Supporting Information:

The Effect of Molecular Structure on Cytotoxicity and Antitumor Activity of PEGylated Nanomedicines

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EXPERIMENTAL METHODS

Characterizations: The details of ^{1}H NMR, DLS and TEM were shown in our previous work.[1,2] The quantification of Dox·HCl, PEG-Dox, was carried by an Agilent 1100 Series HPLC (Agilent Technologies, Palo Alto, CA, USA) coupled to a Triple TOF 5600 mass spectrometer (Sciex, Ontario, Canada) equipped with a TurboIonSpray source (LC-ESI-MS). Data acquisition and integration were controlled by Analyst Software. The chromatography was performed on a PLRP-S 1000A column (150 × 2.1 mm, 8 μm) maintained at room temperature. Gradient elution was 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) with a flow rate of 0.4 mL/min. The gradient elution program: 0-2min 70 % A; 2-5min 70→50 % A; 5-9.6min 5 % A; 9.6-12min 70 % A. Optimal MS parameters were as follows: Nebulizer, heater and curtain gas flow rates 50, 50 and 30 units, respectively; ionspray needle voltage 4500 V; heater gas temperature 500°C. Declustering potential (V) and collision energy (eV) were: 100, 50. In vivo imaging was carried out immediately by fluorescent imaging system (CRI Maestro 500FL).

Synthesis of PEG-AM-Dox (PEG-AM-Dox including PEG400-AM-Dox, PEG550-AM-Dox, PEG750-AM-Dox, PEG2K-AM-Dox, PEG10K-AM-Dox and PEG20K-AM-Dox): The synthesis was according to the previous work.[3,4] PEG400 (400 mg, 1 mmol) was dissolved in methylene chloride (20 mL). Then NPC (201 mg, 1 mmol) and TEA (550 μL, 4 mmol) were added dropwise at 0 ^{o}C. After stirred for 12 h, the mixture was extracted by water three times. The organic phase was treated with

Mg_2SO_4, then filtered and evaporated. The NPC-activated PEG400 (67.8 mg, 0.12mmol) was dissolved in dimethlformamide (DMF, 3 mL). Dox·HCl (58 mg, 0.1 mmol) with TEA (100 μL) was added and then the mixture was stirred for 48 h at room temperature. The mixture was added into distilled water (7 mL) and stirred for 4 h, followed by dialyzing against distilled water with a cellulose membrane (cutoff Mn= 3500) for 3 days. After dialysis, the solution was filtered using disposable 220 nm Millipore filters prior to obtain PEG400-AM-Dox nanoparticles (PEG400-AM-Dox NPs). A part of the solution was freeze-dried. Similar processes were done for PEG550-AM-Dox, PEG750-AM-Dox and PEG2K-Dox NPs.

PEG10K (1000 mg, 0.1 mmol) was dissolved in methylene chloride (20 mL). Then NPC (20.2 mg, 0.1 mmol) and TEA (55.5 μL, 0.4 mmol) were added dropwise at 0 ^oC. After stirred for 12 h, the mixture was extracted by water three times. The organic phase was treated with Mg_2SO_4, then filtered and evaporated. The NPC-activated PEG10K (400 mg, 0.04mmol) was dissolved in dimethlformamide (DMF, 3 mL). Dox·HCl (26 mg, 0.045 mmol) with TEA (50 μL) was added and then the mixture was stirred for 48 h at room temperature. The mixture was added into distilled water (7 mL) and stirred for 4 h, followed by dialyzing against distilled water with a cellulose membrane (cutoff Mn= 14000) for 3 days. After dialysis, the solution was filtered using disposable 220 nm Millipore filters prior to obtain PEG10K-AM-Dox NPs. A part of the solution was freeze-dried. Similar processes were done for PEG20K-AM-Dox.

Synthesis of PEG-HZ-Dox (PEG-HZ-Dox including PEG400-HZ-Dox, PEG550-HZ-Dox, PEG750-HZ-Dox, PEG2K-HZ-Dox, PEG10K-HZ-Dox and PEG20K-HZ-Dox): The synthesis was according to the previous work.[4,5] The NPC-actived PEG400 (200 mg, 0.353 mmol) and Hydrazine hydrate (171.84 μL, 3.53 mmol) were dissolved in CH_2Cl_2 (20 mL) and stirred at room temperature overnight. Then the mixture was extracted with water three times. The organic phase was treated with Mg_2SO_4, then filtered and evaporated. The hydrazone conjugated PEG 400 (16.4 mg, 0.035mmol) was reacted with Dox·HCl (20 mg, 0.035mmol) in the presence of TEA (50 μL) in DMF (3 mL) for 48 h at room temperature. The mixture was added into distilled water (7 mL) and stirred for 4 h, followed by dialyzing against distilled water with a cellulose membrane (cutoff Mn= 3500) for 3 days. After dialysis, the solution was filtered using disposable 220 nm Millipore filters prior to obtain PEG400-HZ-Dox NPs. A part of the solution was freeze-dried. Similar processes were done for PEG550-HZ-Dox, PEG750-HZ-Dox and PEG2K-HZ-Dox.

The NPC-activated PEG10K (400 mg, 0.04 mmol) and Hydrazine hydrate (25 μL, 0.4 mmol) were dissolved in CH_2Cl_2 (20 mL) and stirred at room temperature overnight. Then the mixture was extracted with water three times. The organic phase was treated with Mg_2SO_4, then filtered and evaporated. The hydrazone conjugated PEG 10K (345 mg, 0.0345mmol) was reacted with Dox·HCl (22 mg, 0.038mmol) in the presence of TEA (50 μL) in DMF (3 mL) for 48 h at room temperature. The mixture was added into distilled water (7 mL) and stirred for 4 h, followed by dialyzing against distilled water with a cellulose membrane (cutoff Mn= 14000) for 3

days. After dialysis, the solution was filtered using disposable 220 nm Millipore filters prior to obtain PEG10K-HZ-Dox NPs. A part of the solution was freeze-dried. Similar processes were done for PEG20K-HZ-Dox.

Cell viability assays: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assays against HeLa cells were carried out. Similar experimental methods were shown in our previous work.[3,6,7] Briefly, PEG-Dox NPs and Dox·HCl were added to cell wells to achieve final Dox concentration of 0.1 to 15 μg/mL. The cells were incubated for 24 h, 48 h and 72 h.

Cellular uptake studies: Similar experimental methods and instruments were shown in our previous work.[8-10] The concentration of used PEG-Dox was 8.62 nmol mL^{-1}. The incubation time was 1 h, 6 h, 12 h, respectively. The cells were seeded in 6-well plates at about 200,000 cells per well in 1.5 mL Dulbecco Modified Eagle Medium (DMEM, GIBCO) containing 10% fetal bovine serum, supplemented with 100 U/mL penicillin and 100 U/mL streptomycin, and incubated at 37 ^{o}C in 5 % CO_2 atmosphere for 24 h. Then the cells were washed with PBS and incubated at 37 ^{o}C for additional 1, 6 and 12 h with NPs. Then the culture medium was removed and cells were washed with PBS three times. Thereafter, the cells were fixed with 4% formaldehyde for 10 min at room temperature, and the cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, blue). CLSM images of cells were obtained by Carl Zeiss LSM 710 (Zurich, Switzerland). The operation was based on the instrument manual. Blue fluorescence from Hoechst 33258 was observed through

DAPI channel. Red fluorescence from PEG-Dox was observed through FITC channel. Excitation wavelengths in DAPI channel is 405 nm and FITC channel is 488 nm.

Animal model. All animal experiments were performed in compliance with the Guidelines for the Care and Use for Care and Use of Research Animals established by Jilin University Studies Committee. Female Kunming mice with naked-eye-visible U14 tumor nodules in the armpit of the left anterior limb were applied. Tumor volume = 0.5ab^2, where a represents the major axis, and b represents the minor axis

Sample preparation. Frozen biological samples were allowed to thaw in a water-bath at room temperature. In an aliquot of 50 μL biological sample, 50 μL IS working solution and 200 μL acetonitrile was added to precipitate protein. The mixture was vortex-mixed for 1 min and centrifuged for 5 min at 15000 rpm. 100 μL supernatant was collected and vortex-mixed with 50 μL 0.1% formic acid for 1 min, and 50μL was injected into the LC-MS system.

For quantification of PEGylated doxorubicin and doxorubicin using the MS^{ALL} technique with CID in Quadrupole. Using the MS^{ALL} technique with CID in Quadrupole, precursor ions of all molecules are generated in Q1, fragmented to product ions in Q2 (collision cell),and subjected to TOF separation before being recorded at low CE (precursor ions) and high CE (product ions) in different experiments. In this study, dissociation in Q2 generated a series of high resolution PEG-related product ions at *m/z* 89.0611, 133.0869, 177.1102, 221.1366, 265.1622, 309.1878, and 353.2108 corresponding to fragments containing various numbers of ethylene oxide subunits, doxorubicin-related product ions at *m/z* 321.0838 and

361.0785. Doxorubicin could be determined by doxorubicin related ions; and PEGylated doxorubicin could be determined by PEG related ions.

