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Supporting Information

Self-Folded Redox/Acid Dual-Responsive Nanocarriers for Anticancer Drug Delivery

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Experimental Section

Materials

All chemicals were obtained from commercial sources and used without further purification. Tris(2-aminoethyl)amine, di-t-butyl dicarbonate (Boc_2O), trifluoroacetic acid (TFA), *N*,*O*-bis(trimethylsilyl) acetamide (BSA) and triphosgene were purchased from Sigma Aldrich. *N*,*N*′-diisopropylcarbodiimide (DIPC), triethylamine (Et_3N) and 4-dimethylaminopyridine (DMAP) were obtained from Acros Organics. NHS-PEG_{5000}-NHS, NHS-PEG-SS-PEG-NHS and Folic acid-PEG-ma were purchased from Nanocs Inc. 2-Ethoxypropene was purchased from. Doxorubicin (DOX) was from Alfa Aesar. All the organic solvents for synthesis and analysis were ordered from Fisher Scientific Inc. and used as received.

Methods

Synthesis of graft copolymer.

Synthesis of monomer I. N, *N*-bis(2-aminoethyl)-*N*-[2-(tert-butylcarbamoyl) ethyl-amine] (monomer I) was synthesized as reported. Boc_2O (1 g, 4.6 mmol, chloroform solution) was added dropwise into tris(2-aminoethyl)amine (6.7 g, 46 mmol) dissolved in 300 mL of chloroform at 0^{o}C, under stirring. The reaction was allowed to warm up to room temperature and stirred overnight. The reaction was quenched by 15 mL of DD water. The organic phase was separated after being stirred for 5 min. The aqueous phase was re-extracted with 30 mL chloroform. The combined organic phase was first dried with Na_2SO_4, then concentrated under vacuum to yield crude compound, which was further purified with silica gel chromatography using $CHCl_3$/MeOH/concentrated aqueous NH_4OH (v/v/v, 10/5/1) as eluent. The purified monomer I was given as viscous oil.

Synthesis of monomer IV. AcO-L-Serine (500 mg, 3.4 mmol) was dissolved in 20mL tetrahydrofuran (THF), followed by the addition of triphosgene (392 mg, 1.7 mmol). The mixture was allowed to react under 48 ^{o}C for 2 h. The resulted product solution was first purified with silica gel chromatography using petroleum ether/AcOEt (v/v, 2/1 for the 1st elution, 1/1 for the 2nd elution) as eluent. The product solution was then rotary evaporated to give light yellow oil.

Synthesis of multiblock copolymer by condensation polymerization. Multiblock copolymerwas synthesized via one-pot polymerization. Briefly, monomer I, NHS-PEG_{5000}-NHS (monomer II) and NHS-PEG-SS-PEG-NHS (monomer III) were pre-dried in $CaCl_2$ desiccator for two days before synthesis. NHS-PEG_{5000}-NHS (200 mg, 40 μmol) and NHS-PEG-SS-PEG-NHS (14.8 mg, 13 μmol) were dissolved in 2 mL DMF, followed by the addition of 132 μL monomer I (13.8 mg, 50 μmol) solution in DMF under N_2 atmosphere. The reaction was initiated by adding 22.3 μL of Et_3N. Two days later, the reaction mixture was poured into 50 mL diethyl ether. The precipitate was washed twice with diethyl ether, dried in vacuum and yield as white powder.

TMS activation of linear multi-block copolymer. To remove the protecting group Boc, The above resulting copolymer was dissolved in 10 mL of dichloromethane (DCM) and TFA mixture (v/v, 1/1). The scavenger, phenol (70 mg), was also added into the mixture to minimize the side reaction. The reaction mixture was stirred at room temperature for 1 hour. The organic solvent was evaporated in vacuum and the residue was washed with diethyl ether. After removing the remaining organic solvent in vacuum, the residue was dissolved in water and dialysis against DD water with cellulose tubing (MWCO: 12 kDa). The resulting solution was lyophilized and dried in $CaCl_2$ desiccator. The amine groups on the multiblock copolymer were activated with TMS as initiator for graft polymerization. The dry polymer (100 mg) was mixed with 5 mL of anhydrous benzene. After polymer was completely dissolved, 2 mL of *N*,*O*-bis(trimethylsilyl) acetamide (BSA) was added. The reaction was stirred at room temperature for 24 h. Anhydrous hexane (10 mL) was added into the reaction mixture to precipitate the product. The white precipitate was washed three times with anhydrous hexane and dried under vacuum to yeild TMS-activated multi-block copolymer.

