Supporting Information

Dual Responsive Supramolecular Nanogels for Intracellular Drug Delivery

Xiaofei Chen,[a] Li Chen,[a]* Xuemei Yao,[a] Zhe Zhang,[a] Chaoliang He,[b]* Jingping Zhang,[a] and Xuesi Chen[b]

Materials

Dextran (Dex, M_n=40 kDa) was obtained from Sigma-Aldrich and used as received. 5-Benzimidazolecarboxylic acid (96%), benzoic acid (99.5%), glutathione (GSH), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl), 4-dimethylaminopyridine (DMAP) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich and used directly. Thiol-β-cyclodextrin (98.5%) was bought from Shandong Binzhou Zhiyuan Bio-Technology Co., Ltd). Doxorubicin hydrochloride (DOX·HCl) was purchased from Zhejieng Hisun Pharmaceutical Co.,Ltd. Dimethyl sulfoxide (DMSO) was dried over calcium hydride (CaH_2) and purified by vacuum distillation with CaH_2. All the other reagents and solvents were purchased from Sinopharm Chemical Reagent Co., Ltd and used as obtained.

Characterizations

^{1}H NMR spectra were recorded on a Bruker AV 400 NMR spectrometer in dimethyl sulfoxide-d_6 (DMSO-d_6). FT-IR spectra were recorded on a Bio-Rad Win-IR instrument using the potassium bromide (KBr) method. Transmission electron microscopy (TEM) measurements were performed on a JEOL JEM-1011 transmission electron microscope with an accelerating voltage of 100 Kv. A drop of the nanogel solution (0.1 g L^{-1}) was deposited onto a 230 mesh copper grid coated with carbon and allowed to dry in air at 25 ^{o}C before measurements. Dynamic laser scattering (DLS) measurements were performed on a WyattQELS instrument with a vertically

polarized He-Ne laser (DAWN EOS, Wyatt Technology). The scattering angle was fixed at 90°.

Synthesis of dextran-graft-benzimidazole (Dex-*g*-BM)

5-Benzimidazolecarboxylic acid (0.35 g, 2.160 mmol), Dex (0.5 g, 0.0125 mmol), EDC·HCl (0.825 g, 4.304 mmol), DMAP (0.056 g, 0.459 mmol) were dissolved in 40 mL of DMSO in a glass ampoule with a magnetic bar. The reaction was performed at 25 ^{o}C for 48 h. Then, the solvent and unreacted substances were removed by dialysis against deionized water for 72 h. The solution was lyophilized to give the product Dex-*g*-BM as a pink solid (yield: 79.6%).

Synthesis of dextran-graft-benzoic acid (Dex-*g*-BA)

Benzoic acid (0.26 g, 2.131 mmol), Dex (0.5 g, 0.0125 mmol), EDC·HCl (0.76 g, 3.979 mmol), DMAP (0.035 g, 0.287 mmol) were dissolved in 35 mL of DMSO in a glass ampoule with a magnetic bar. The reaction was performed at 25 ^{o}C for 48 h. Then, the solvent and unreacted substances were removed by dialysis against deionized water for 72 h. The solution was lyophilized to give the product Dex-*g*-BM as a pink solid (yield: 82.4%).

Preparation of disulfide-core-cross-linked Dex-*g*-BM supramolecular nanogels (SNG)

The crosslinking is based on the supramolecular interactions between the thiol-β-cyclodextrin (β-CD-SH) and BM. Typically, Dex-*g*-BM (0.1 g, 0.00228 mmol) and β-CD-SH (0.04328 g, 0.03435 mmol) were dissolved in 100 mL of pH 7.4 PBS and added oxygen for 120 min. After stirring for 36 h at room temperature, the solution was dialyzed against deionized water for 48 h. Then the solution was lyophilized (yield: 88.6%). (Table S1)