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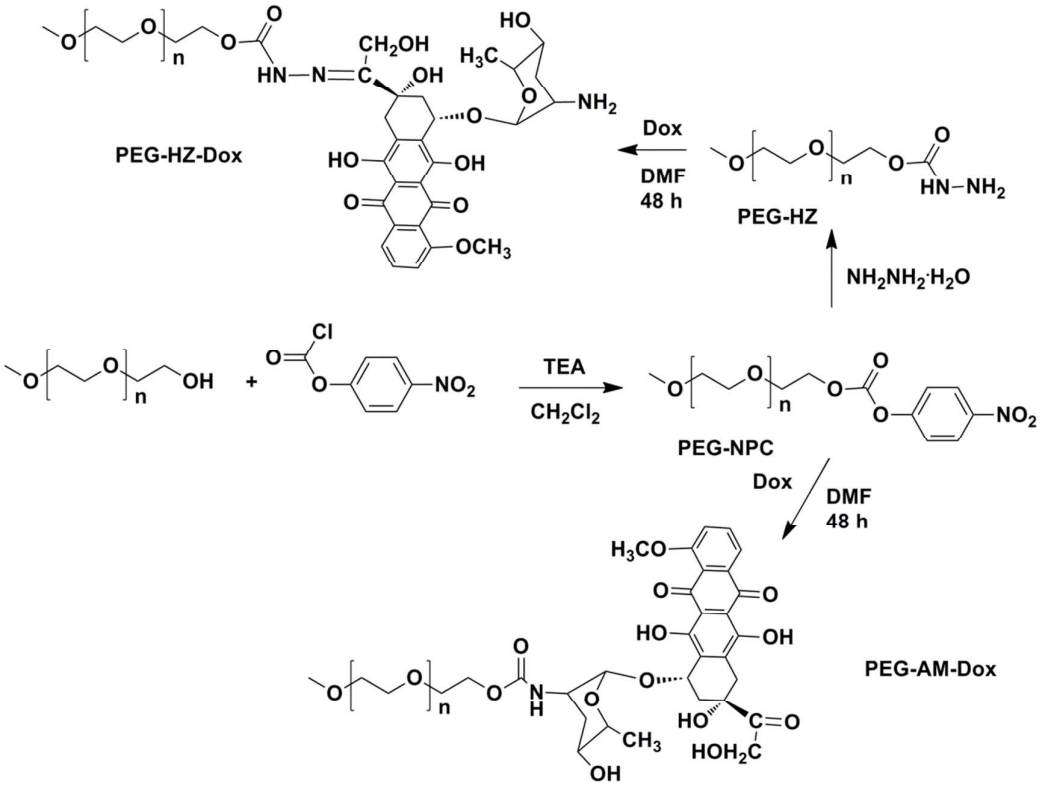

Scheme S1. Synthesis of PEG-AM-Dox and PEG-HZ-Dox.

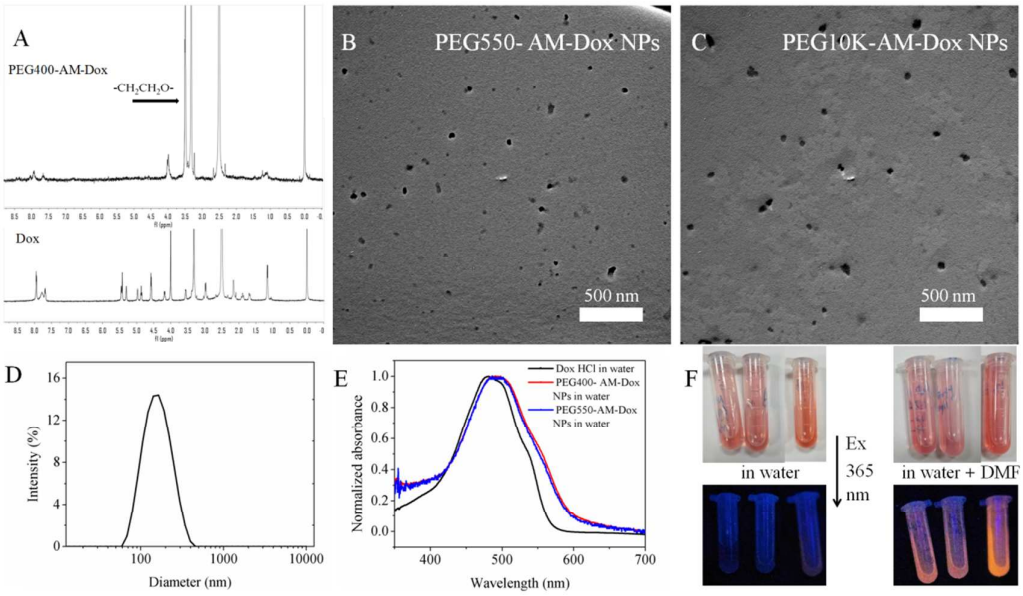

Figure S1. Various characterizations of PEG-Dox: A) The ^{1}H NMR spectra of PEG400-AM-Dox and Dox. TEM images of B) PEG550-AM-Dox NPs and C)

PEG10K-AM-Dox NPs. D) The size and polydispersity of PEG10K-AM-Dox NPs measured by DLS. E) UV-vis absorption spectra of Dox·HCl, PEG400-AM-Dox NPs and PEG550-AM-Dox NPs in water. F) The fluorescence of PEG400-AM-Dox NPs, PEG550-AM-Dox NPs and PEG10K-AM-Dox NPs in water (left) and in water + DMF (v/v, 1:1, right) under 365-nm light irradiation.

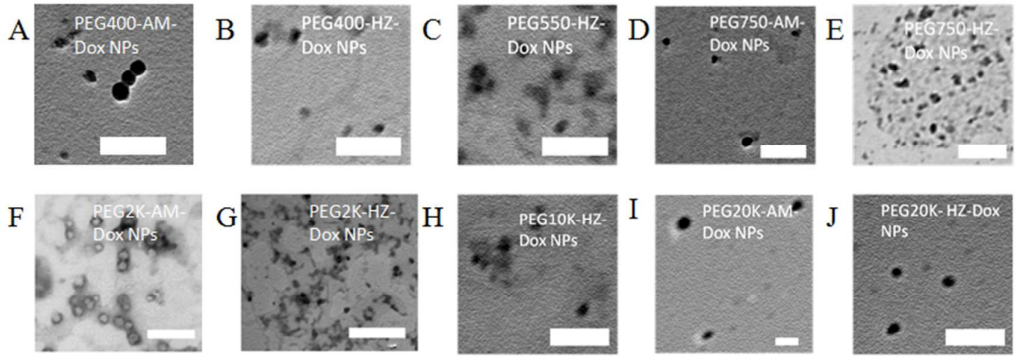

Figure S2. TEM images of A) PEG400-AM-Dox NPs, B) PEG400-HZ-Dox NPs, C) PEG550-HZ-Dox NPs, D) PEG750-AM-Dox NPs, E) PEG750-HZ-Dox NPs, F) PEG2K-Dox NPs, G) PEG2K-HZ-Dox NPs, H) PEG10K-HZ-Dox NPs, I) PEG20K-AM-Dox NPs and J) PEG20K-HZ-Dox NPs. Scale bar, 500 nm.

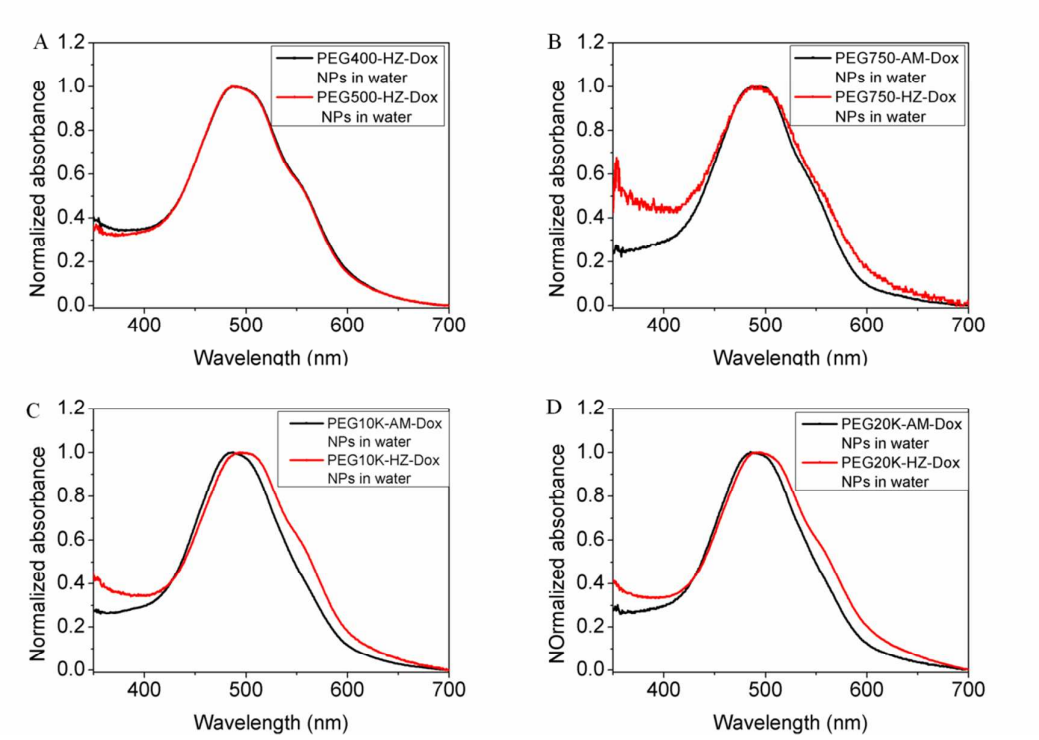

Figure S3. UV-vis absorption spectra of A) PEG400-HZ-Dox NPs and PEG550-HZ-Dox NPs in water, B) PEG750-AM-Dox NPs and PEG750-HZ-Dox NPs in water, C) PEG10K-AM-Dox NPs and PEG10K-HZ-Dox NPs in water and D) PEG20K-AM-Dox NPs and PEG20K-HZ-Dox NPs in water.