Graft polymerization. The Ring-Opening Polymerization of the side chain was initiated by N-TMS Amines. Briefly, monomer IV (127 mg) and TMS-activated multi-block copolymer (97.3 mg) was suspended in 5 mL of dry chloroform. The yellow suspension gradually turned clear after adding 20 μL of Et_3N. The reaction gradually became turbid over time, indicating the successful polymerization. Forty eight hours later, the reaction mixture was poured into 30 mL of diethyl ether to precipitate the product. After washing three times with diethyl ether, the precipitate was dried under vacuum to give graft copolymer. The yielded graft copolymer was dissolved in water with the addition of LiOH. The hydrolytic was then dried under vacuum.

Modification of side chains. 2-Ethoxypropene was added to the DMSO solution of graft copolymer with the existence of PPTS under magenitic stirring. After reacting for 1 h, the reaction process was quenched by TEA.

Preparation and characterization of DOX/RAD-NCs.

To obtain self-folded RAD-NCs, 1 mL of the graft copolymer solution (dissolved in DMSO) was drop-wisely injected ($2mL \cdot h^{-1}$) into 4 mL of DD water under vigorously magnetic stirring. The RAD-NCs solution was then transferred into centrifuge tube and sonicated for 30 cycles (1 s each with a duty cycle of 20%). The homogenous RAD-NCs solution was dialyzed against DD water for 48 hours at room temperature in order to completely get rid of organic solvent DMSO. The DD water was frequently changed during the dialysis process. The folded RAD-NCs were stored at 4 ^oC for further characterization. In order to encapsulate DOX into RAD-NCs, DOX·HCl was added into DMSO together with TEA under stirring. 1 mL of graft copolymer solution was then added into the above reaction mixture. The resulting homogenous solution was injected slowly into DD water as described above.

***In vitro* drug release.**

The release profiles of DOX were assayed using dialysis method in PBS buffer as reported. [50] 400 μL DOX/RAD-NCs solution with 14 mg of DOX was added into a dialysis tube (10 K MWCO) (Slide-A-Lyzer, Thermo Scientific) against 14 ml of PBS buffer solution with different pH (7.4 and 5.0) and different concentrations of GSH (0, 1 mM and 10 mM). The dialysis tube was immerged in 37 °C water bath. At predetermined time intervals, the total buffer solution was withdrawn, followed by replacing with 14 ml of fresh buffer solution with the same pH and GSH concentration. The fluorescence intensity of released DOX was measured at 596nm with an excitation wavelength of 480 nm by microplate reader (Infinite M200 PRO, Tecan).

Insertion of target ligand.

GSH (0.5 mM) was added into 2 mL DOX/RAD-NCs solution under magnetic stirring for 5 min, dialyzed against DD water with cellulose tubing (MWCO: 12 kDa), followed by the adding of folic acid-PEG-maleimide (1 mg, 0.5 μmol). After stirred for 1 h, the reaction mixture was dialysed against DD water with cellulose tubing (MWCO: 12 kDa).

Cell culture.

HeLa cells were obtained from Tissue Culture Facility of UNC Lineberger Comprehensive Cancer Center and cultured in DMEM with 10% (v:v) fetal bovine serum (FBS), 100U • ml^{-1} penicillin and 100 $mg \cdot ml^{-1}$ streptomycin in an incubator (Thermo Scientific) at 37 ℃ under an atmosphere of 5% CO_2 and 90% relative humidity. The cells were sub-cultivated approximately every 3 days at 80% confluence using 0.25% (w: v) trypsin at a split ratio of 1:5.