***In vitro* drug loading and release**

Doxorubicin (DOX) was used as a model drug for *in vitro* drug loading and release. DOX loaded in nanogels were prepared by a simple dialysis technique. Typically, nanogels (20.0 mg), drug (4 mg) were mixed in 2.0 mL of DMSO. The mixture was stirred at room temperature for 24 h and then added dropwise into 20.0 mL of PBS at pH 7.4. The DMSO was removed by dialysis against deionized water at pH 7.4 for 24

h. The dialysis medium was refreshed four times and the whole procedure was performed in the dark. Finally, the solution was filtered and lyophilized. To determine the drug loading content (DLC) and drug loading efficiency (DLE), the drug-loaded nanogels were dissolved in DMSO and analyzed by fluorescene measurement (Perkin-Elmer LS50B luminescence spectrometer) using a standard curve method. The DLC and DLE of drug-loaded nanogels were calculated according to eqn (1) and (2), respectively:

DLC (wt %) = amount of drug in nanogels/amount of drug loaded nanogels× 100 (1)

DLE (wt %) = amount of drug in nanogels/total amount of feeding drug× 100 (2)

In vitro drug release profiles of drug-loaded nanogels were investigated in PBS (at pH 5.5, 6.8 or 7.4) without or with 10 mM GSH. The pre-weighed freeze-dried DOX loaded nanogels were suspended in 3 mL of release medium and transferred into a dialysis bag (MWCO 3500 Da). The release experiment was initiated by placing the end-sealed dialysis bag into 50 mL of release medium at 37 ^{o}C with continuous shaking at 70 rpm. At predetermined intervals, 2 mL of external release medium was taken out and an equal volume of fresh release medium was replenished. The amount of released DOX was determined by using fluorescence measurement. The release experiments were conducted in triplicate.

Intracellular drug release

The cellular uptake and intracellular release behaviors of DOX-loaded nanogels were assessed by confocal laser scanning microscopy (CLSM) and flow cytometric analyses on HepG2 cells.

CLSM

For CLSM study, HepG2 cells were seeded in 6-well plates at a density of 2 × 10^5 cells per well in 2.0 mL of complete Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum, supplemented with 50 IU mL^{-1} penicillin and 50 IU mL^{-1} streptomycin, and cultured for 24 h, and then treated with 10 mM GSH for 2

h. Cells were washed by PBS and incubated at 37℃ for additional 3 h with DOX-loaded nanogels at a final DOX concentration of 10.0 mg L^{-1} in complete DMEM. Then, the culture medium was removed and cells were washed with PBS thrice. Thereafter, the cells were fixed with 4 % paraformaldehyde for 30 min at room temperature, and the cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue) for 20 min. CLSM images of cells were obtained through confocal microscope (Olympus FluoView 1000). The excitation wavelengths of DAPI and DOX were 405 and 488 nm, emission windows were 425-475 nm and 564-620 nm, repectively.

Flow cytometric analyses

HepG2 cells were seeded in 6-well plates at 2×10^5 cells per well in 2.0 mL of complete DMEM, and cultured for 24 h, and then treated with 10 mM GSH for 2 h. The cells were then washed by PBS and incubated at 37 °C for additional 3 h with DOX-loaded nanogels at a final DOX concentration of 10.0 mg L^{-1} in complete DMEM. Thereafter, the culture medium was removed and the cells were washed with PBS thrice and treated with trypsin. Then, 2.0 mL of PBS was added to each culture well, and the solutions were centrifuged for 4 min at 3000 rpm. After the removal of supernatants, the cells were resuspended in 0.3 mL of PBS. Data for 1×10^4 gated events were collected, and analysis was performed by flow cytometer (Beckman, California, USA).

Cell viability assays

The relative cytotoxicities of nanogels against HepG2 or Hela cells were evaluated *in vitro* by a standard MTT assay. The cells were seeded in 96-well plates at 1×10^4 cells per well in 200.0 μL of complete DMEM and incubated at 37 °C in 5 % CO_2 atmosphere for 24 h. The culture medium was then removed and nanogels solutions in complete DMEM at different concentrations (0-50 mg L^{-1}) were added. The cells were subjected to MTT assay after being incubated for additional 24 h. The absorbance of the solution was measured on a Bio-Rad 680 microplate reader at 490 nm. Cell viability (%) was calculated based on eqn (3):

$$\text{Cell viability (\%)} = A_{sample} / A_{control} \times 100 \quad (3)$$

Where, A_{sample} and $A_{control}$ represent the absorbances of the sample and control wells, respectively.