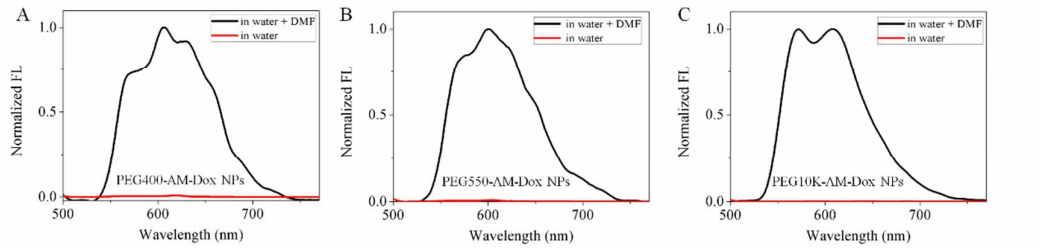

Figure S4. The fluorescence spectra of A) PEG400-AM-Dox NPs, B) PEG550-AM-Dox NPs and C) PEG10K-AM-Dox NPs in water and in water + DMF (v/v, 1:1).

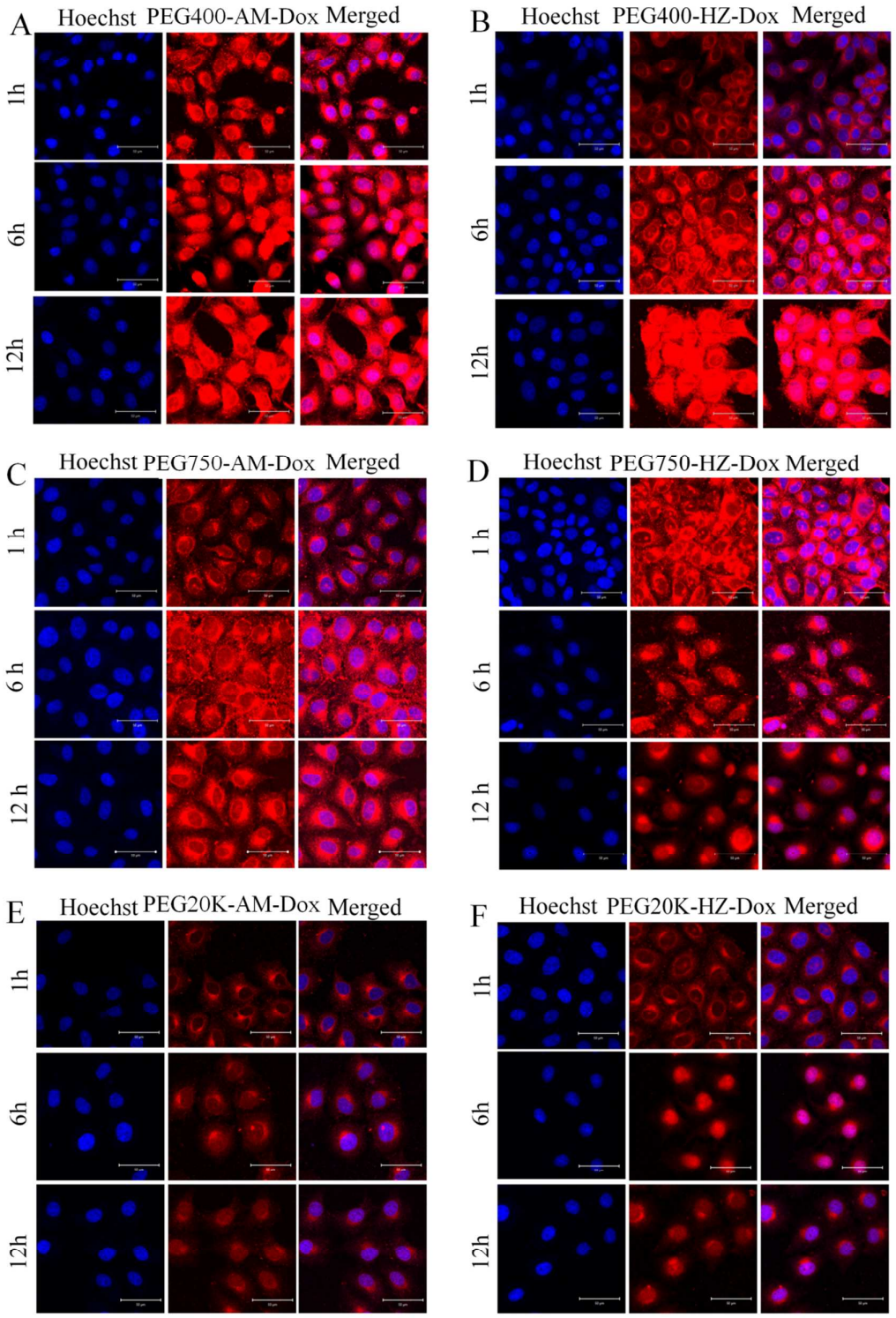

Figure S5. Representative CLSM images of HeLa cells incubated with A) PEG440-AM-Dox NPs, B) PEG440-HZ-Dox NPs, C) PEG750-AM-Dox NPs, D)

PEG750-HZ-Dox NPs, E) PEG20K-AM-Dox NPs and F) PEG20K-HZ-Dox NPs for 1, 6 and 12h. For each panel, the images from left to right show cell nuclei stained by Hoechst (blue), PEG-Dox and PEG-HZ-Dox fluorescence in cells (green), and overlays of both images. Scale bar, 50 μm.

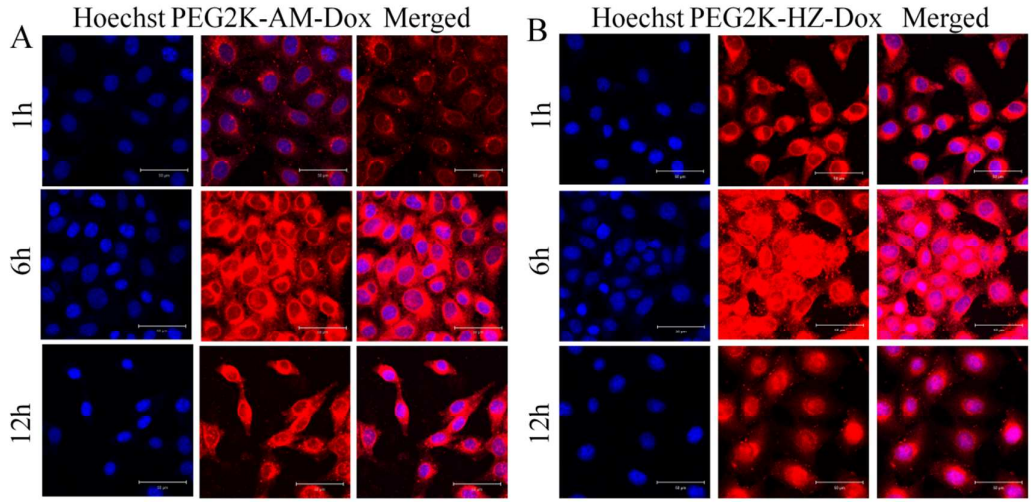

Figure S6. Representative CLSM images of HeLa cells incubated with A) PEG2K-AM-Dox NPs, B) PEG2K-HZ-Dox NPs for 1, 6 and 12h. For each panel, the images from left to right show cell nuclei stained by Hoechst (blue), PEG-Dox fluorescence in cells (green), and overlays of both images. Scale bar, 50 μm.

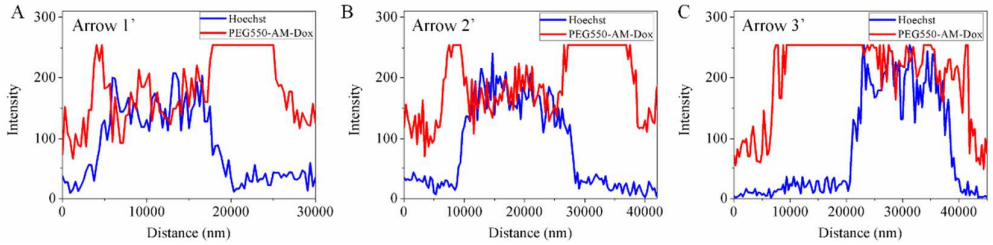

Figure S7. A, B and C) The trend of fluorescent intensity in a cell along the direction of the arrows marked in Figure 1A. The cells were incubated with PEG-AM-Dox NPs

for 1, 6 and 12 h, respectively.