Cellular uptake mechanism.

HeLa cells (1×10^5 cells per well) were seeded in 6-well plates. After culture for 48 h, the cells were pre-incubated with several specific inhibitors for various kinds of endocytosis [inhibitor of clathrin-mediated endocytosis: sucrose (SUC, 450 mM) and chlorpromazine (CPZ, 10 μM); inhibitor of caveolin-mediated endocytosis: nystatin (NYS, 25 μg mL^{-1}); inhibitor of macropinocytosis: amiloride (AMI, 1 mM); inhibitor of lipid raft: methyl-*β*-cyclodextrin (MCD, 3 mM)] for 1 h at 37 °C, respectively. Afterward, the cells were incubated with DOX/RAD-NCs at a DOX concentration of 1 mg/L in the presence of inibitors for additional 2 h. After washing the cells by PBS twice, the fluorescence intensity of DOX in the cells and the cell proteins were measured, respectively.

Intracellular trafficking.

HeLa cells (1 × 10^5 cells per well) were seeded in a confocal microscopy dish (MatTek). After culture for 24 h, the cells were incubated with DOX/RAD-NCs and DOX/FA-RAD-NCs (2 mM Dox concentration) at 37 °C for 1 h and 4 h, respectively, and then washed by PBS twice. Subsequently, the cells were stained by LyosTracker Green (50 nM) (Life Technologies) at 37 °C for 30 min and Hoechst 33342 (1 $mg \cdot ml^{-1}$) (Life Technologies) at 37 °C for 10 min. Finally, the cells were washed by PBS twice and immediately observed using CLSM (LSM 710, Zeiss).

***In vitro* cytotoxicity.**

HeLa cells (1×10^4 cells per well) were seeded in 96-well plates. After 24 h culture, the cells were exposed to the PBS buffer, free DOX solution, DOX/RAD-NCs and DOX/FA-RAD-NCs with different concentrations of DOX for 24 h and 48 h, respectively. 20 μL MTT solution (5 $mg \cdot mL^{-1}$) was then added to each well. After 4 h of incubation, the medium was removed, and 150 ml DMSO was added to each well. The absorbance was measured at a test wavelength of 570 nm and a reference wavelength of 630 nm by microplate reader (Infinite M200 PRO, Tecan).

