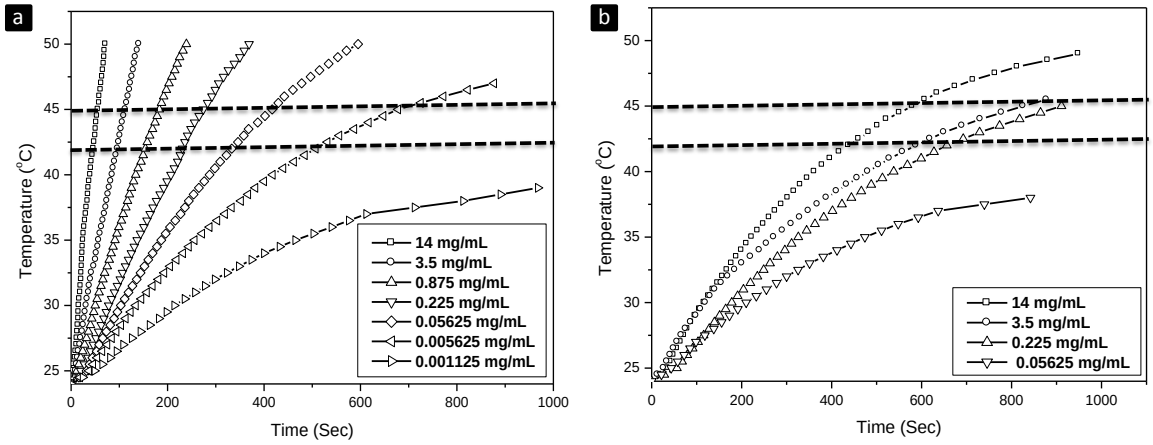

Figure S9. Plots of increase in temperature *versus* time for suspensions with different concentrations of **a)** 11 nm FeCo/C NPs and **b)** 11 nm Fe_3O_4 NPs under the 334 kHz magnetic field.

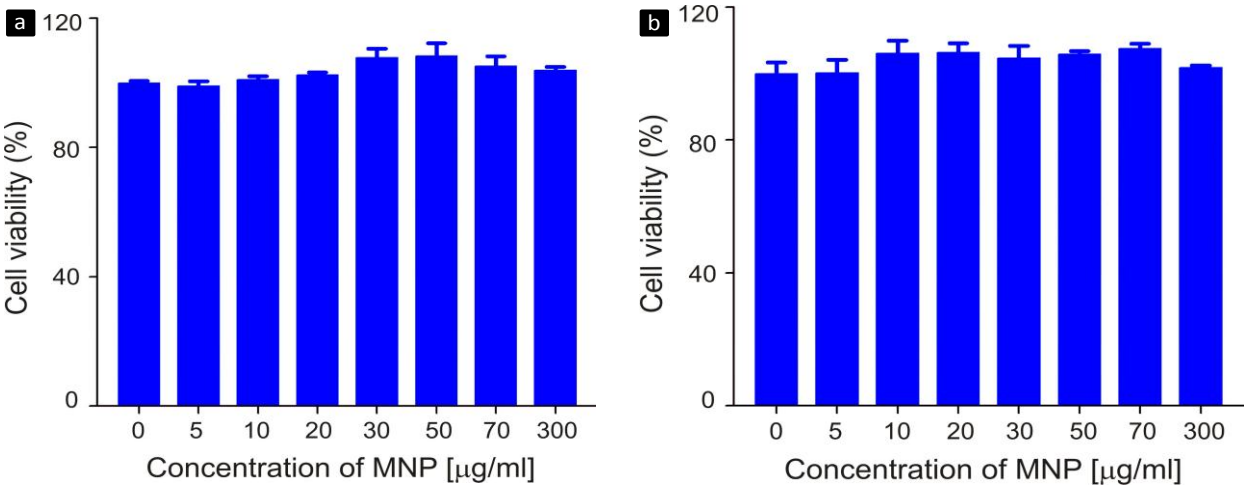

Figure S10. Toxicity assay using MTS for various concentrations of 11 nm FeCo/C NPs in a) U87-EGFP and **b)** U87-EGFRvIII cell lines.MTS assayed wells were quantified with UV absorbance (490 nm) and converted to cell viability. This study shows that there was no FeCo/C MNP-associated cellular toxicity within the concentration range tested.