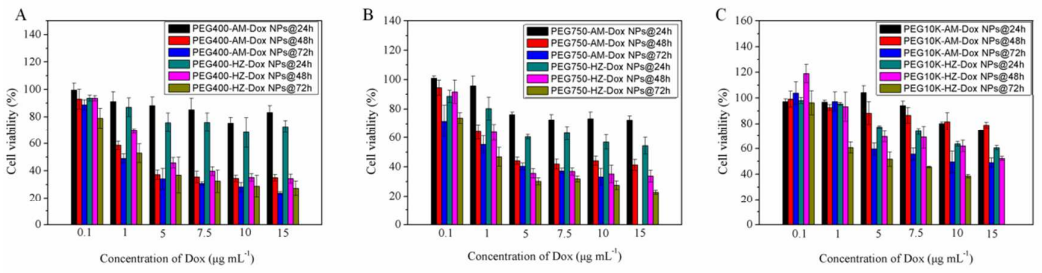

Figure S8. Cell viability of HeLa cells incubated with A) PEG400-AM-Dox NPs and PEG400-HZ-Dox NPs, B) PEG750-AM-Dox NPs and PEG750-HZ-Dox NPs and C) PEG10K-AM-Dox NPs and PEG10K-HZ-Dox for 24, 48, 72 h.

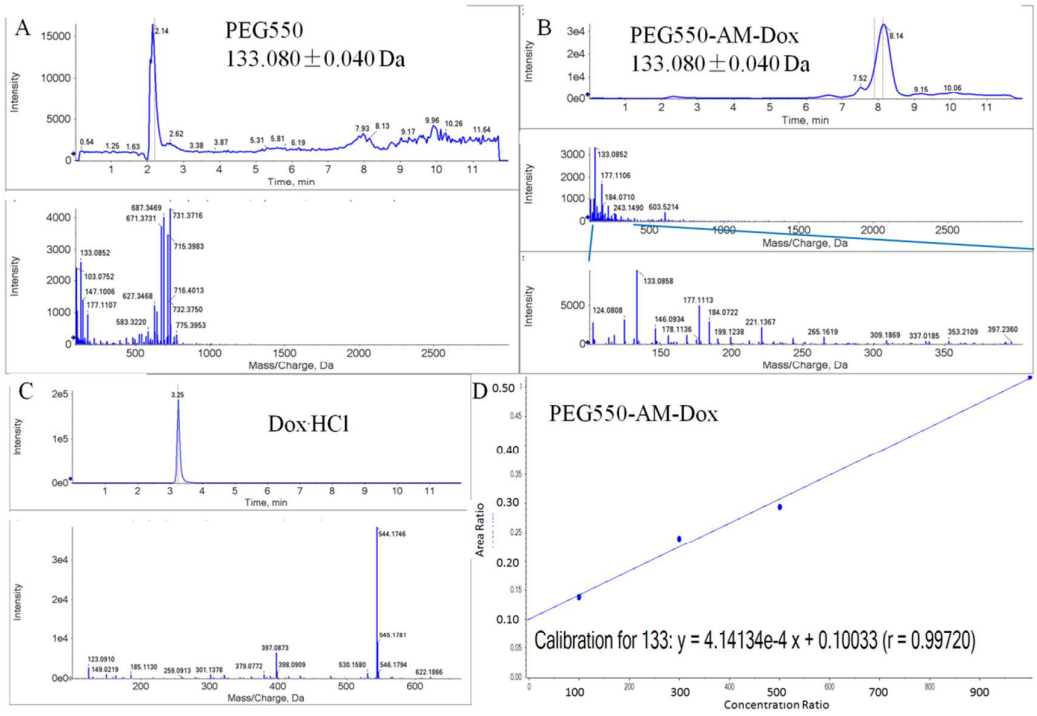

Figure S9. LC-MS/MS of A) PEG550, B) PEG550-Dox and C) Dox·HCl. D) The quantitative standard curves of PEG550-AM-Dox.

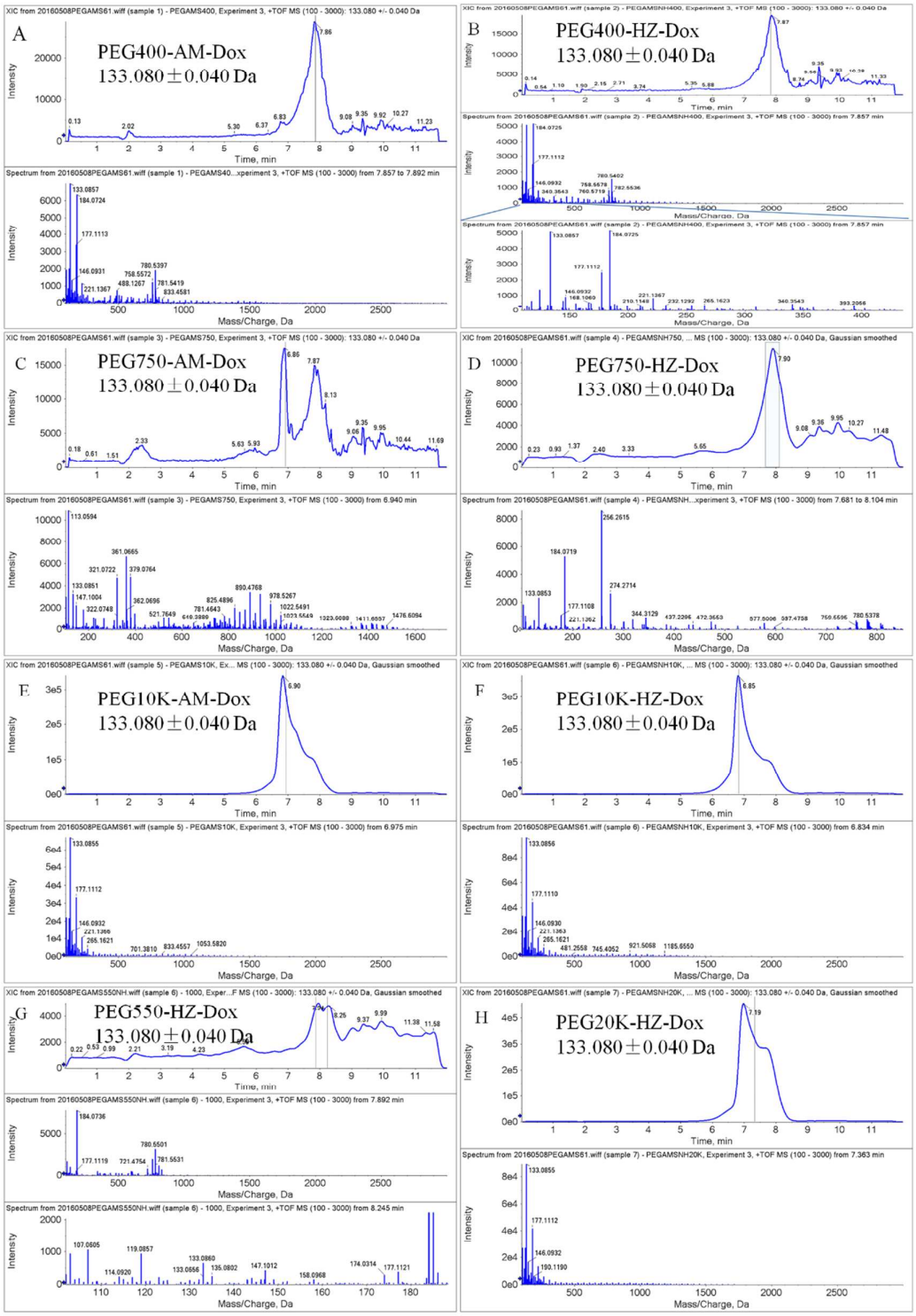

Figure S10. The LC-MS/MS of PEG-AM-Dox and PEG-HZ-Dox.

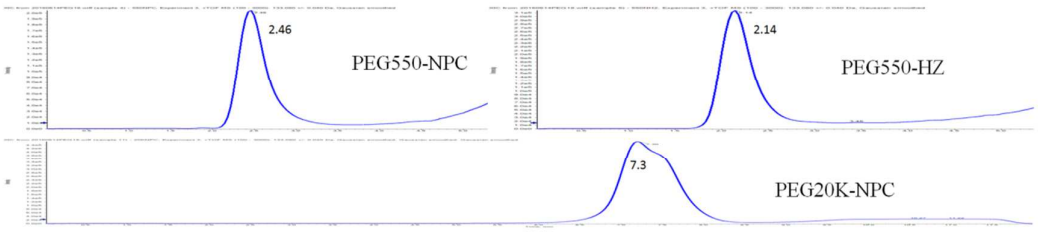

Figure S11. The HPLC of PEG550-NPC, PEG550-HZ and PEG20K-NPC according to m/z 133.080 ± 0.040 Da.