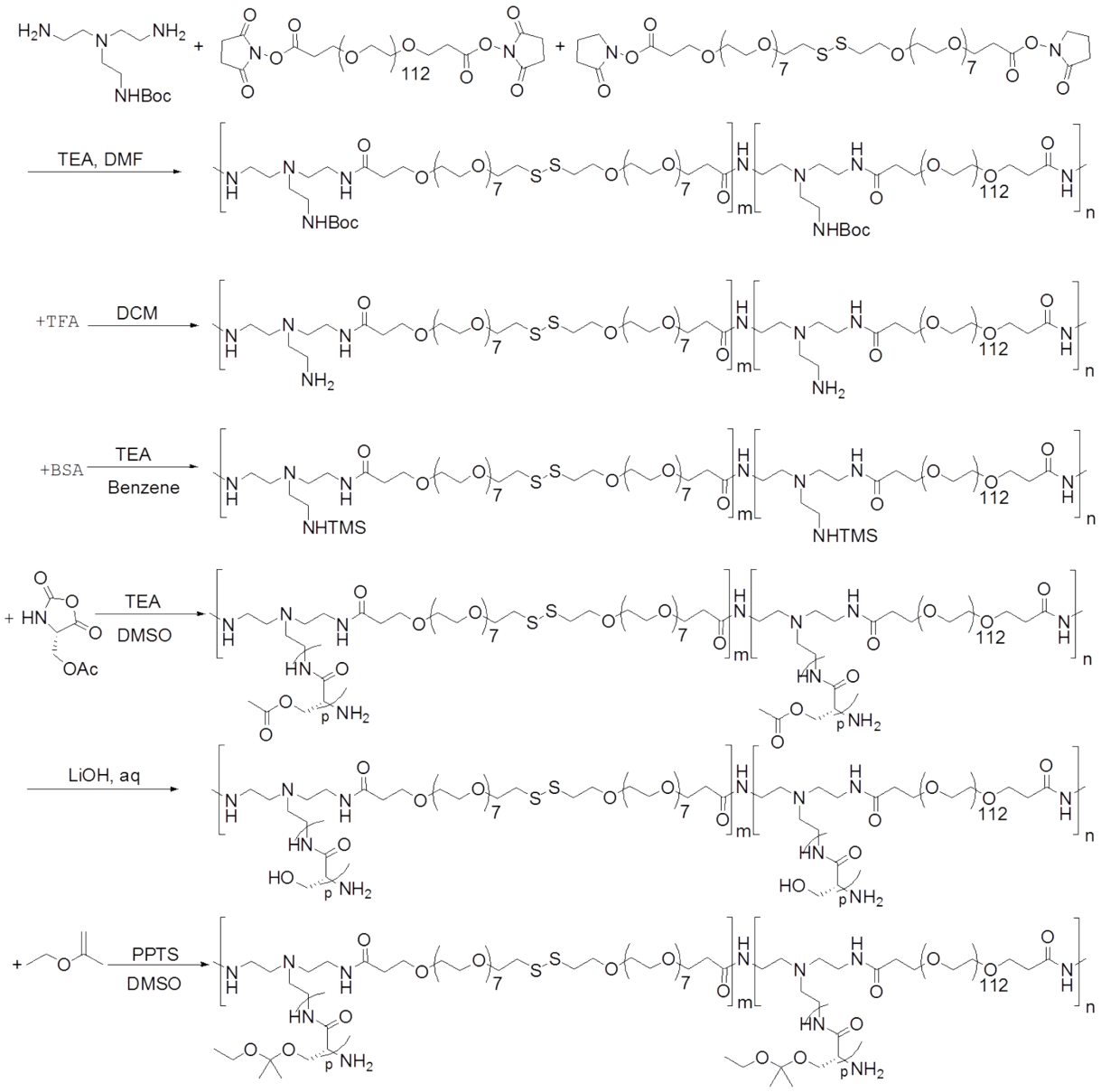

Figure S1. Synthesis route of graft copolymer. Monomer I, monomer II and monomer III were firstly condensed into a linear multiblock copolymer by NHS mediated amidation. After liberating the third amino group of monomer I, the side chains were gradually elongated on the main chain *via* a TMS controlled ROP of monomer IV. The side chains were then modified with 2-ethoxypropene into the target graft copolymer capable of self-folding.

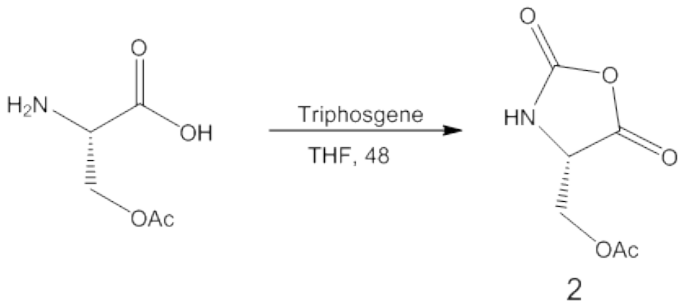

Figure S2. Synthesis route of AcO-L-Serine-NCA (monomer IV).