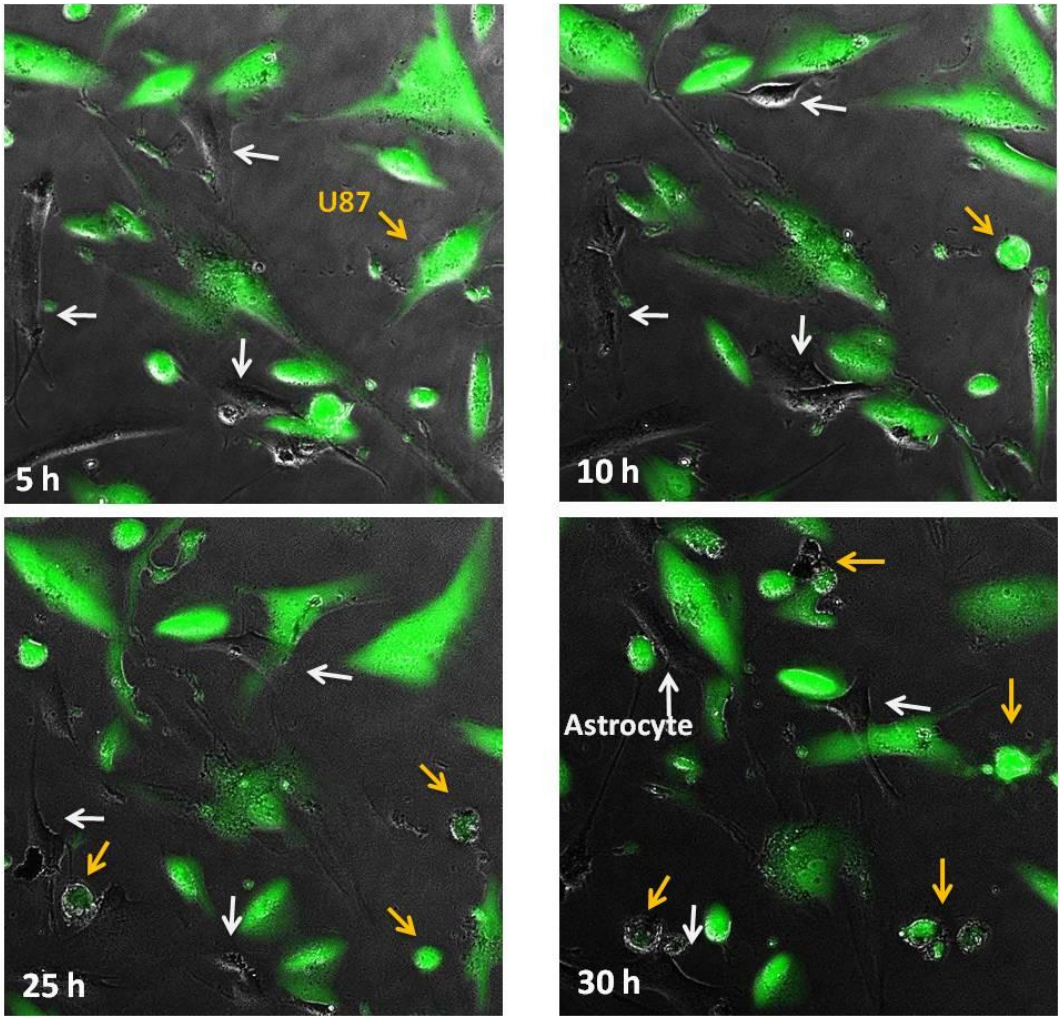

Figure S11. Targeted hyperthermia using FeCo/C NPs in co-cultures of U87-EGFP and astrocytes. U87-EGFP cells were co-cultured with astrocytes and 11 nm FeCo/C NPs with EGFR antibody were selectively delivered to U87-EGFP at a nanoparticle concentration of 10 µg/mL. After 15 minutes of hyperthermia treatment, cells were monitored to check the hyperthermia effect up to 30 h. Images show that U87-EGFP cells are in the process of apoptosis but the astrocytes are still viable.