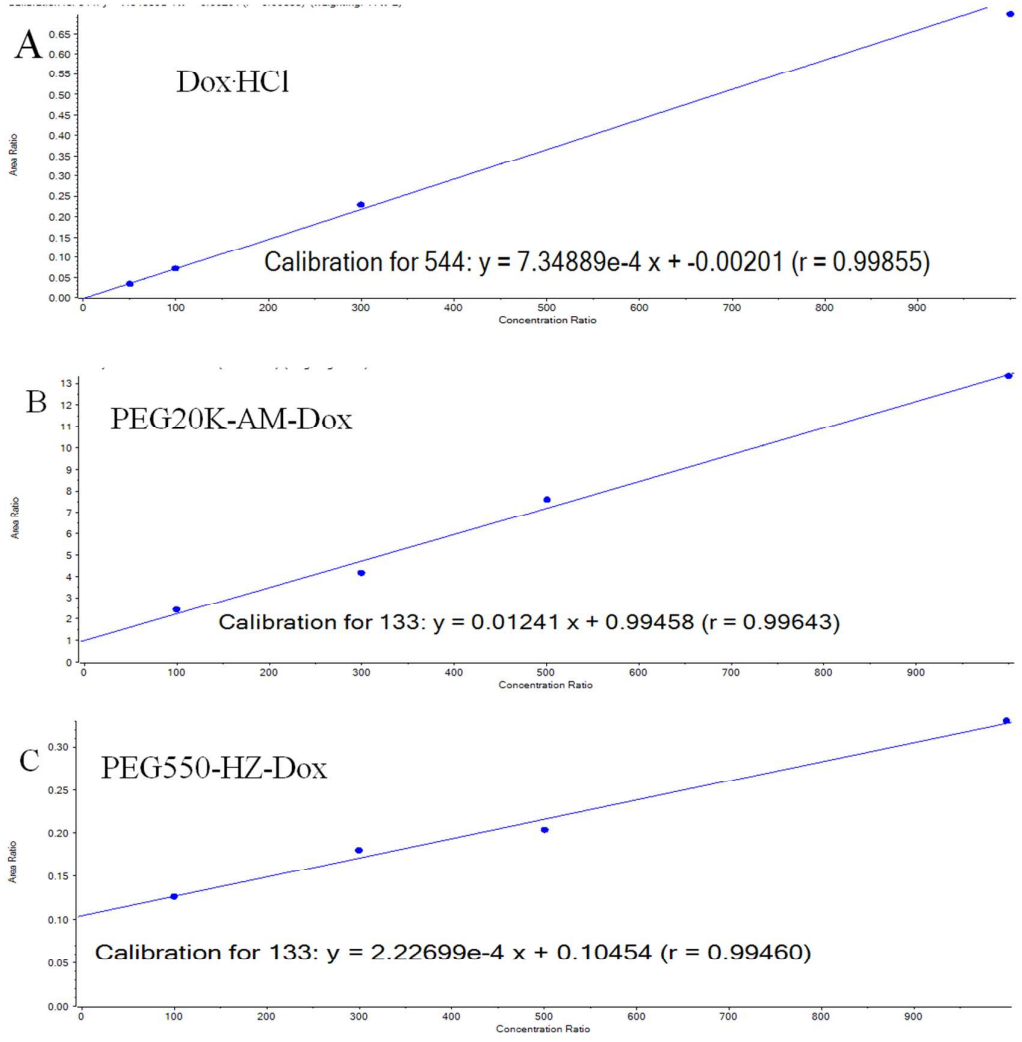

Figure S12. The quantitative standard curves of Dox·HCl, PEG20K-AM-Dox and PEG550-HZ-Dox.

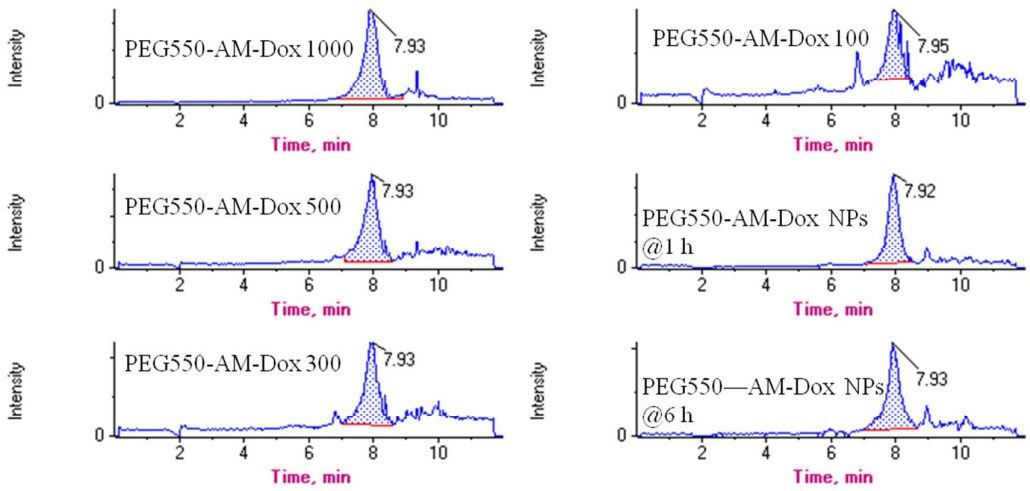

Figure S13. HPLC of PEG550-AM-Dox at Dox concentrations of 1000, 500, 300, 100 ng $mL^{-1}$and extract of HeLa cells treated with PEG550-AM-Dox of 8.62 nmol $mL^{-1}$for 1 h and 6 h.

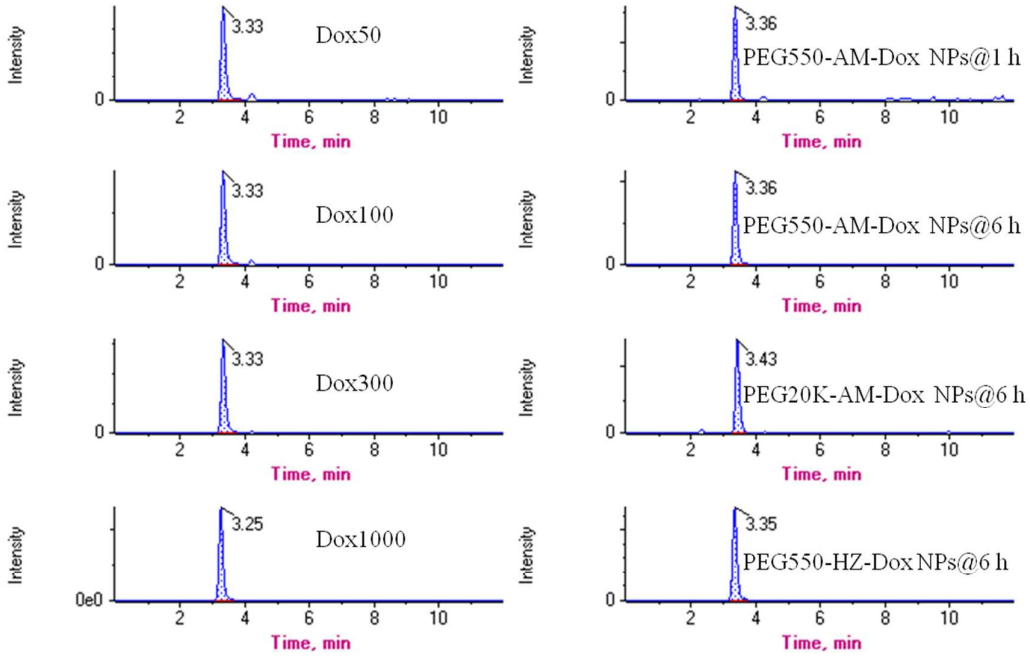

Figure S14. HPLC of Dox at different concentrations and Dox released from different PEG-Dox NPs in HeLa cells for different time for quantification. Dox 1000, 500, 300 and 100 meant that the Dox concentration was 1000 ng mL^{-1}, 500 ng mL^{-1}, 300 ng mL^{-1} and 100 ng mL^{-1}.

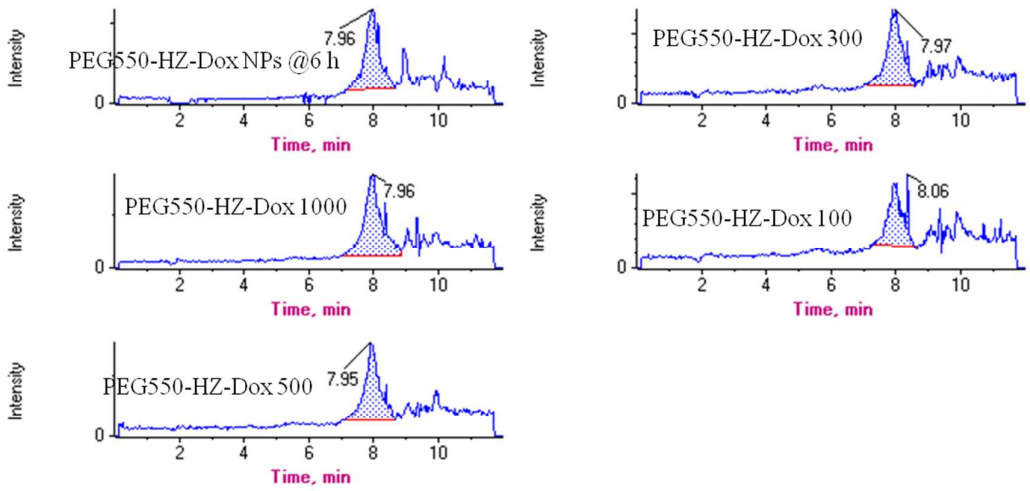

Figure S15. HPLC of PEG550-HZ-Dox at different concentrations and PEG550-HZ-Dox in HeLa cells for 6 h. PEG550-HZ-Dox 1000, 500, 300 and 100 meant that the equivalent Dox concentration was 1000 ng mL^{-1}, 500 ng mL^{-1}, 300 ng mL^{-1} and 100 ng mL^{-1}.