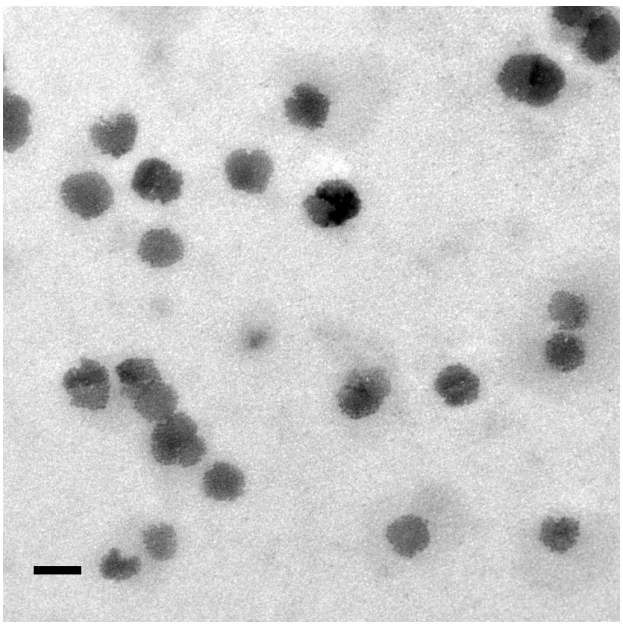

Figure S3. Representative TEM images of DOX/RAD-NCs. Scale bar 200 nm.

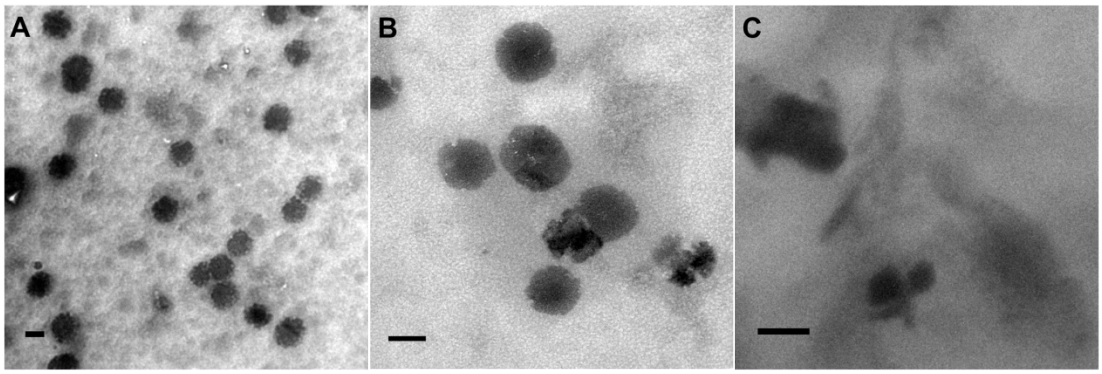

Figure S4. Representative TEM images of A) Folic-acid conjugated RAD-NCs. B) DOX/ FA-RAD-NCs. C) FA-RAD-NCs treated with GSH and acid (pH 5.0). Scale bars 100 nm.