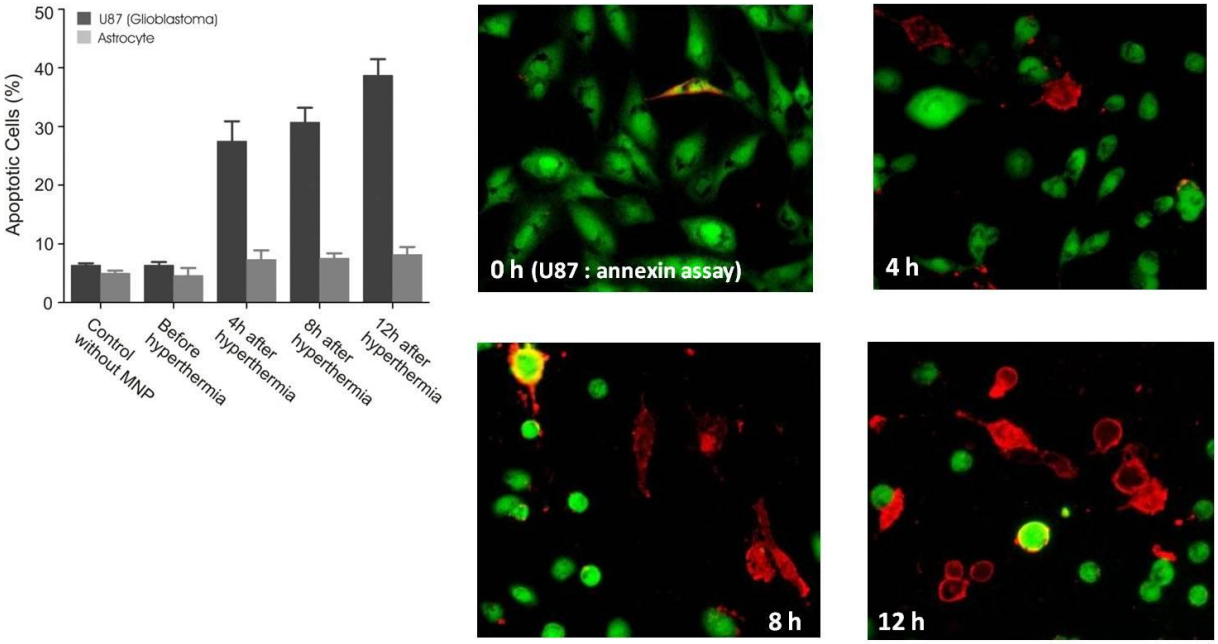

Figure S12. Annexin-V assay for quantification of apoptotic cells in co-cultures of U87-EGFP cells and astrocytes. Apoptotic cells are quantified with annexin-V assay after treatment with 30 µg/mL EGFR-conjugated FeCo/C NPs and subsequent hyperthermia at 334 kHz for 15 min. Astrocytes are not damaged by the FeCo/C, however 40% of U87-EGFP cells are in an early apoptotic process 12 hours after hyperthermia.