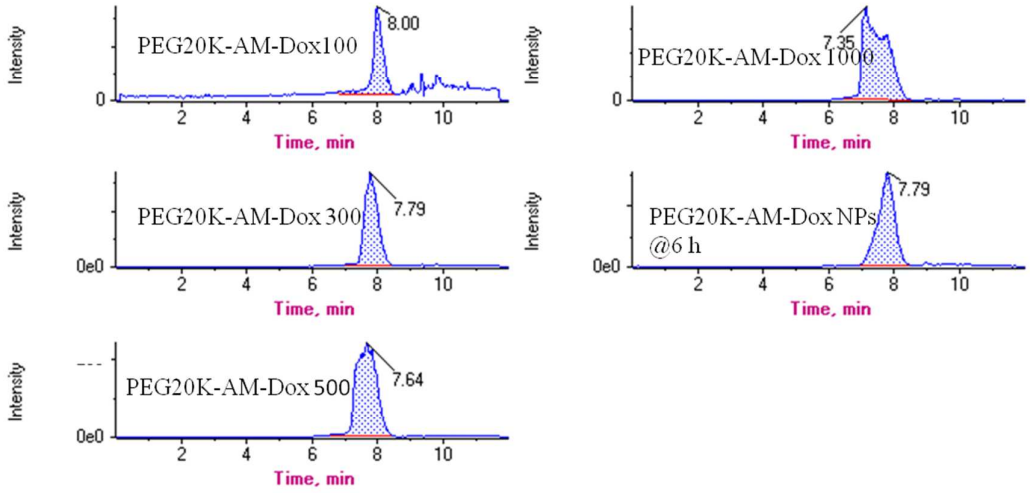

Figure S16. HPLC of PEG20K-AM-Dox at different concentrations and PEG20K-AM-Dox in HeLa cells for 6 h. PEG20K-AM-Dox 1000, 500, 300 and 100 meant that the equivalent Dox concentration was 1000 ng mL^{-1}, 500 ng mL^{-1}, 300 ng mL^{-1} and 100 ng mL^{-1}.

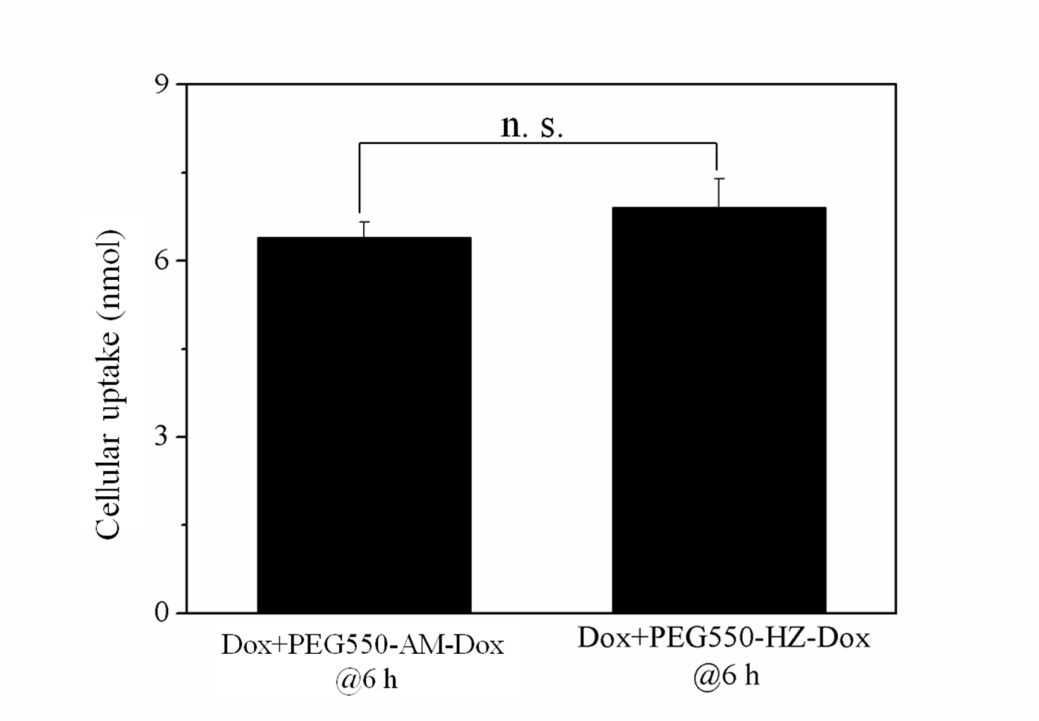

Figure S17. The total of Dox+PEG550AM-Dox and Dox+PEG550-HZ-Dox in HeLa cells incubated for 6 h. n.s. P >0.05.

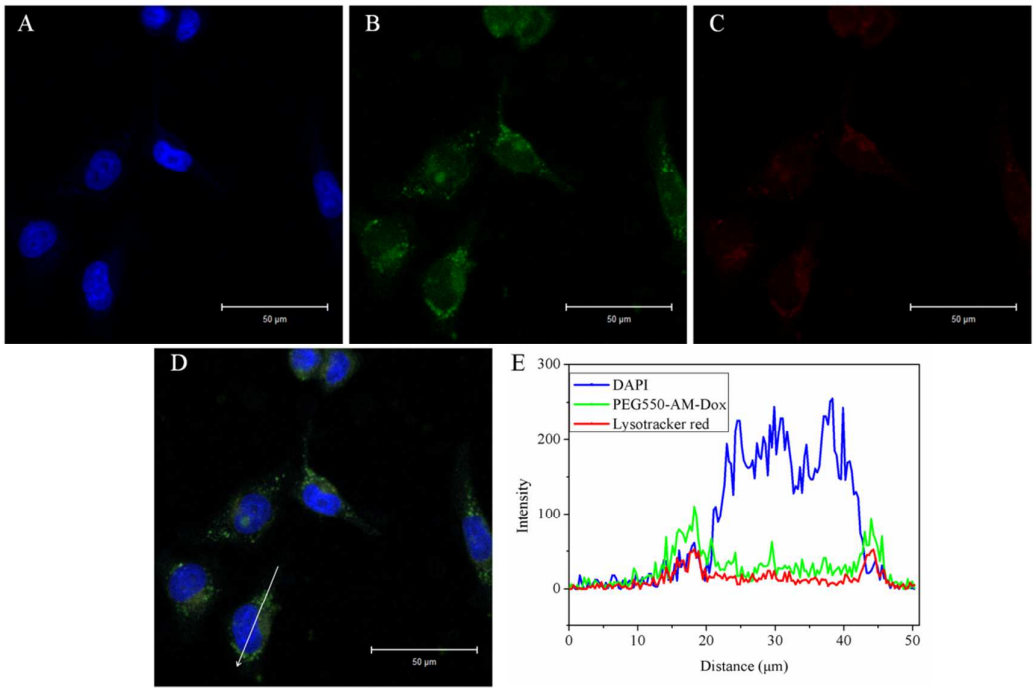

Figure S18. CLSM images of HeLa cells incubated with PEG550-AM-Dox NPs (3.4 nmol mL^{-1}) for 6 h. the images show cell nuclei stained by A) Hoechst 33258 (blue),

B) PEG550-AM-Dox (green), C) Lysotracker red (red) and D) overlay of all images. E) The trend of fluorescent intensity in a cell along the direction of the arrows marked in D). Blue fluorescence from Hoechst 33258, green fluorescence from PEG550-AM-Dox and red fluorescence from Lysotracker red were observed through DAPI channel, FITC channel and Rhodamine B channel, respectively. Scale bar, 50 μm.