The cytotoxicities of DOX-loaded nanogels against HepG2 and HeLa cells were also evaluated *in vitro* by a MTT assay. Similarly, cells were seeded into 96-well plates at 1×10^4 cells per well in 200.0 μL of complete DMEM and further incubated for 24 h. After washing cells with PBS, 180.0 μL of complete DMEM and 20.0 μL of DOX-loaded nanogel solutions in PBS were added to form culture media with different DOX concentrations (0-10.0 mg L^{-1} DOX). The cells were subjected to MTT assay after being incubated for 24, 48 and 72 h. The absorbance of the solution was measured on a Bio-Rad 680 microplate reader at 490 nm. Cell viability (%) was also calculated based on eqn (3).

Table S1. Characterizations of Nanogels

Entry	Sample (mg)	β-CD-SH [a] (mg)	R_h [b] (nm)	DLC (wt. %)	DLE (wt. %)	CMC [c] (mg mL^{-1})
SNG-1	Dex-BM (100)	43.28	136	4.96	26.41	0.067
SNG-2	Dex-BM (100)	86.57	131	5.75	38.79	0.082
NG	Dex-BA (100)	48.98	129	6.42	43.56	0.041

[a] The crosslinked feed ratios were calculated from eqn (4).

[b] Determined at pH 7.4

[c] Determined in PBS at pH 7.4

Crosslinked feed ratios (mg) = $a / (b + r*M_x)$ eqn (4)

Where a represents 100 mg dextran, b represents the mole mass of every unit of dextran, r represents the molar ratio of BM or BA to Dex, M_x represents the mole mass of BM or BA, respectively.