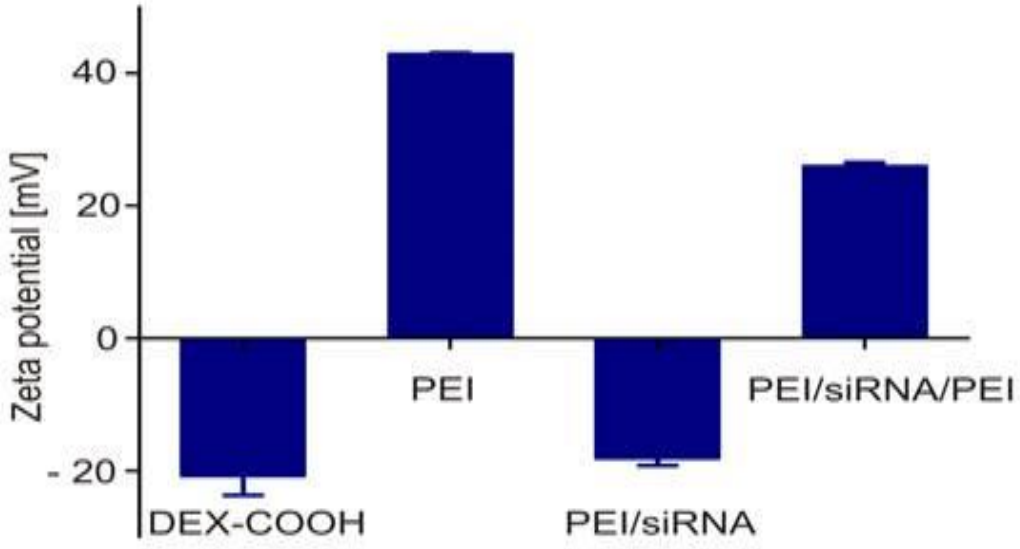

Figure S13. Zeta potential measurements of PEI-siRNA-PEI-FeCo/C MNPs to ensure appropriate layer-by-layer coating. The zeta potential value increased from -20 mV to 40 mv after the coating of a single layer of PEI on carboxydextran-coated FeCo/C NPs. Upon complexation of negatively charged siRNA molecules, the zeta potential value again dropped to -15 mv. Further, on coating a second layer of PEI on the NPs, this value again became positive, thereby indicating a successful layer-by-layer coating of siRNA and PEI on the FeCo/C-dextran NPs.