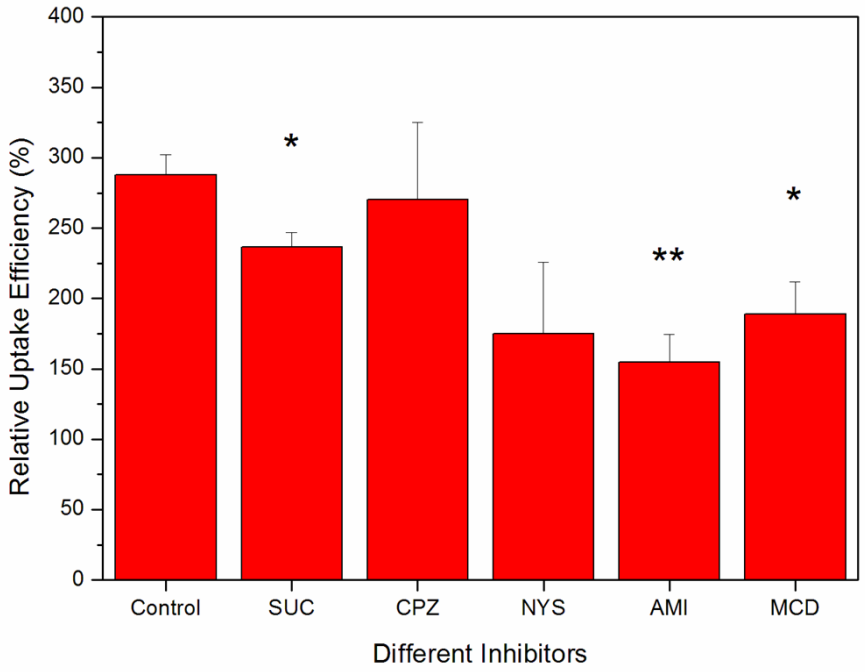

Figure S5. Relative uptake efficiency of DOX/RAD-NCs on HeLa cells in the presence of various endocytosis inhibitors. Error bars indicate s.d. (n = 3). *P < 0.05, **P < 0.01 compared with the control group (two-tailed Student's t-test). Inhibitor of clathrin-mediated endocytosis: sucrose (SUC) and chlorpromazine (CPZ); inhibitor of caveolin-mediated endocytosis: nystatin (NYS); inhibitor of macropinocytosis: amiloride (AMI); inhibitor of lipid raft: methyl-β-cyclodextrin (MCD). Compared with the cellular uptake of DOX/RAD-NCs without inhibitors as a control, the significant decrease in uptake of DOX/RAD-NCs with inhibitors confirmed the corresponding endocytosis pathways of the nanocarriers.

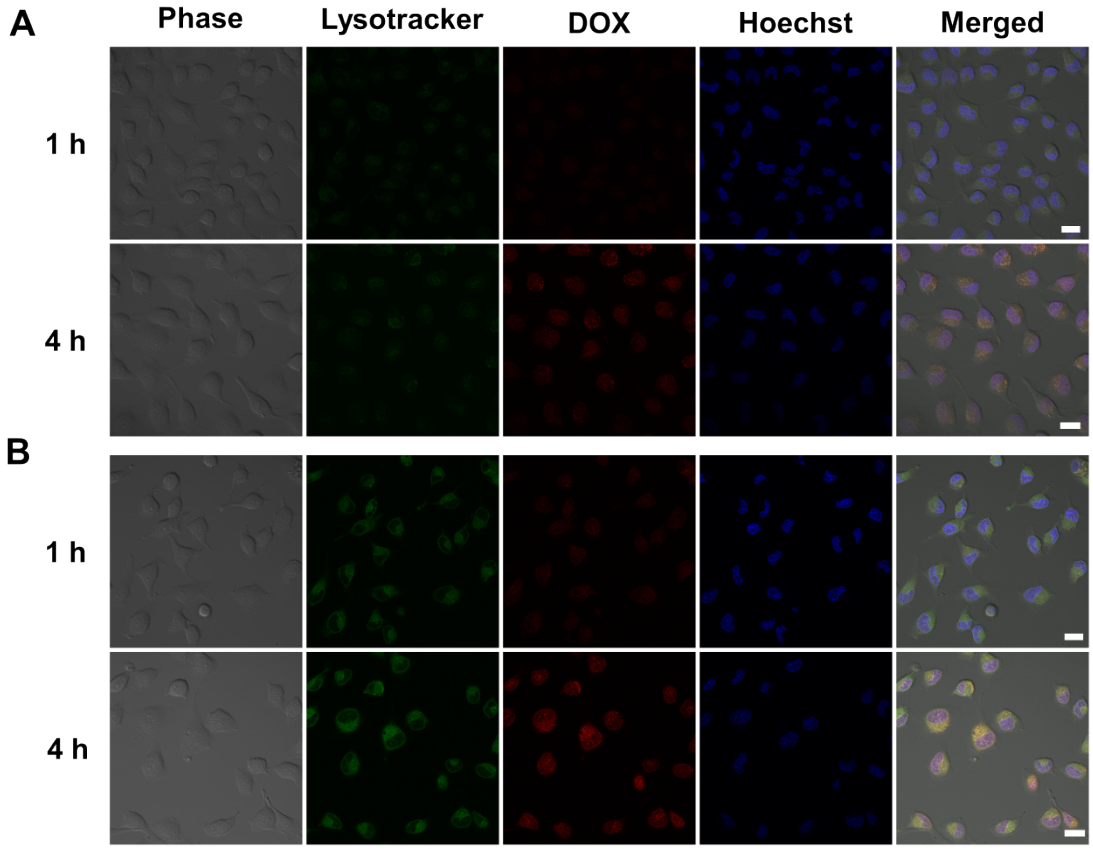

Figure S6. Intracellular trafficking of DOX/RAD-NCs (A) and DOX/FA-RAD-NCs (B) on HeLa cell line observed by CLSM (large cell numbers). The late endosomes and lysosomes were stained by LysoTracker Green, and the nuclei were stained by Hoechst 33342. Scale bar: 20 μm.