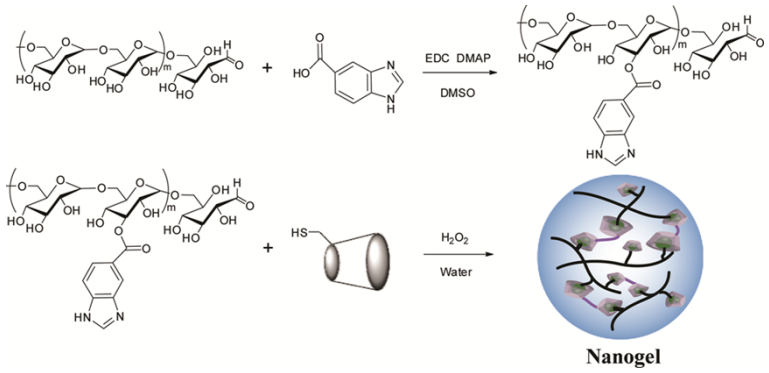

Fig. S1 Synthetic route for SNG

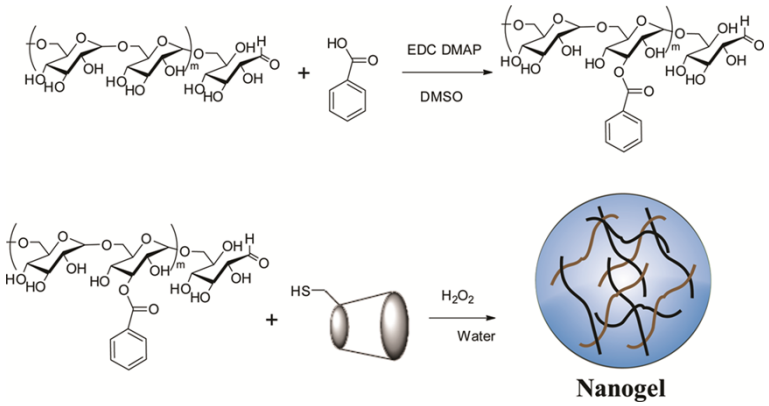

Fig. S2 Synthetic route for NG

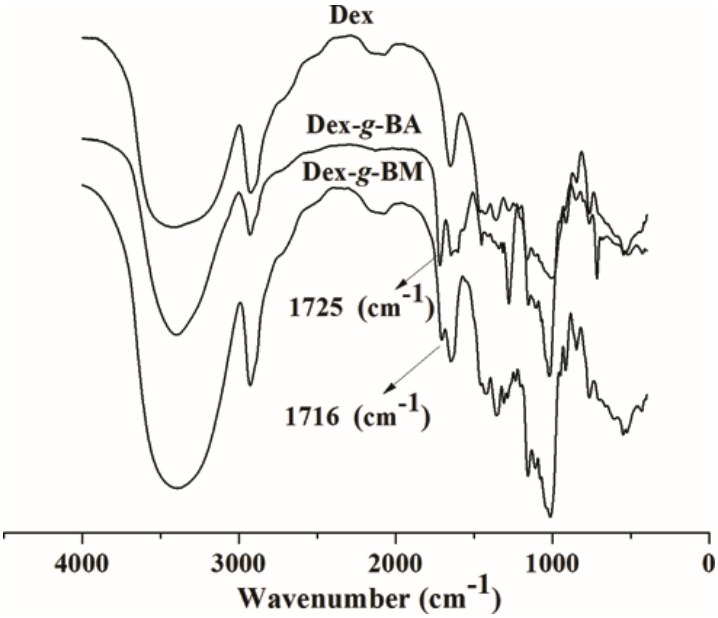

Fig. S3 FT-IR spectra of Dex, Dex-*g*-BA and Dex-*g*-BM

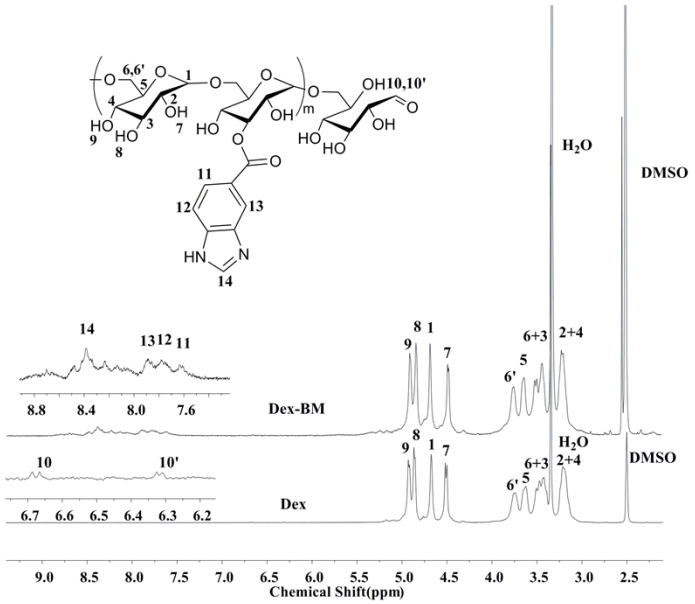

Fig. S4 [1]H NMR spectra of Dex-*g*-BM

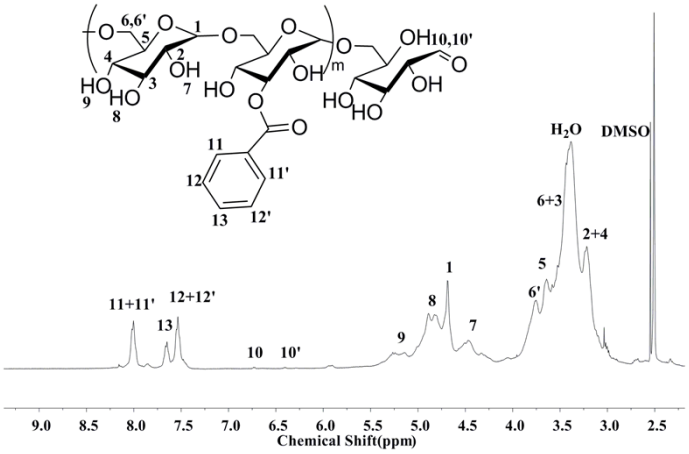

Fig. S5 [1]H NMR spectrum of Dex-*g*-BA