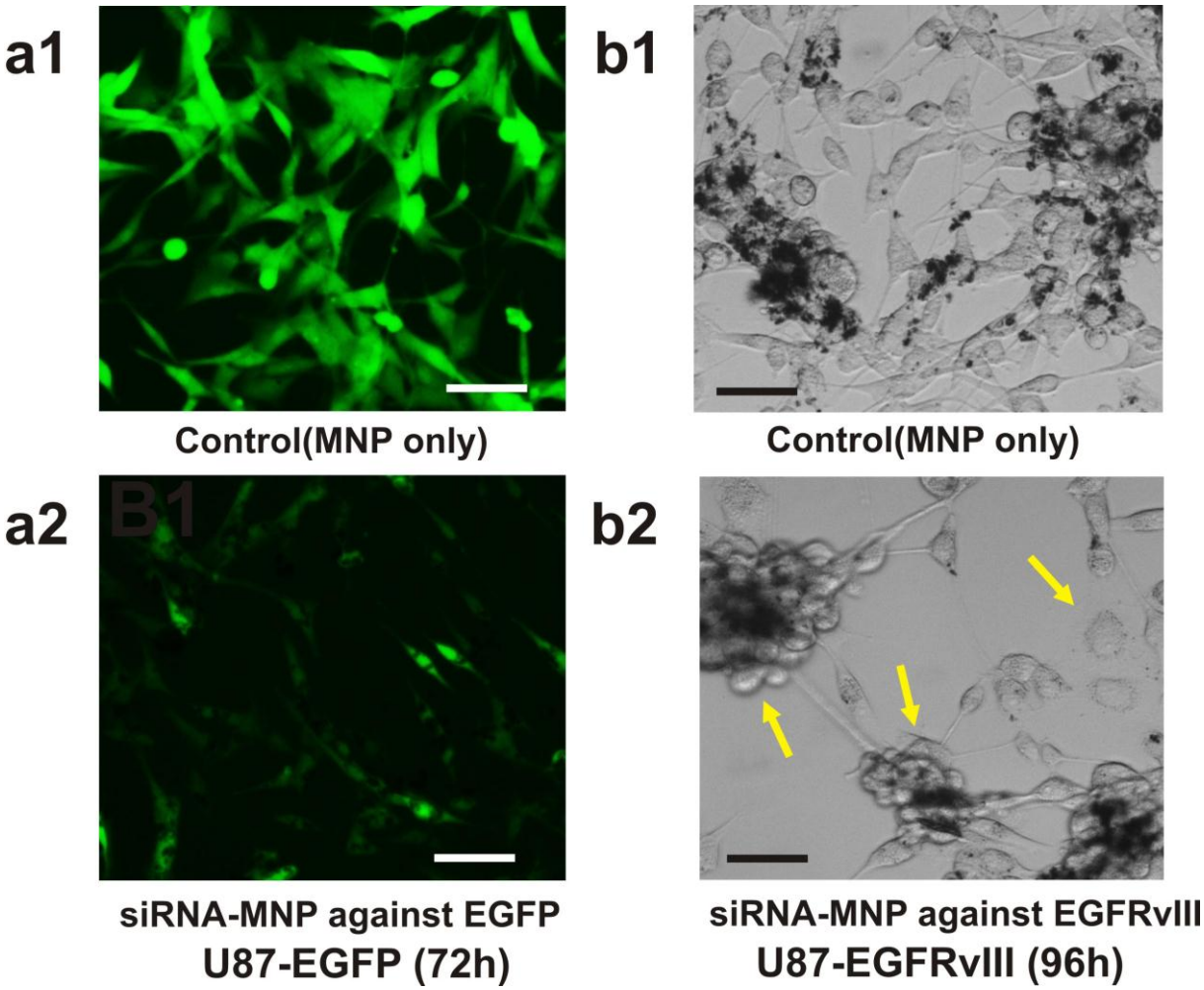

Figure S14. siRNA-mediated gene suppression using FeCo/C-siRNA conjugates in U87 cells a) Knockdown of EGFP in U87-EGFP cells using FeCo/C NPs (**a1**) as compared to controls (**a2**) 72 hours post-transfection and **b**) Effect of knockdown of the EGFRvIII oncogene in U87-EGFRvIII cells using FeCo/C NPs (**b2**) as compared to controls (**b1**). The siRNA-mediated knockdown of EGFRvIII (marked by yellow arrows) leads to a morphological change indicating the induction of apoptosis.

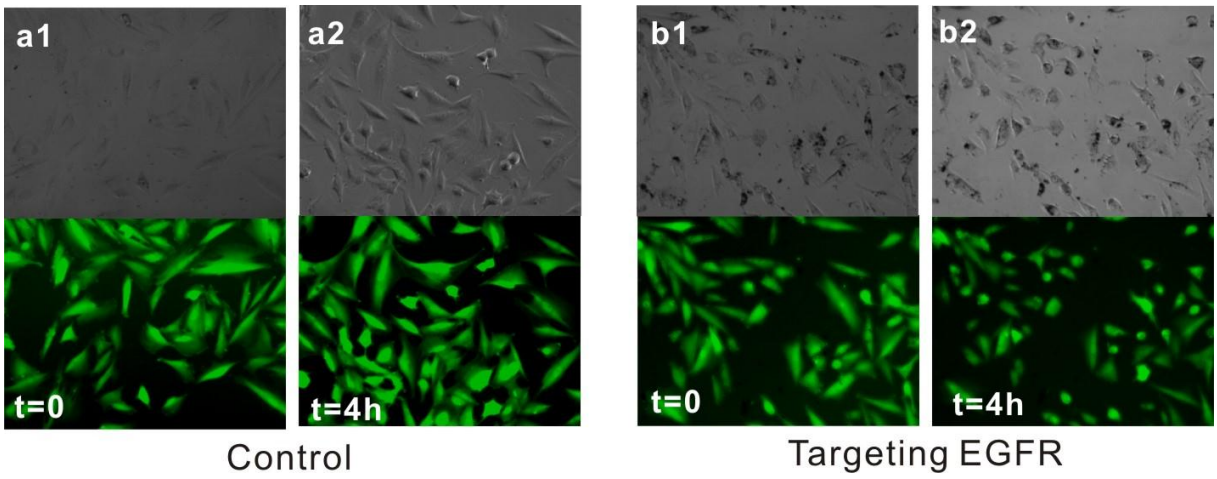

Figure S15. Effect of targeting on the *in vitro* hyperthermal efficiency of the FeCo/C NPs **a)** Phase contrast and fluorescence images of U87-EGFP cells incubated with FeCo/C NPs without EGFR antibody in absence (**a1**) and presence (**a2**) of magnetic field. **b)** In presence of EGFR antibody the cells readily take up the FeCo/C NPs. However, the cell viability is intact in absence of magnetic field (**b2**) as compared to control. On hyperthermal treatment, the cell viability decreases significantly after 4 h.