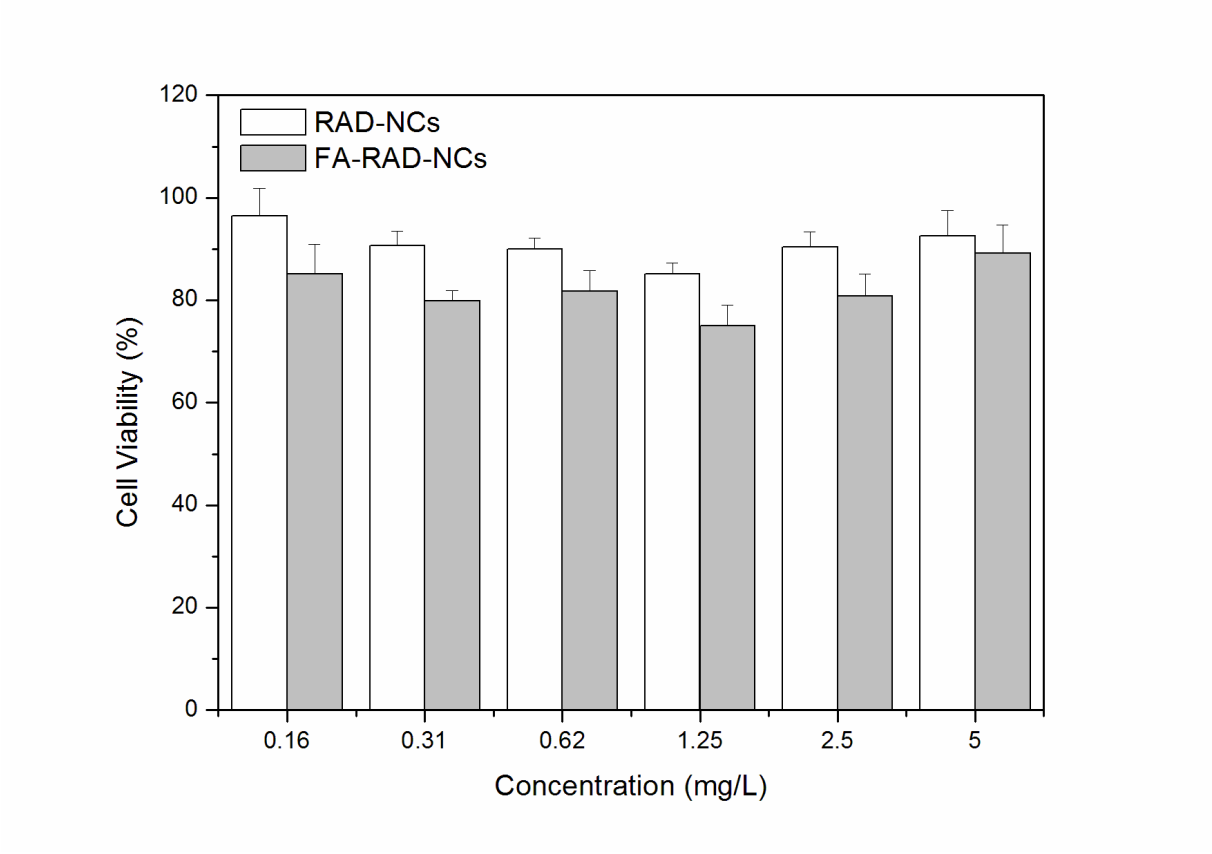

Figure S7. Cytotoxicity study of empty RAD-NCs and FA-RAD-NCs after 48 hour culture with HeLa cells. The horizontal ordinate indicates the concentration of RAD-NCs and FA-RAD-NCs. Error bars indicate s.d. (n=4).