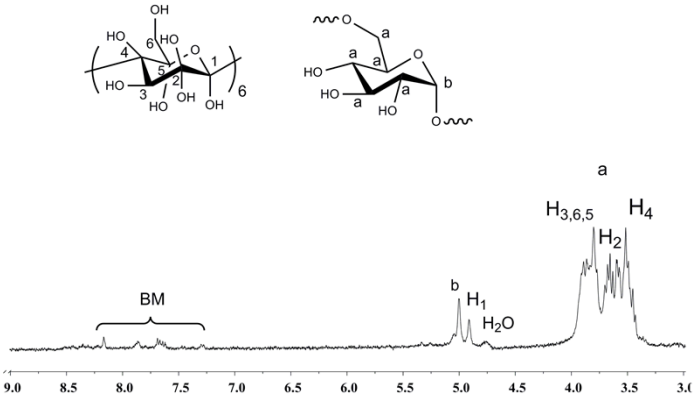

Fig. S6 [1]H MR spectrum of SNG-1

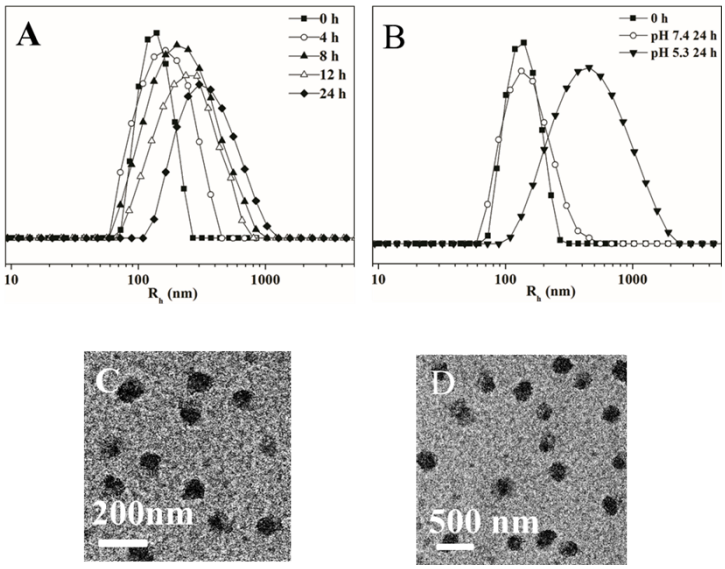

Fig. S7 The hydrodynamic radii (R_h) of SNG-1 in PBS at pH 7.4 treated with GSH for different intervals (A), the hydrodynamic radii (R_h) of SNG-1 in PBS at different pH (B). TEM micrographs of SNG-1 at pH 7.4 (C) and at pH 7.4 with GSH (D), respectively.