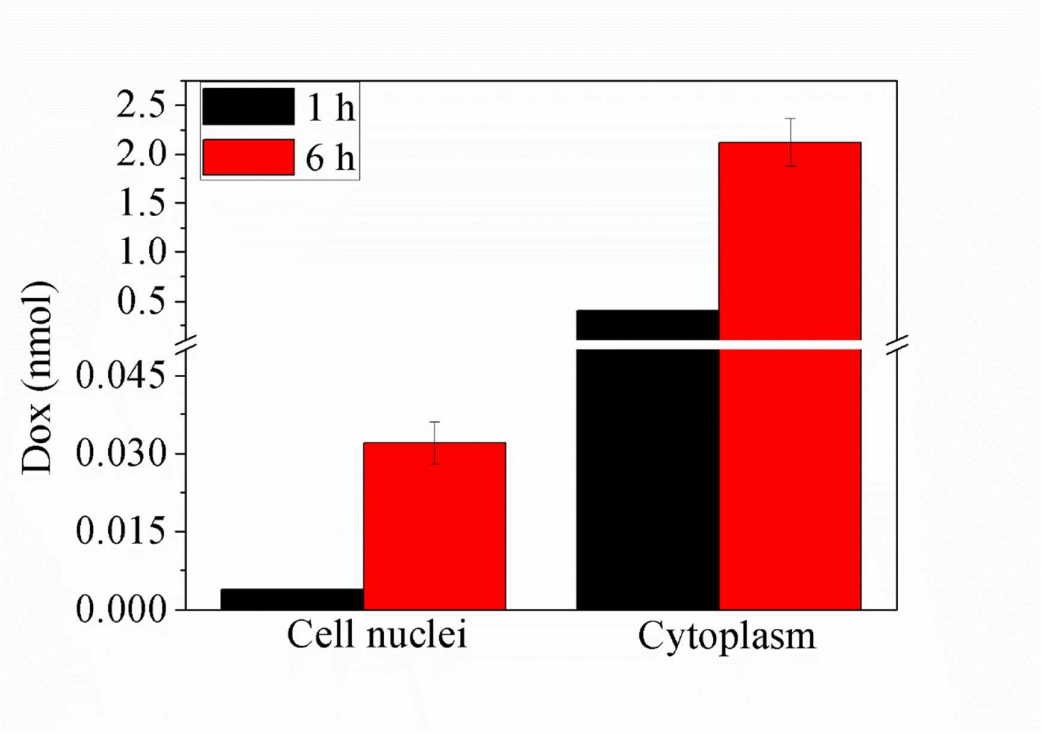

Figure S19. Dox in cell nuclei and cytoplasm of HeLa cells incubated with PEG550-HZ-Dox NPs for 1 and 6 h.

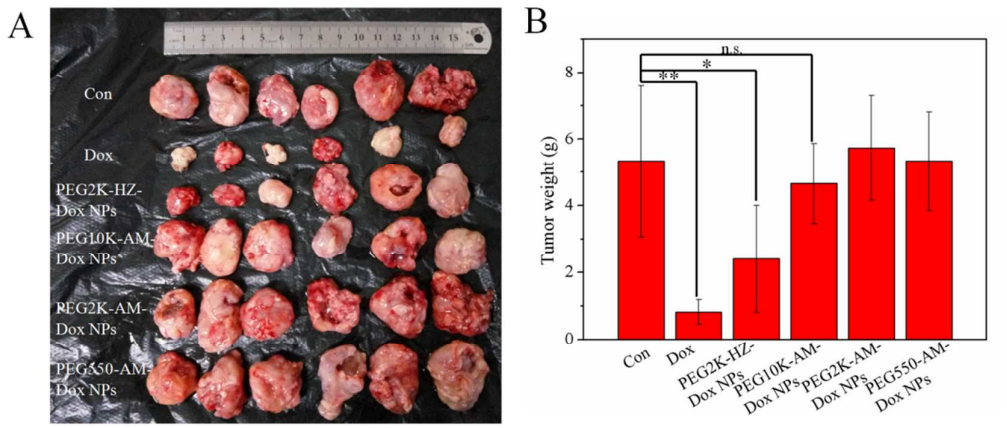

Figure S20. A) The representative tumors of mice. B) The tumor weight of mice.

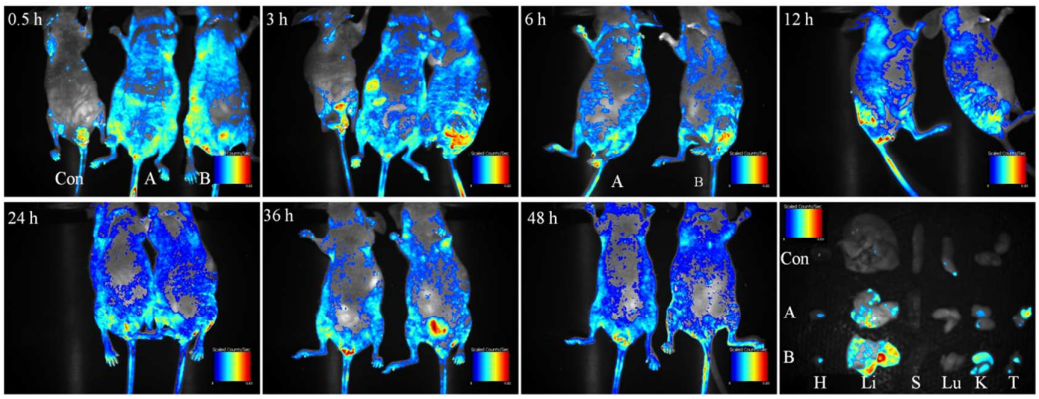

Figure S21. Fluorescence imaging in vivo of HeLa-tumor bearing Male Balb/C nude mice. The mice were injected with (con) Dox, (A) PEG2000-AM-Dox NPs and (B) PEG2K-HZ-Dox NPs intravenously (equivalent Dox: 5 mg kg^{-1}). H (heart), Li (liver), S (spleen), Lu (lung), K (kidney), G (female genitalia) and T (tumor).

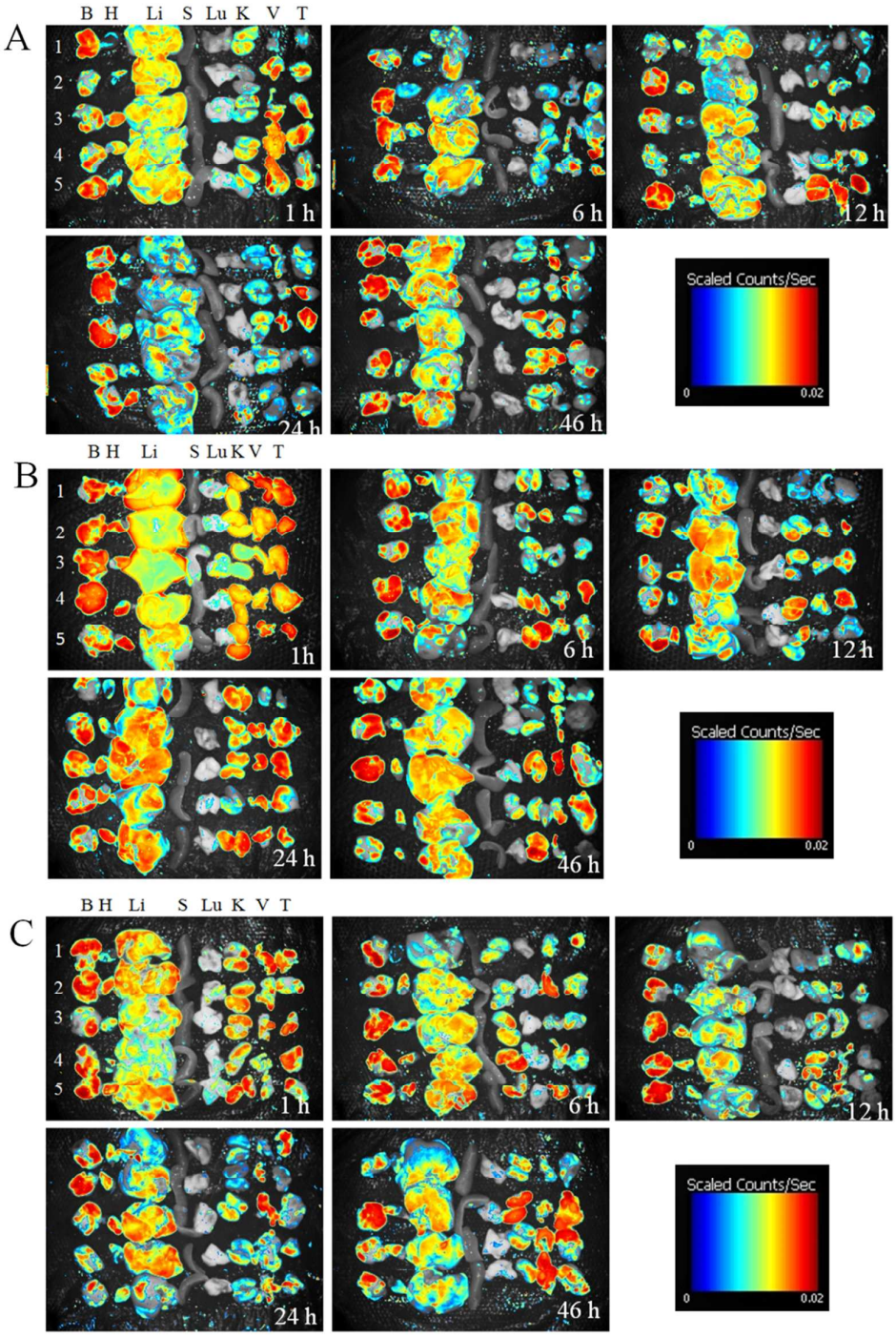

Figure S22. Fluorescence imaging of main organs ex vivo. Mice were treated with A) Dox, B) PEG550-AM-Dox NPs and C) PEG10K-AM-Dox NPs. B (brain), H (heart), Li (liver), S (spleen), Lu (lung), K (kidney), G (female genitalia) and T (tumor).