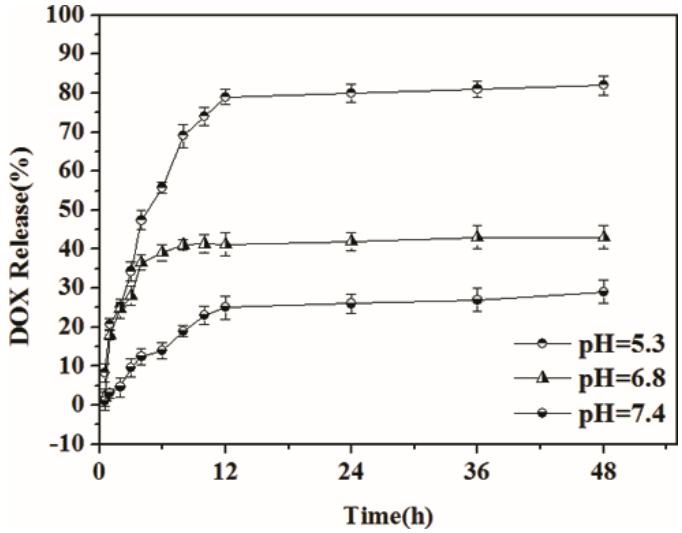

Fig. S8 *In vitro* DOX release profiles for DOX-loaded SNG-2 in PBS at 37℃

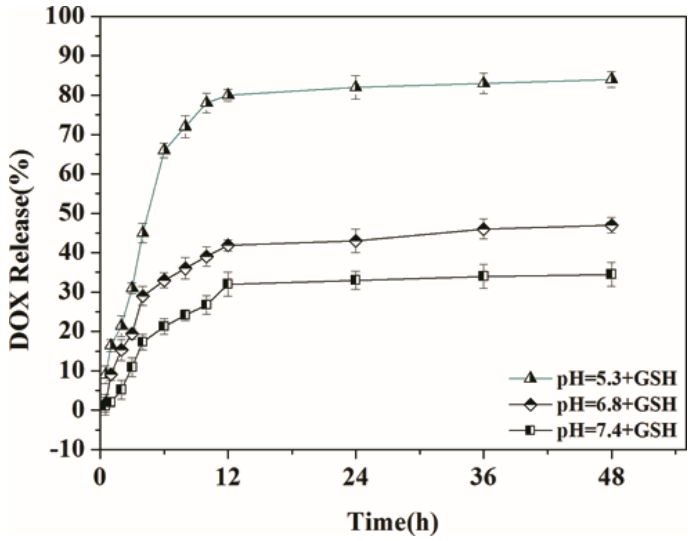

Fig. S9 *In vitro* DOX release profiles for DOX-loaded SNG-2 in PBS at 37℃ with GSH

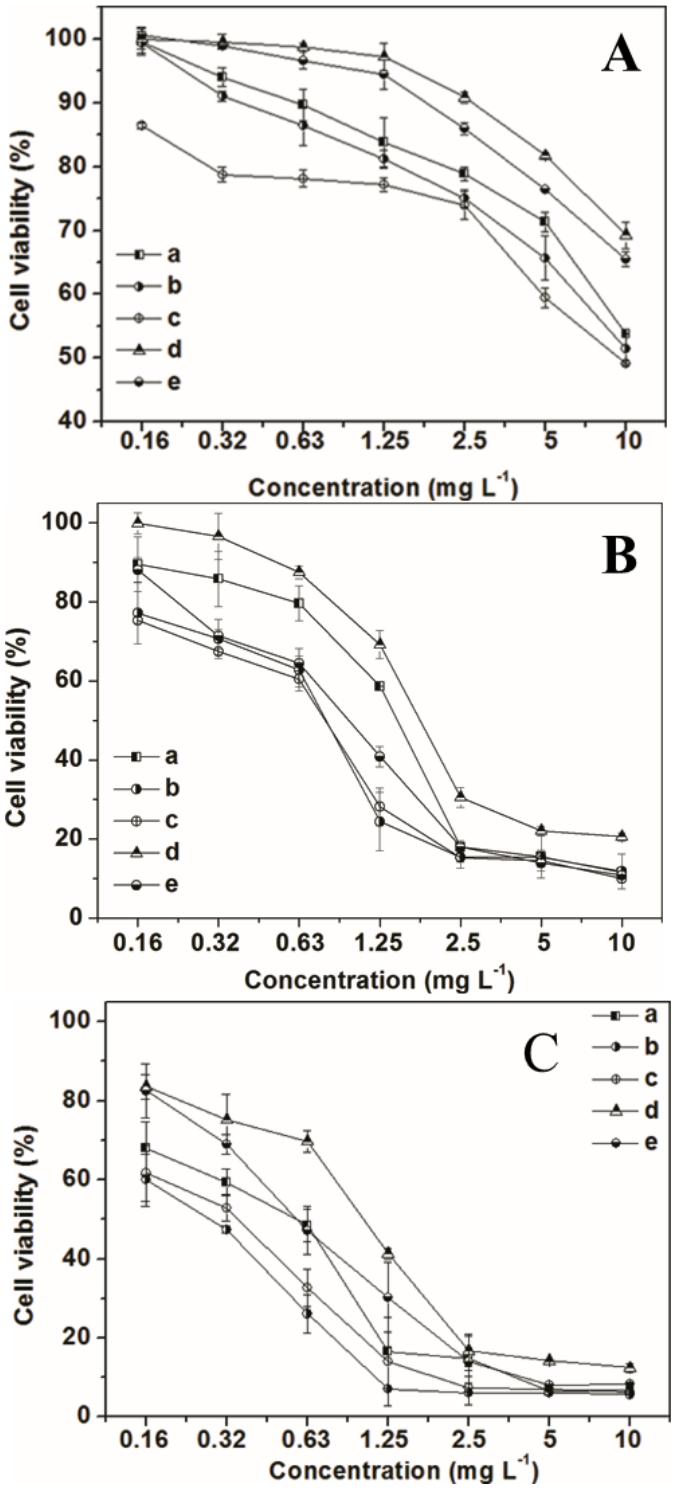

Fig. S10 Cytotoxicities of DOX-loaded SNG-1 (a), SNG-1 with GSH (b), DOX (c), DOX-loaded NG (d) and DOX-loaded NG with GSH (e) toward HeLa cells after incubation for 24 h (A), 48 h (B), and 72 h (C).