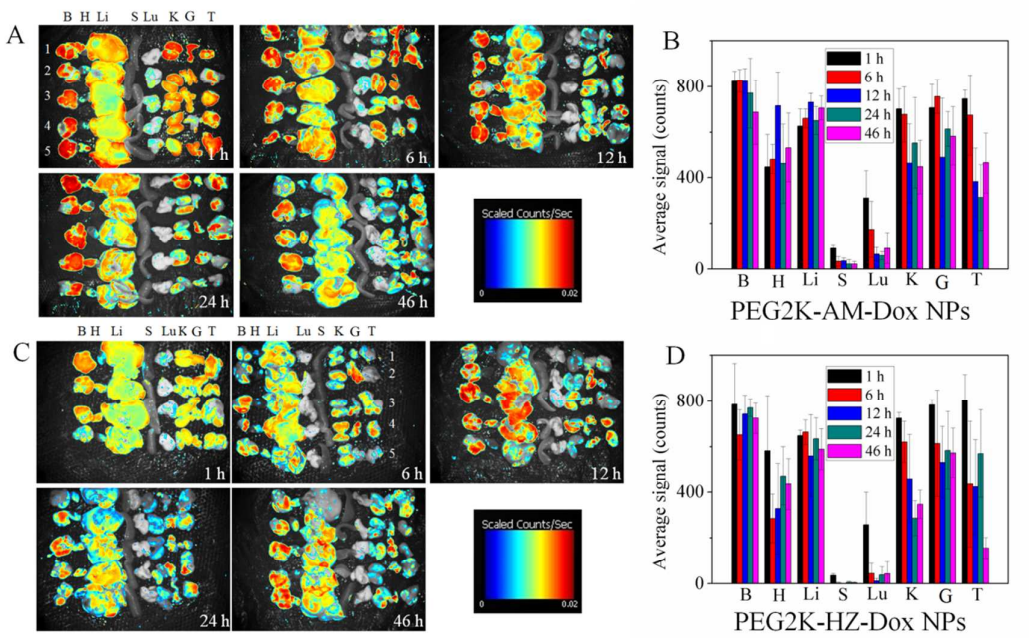

Figure S23. Fluorescence imaging and fluorescence intensity of main organs mice were treated with A and B)PEG2K-AM-Dox NPs, C and D)PEG550-HZ-Dox NPs. B (brain), H (heart), Li (liver), S (spleen), Lu (lung), K (kidney), G (female genitalia) and T (tumor).

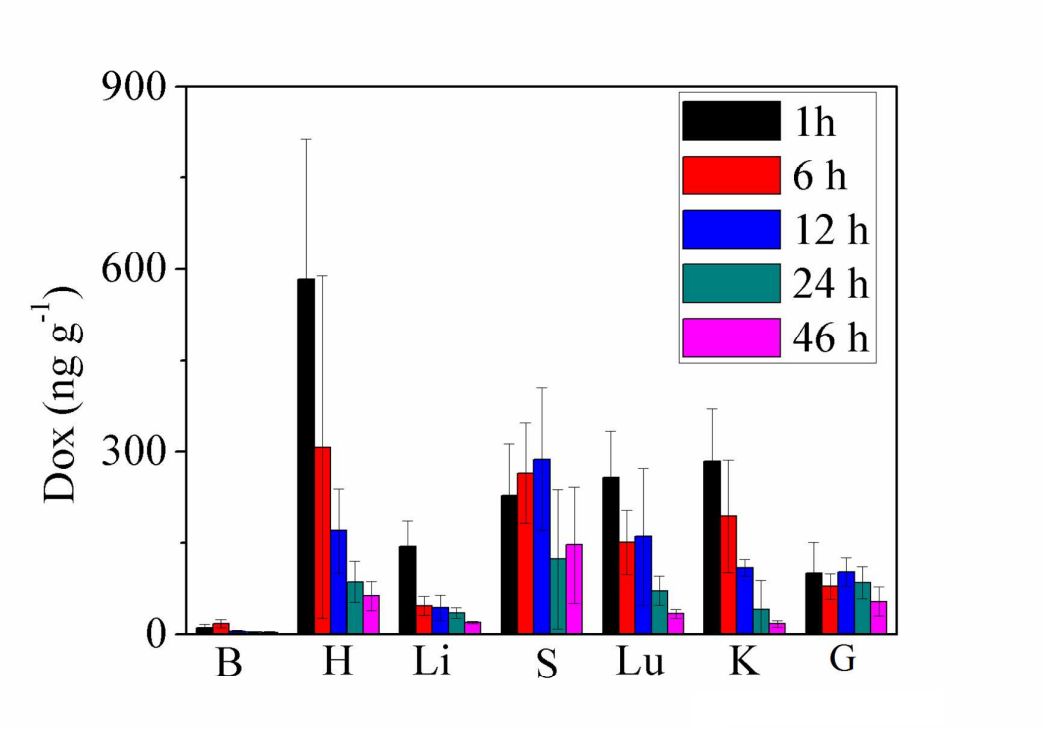

Figure S24. The biodistribution of Dox when mice were treated with Dox.

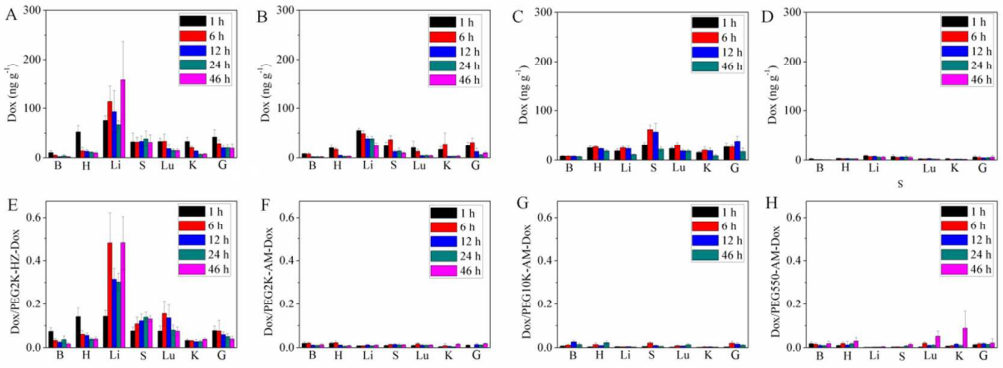

Figure S25. The biodistribution of Dox released from A) PEG2K-HZ-Dox NPs, B) PEG2K-AM-Dox NPs , C) PEG10K-Dox NPs and D) PEG550-AM-Dox NPs. The rate of Dox and E) PEG2K-HZ-Dox, F) PEG2K-AM-Dox, G) PEG10K-AM-Dox and H) PEG550-AM-Dox in main organs.

Table S1. The size and PdI of NPs

Samples	Dox content (%)[a]	Diameter (nm)[b]	PdI[b]
PEG400-AM-Dox NPs	55.96	76.65 ± 2.70	0.29 ± 0.01
PEG400-HZ-Dox NPs	53.58	106.67 ± 2.36	0.12 ± 0.02
PEG550-AM-Dox NPs	48.46	60.85 ± 1.66	0.26 ± 0.01
PEG550-HZ-Dox NPs	46.50	105.50 ± 3.44	0.12 ± 0.03
PEG750-AM-Dox NPs	41.11	41.78 ± 0.06	0.25 ± 0.01
PEG750-HZ-Dox NPs	39.53	51.67 ± 0.54	0.28 ± 0.02
PEG2K-AM-Dox NPs	21.03	101.09 ± 2.48	0.29 ± 0.01
PEG2K-HZ-Dox NPs	20.41	108.19 ± 3.34	0.30 ± 0.02
PEG10K-AM-Dox NPs	5.13	146.50 ± 2.63	0.16 ± 0.01
PEG10K-HZ-Dox NPs	4.98	129.93 ± 2.80	0.32 ± 0.03
PEG20K-AM-Dox NPs	2.64	153.60 ± 2.62	0.15 ± 0.02
PEG20K-HZ-Dox NPs	2.56	94.97 ± 3.01	0.24 ± 0.01

[a]Dox content (wt %) = Molecular weight of Dox/(Molecular weights of Dox, linker and PEG)*100 %. [b] Diameter and PdI were measured by DLS.

Table S2. Quantification of released Dox and PEG-Dox

Samples	Dox released from NPs (nmol per 2×10^6 cells)	PEG-Dox uptake(nmol per 2×10^6 cells)
PEG20K-AM-Dox NPs@6 h	0.173 ± 0.012	0.967 ± 0.092
PEG550-AM-Dox NPs@1 h	0.277 ± 0.010	4.18 ± 0.323
PEG550-AM-Dox NPs@6 h	0.849 ± 0.023	5.55 ± 0.278
PEG550-HZ-Dox NPs@6 h	1.04 ± 0.037	5.87 ± 0.521