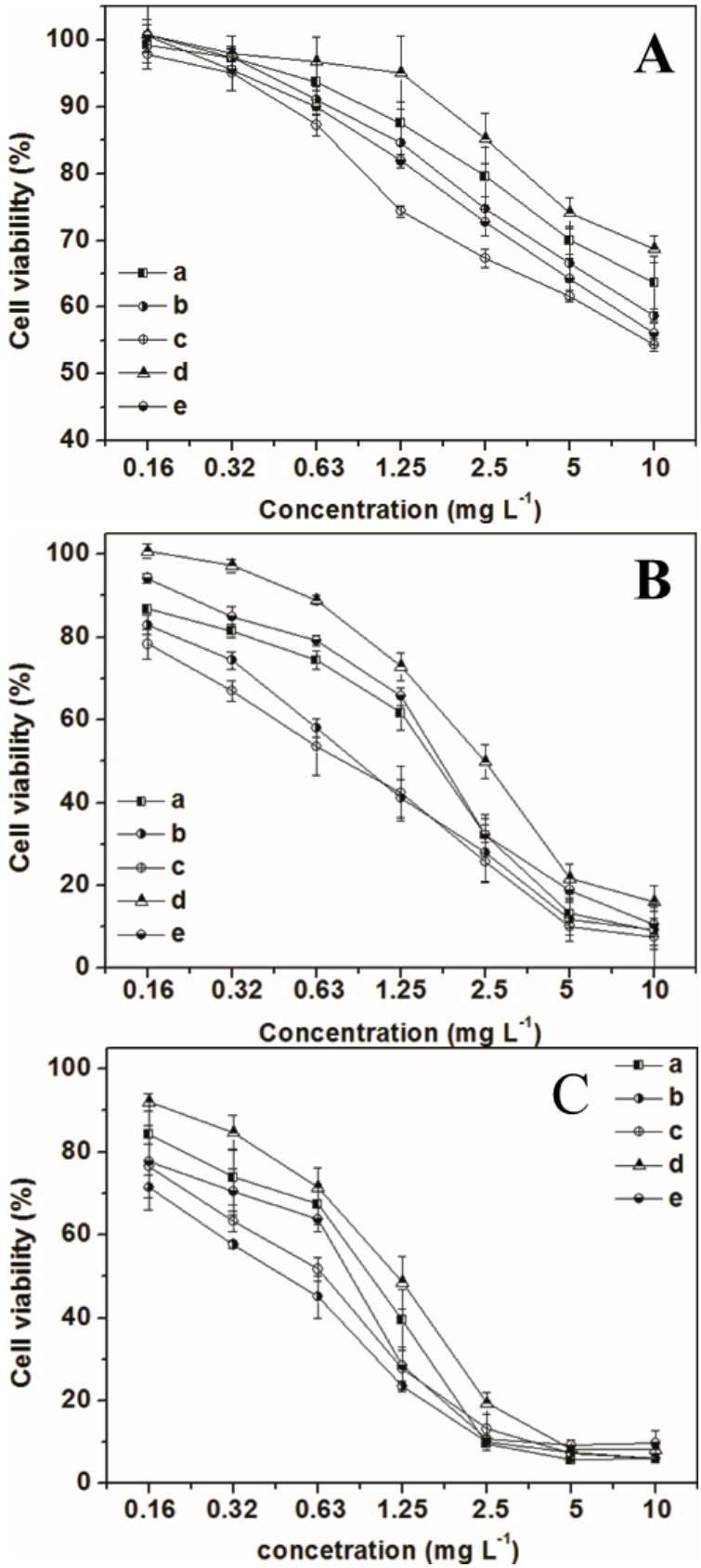

Fig. S11 Cytotoxicities of DOX-loaded SNG-1 (a), SNG-1 with GSH (b), DOX (c), DOX-loaded NG (d) and DOX-loaded NG with GSH (e) toward HepG2 cells after incubation for 24 h (A), 48 h (B), and 72 h (C).

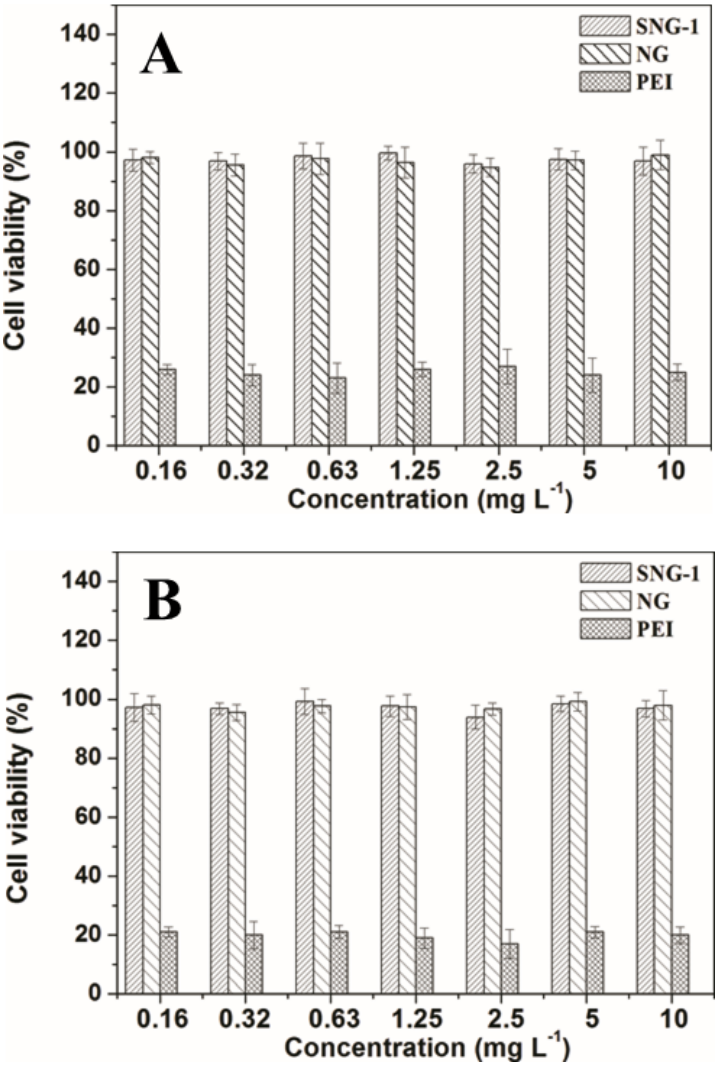

Fig S12 Cytotoxicities of SNG-1, NG and PEI toward HeLa (A) and HepG2 (B) cells after incubation for 72 h.

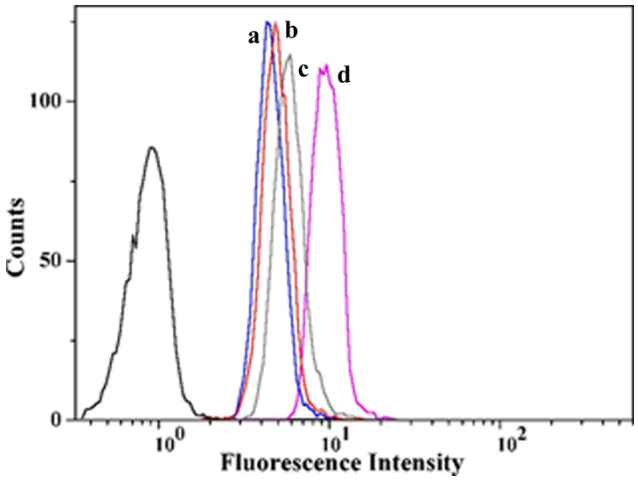

Fig S13 Flow cytometric profiles of HepG2 cells incubated with DOX-loaded nanogels for 3 h: cells incubated with NG (a), cells pretreated with 10mM GSH incubated with NG (b), cells incubated with SNG-1(c) and cells pretreated with 10mM GSH incubated with SNG-1 (d).