Electronic Supplementary Material (ESI) for Polymer Chemistry

Supporting Information

Construction and application of pH-triggered cleavable hyperbranched polyacylhydrazone for drug delivery

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1. Synthesis of 1-(2-aminoethyl) piperazine tri-methyl propionate (AEP-ester).

1-(2-Aminoethyl) piperazine (AEP, 15.0 g, 116.0 mmol) in 12 mL methanol was slowly dropped into methyl acrylate (MA) solution (35.0 g in 50 mL methanol) during 2 h at 0 ^{o}C, and the mixture was continuously stirred for 48 h. After the reaction, the solvent was removed by a rotatory evaporator and dried in vacuum for 24 h to obtain colorless liquids (45.0 g, yield 99%).

^{1}H NMR (400 MHz, $CDCl_3$, 298 K) δ ppm: 3.65 (s, 3H, -COOC*H*$_3$), 3.64 (s, 6H, -COOC*H*$_3$), 2.77 (t, 4H, -C*H*$_2$CH$_2$COOCH$_3$), 2.67 (t, 2H, -CH$_2$C*H*$_2$COOCH$_3$), 2.56 (t, 2H, piperazine-C*H*$_2$CH$_2$N-), 2.48 (t, 2H, -C*H*$_2$CH$_2$COOCH$_3$), 2.42 (t, 6H, -CH$_2$C*H*$_2$COOCH$_3$, piperazine-CH$_2$C*H*$_2$N-), 2.46 (br, 8H, piperazine).

^{13}C NMR (400 MHz, $CDCl_3$) δ ppm: 173.2, 173.0 (CH$_3$O*C*O-), 56.9 (piperazine-*C*H$_2$*C*H$_2$N-), 53.7, 53.6 (piperazine), 52.1 (piperazine-*C*H$_2$-, piperazine-CH$_2$CH$_2$N*C*H$_2$-), 51.5, 50.9 (COO*C*H$_3$), 31.1, 33.4 (-*C*H$_2$COO-).

IR (cm^{-1}): 2954 ($\nu_{as\ CH2,\ CH3}$), 2817 ($\nu_{s\ CH2,\ CH3}$), 1742 ($\nu_{C=O}$), 1579, 1439 (ν_{C-N}), 1257, 1289 (ν_{C-N}, δ_{NH}).

2. Synthesis of 1-(2-aminoethyl) piperazine tri-propionylhydrazine (AEP-NHNH$_2$).

AEP-ester (38.7 g, 100.0 mmol) was dissolved in 50 mL methanol, and hydrazine hydrate (22.5 g, 450 mmol) was slowly injected. Then, the mixture was stirred at 60 ^{o}C for 5 h. Both solvent and residue hydrazine hydrate were removed by a rotatory evaporator. After drying in vacuum at 60 ^{o}C for 48 h, white solid of AEP-NHNH$_2$ was obtained (39.0 g, yield 99%).

^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ ppm: 8.98 (s, 1H, -CON*H*NH$_2$), 8.96 (s, 2H, -CON*H*NH$_2$), 4.09 (br, 6H, -CONHN*H*$_2$), 2.58 (t, 4H, -C*H*$_2$CH$_2$CONHNH$_2$), 2.45 (2H, -C*H*$_2$CH$_2$CONHNH$_2$), 2.42 (t, 2H, piperazine-C*H*$_2$CH$_2$N), 2.33 (br, 8H, piperazine), 2.26 (t, 2H, piperazine-CH$_2$C*H*$_2$N), 2.14 (t, 2H, -CH$_2$C*H*$_2$CONHNH$_2$), 2.10 (t, 4H, -CH$_2$C*H*$_2$CONHNH$_2$).

^{13}C NMR (400 MHz, DMSO-*d*$_6$) δ ppm: 171.6, 171.3 (H$_2$NHN*C*O-), 56.7, 56.4 (piperazine-*C*H$_2$CH$_2$N-

), 53.7, 53.1 (piperazine), 50.9, 50.3 (piperazine-*C*H_2-, piperazine-$CH_2CH_2NCH_2$-), 32.3, 32.0 (-*C*H_2CO-).

IR (cm^{-1}): 3420 ($\nu_{as\ NH2}$), 3312 ($\nu_{s\ NH2}$), 3053 ($\nu_{\ NH}$), 2954, 2927 ($\nu_{as\ CH2}$), 2843 ($\nu_{s\ CH2}$), 1643 ($\nu_{\ C=O}$), 1541 ($\delta_{\ NH}$, $\delta_{\ NH2}$, $\nu_{\ C-N-C}$), 1466, 1360 ($\delta_{\ NH}$, $\delta_{\ NH2}$, $\nu_{\ C-N}$), 1313 ($\nu_{\ C-N}$), 1157, 1124, 1012 ($\nu_{\ C-N-C}$).

3. Synthesis of hyperbranched polyacylhydrazone (HPAH).

HPAHs with different branched architecture were prepared by traditional A_2+B_3 polycondensation of diketone and trihydrazine with a feeding mole ratio of 1/1, 1.2/1 and 1.5/1. A typical procedure with 1/1 feeding mole ratio is as follows: AEP-$NHNH_2$ (1.55 g, 4.00 mmol) was dissolved in 10 mL of absolute ethanol. The solution was bubbled with N_2 for 20 min, followed by injecting 2,3-butanedione (BD, 348 μL, 4.00 mmol). The mixture was vigorously stirred for 24 h at 60 ^oC. Then, the solvent was removed under vacuum. The residues were dissolved in water and exhaustively dialyzed against distilled water for 2 days using dialysis tubing (MWCO, 1 kDa), followed by lyophilization to give the polymer, resulting in a yellowish solid (0.58 g, yield 30%).

^1H NMR (400 MHz, DMSO-d_6) δ ppm: 10.99-10.11 (m, CON*H*N=C), 9.29, 8.98 (m, CON*H*NH_2), 4.12 (br, -CONHN*H*$_2$), 3.01-2.21, 2.21-1.74 (m, -C*H*$_2$-), 1.21, 1.14 (s, -C*H*$_3$).

^{13}C NMR (400 MHz, DMSO-d_6) δ ppm: 174.67-174.32 (*C*O$NHNH_2$), 172.11-171.11, 169.52-168.24 (*C*ONHN=C), 155.01-147.96, 143.63-142.67 (NHN=*C*), 58.43-44.62, 32.59-30.68 (*C*H_2), 23.29-21.17, 13.97-9.31 (*C*H_3).

IR (cm^{-1}): 3423 ($\nu_{as\ NH2}$), 3248 ($\nu_{s\ NH2}$), 3159 ($\nu_{as\ NH}$), 3048 ($\nu_{s\ NH}$), 2945 ($\nu_{as\ CH2}$), 2819 ($\nu_{s\ CH2}$), 1670 ($\nu_{\ C=O,\ C=N}$), 1529, 1380 ($\delta_{\ CH3}$), 1142, 1051, 1003 ($\nu_{\ C-N-C}$).

4. Synthesis of DOX-conjugated hyperbranched polyacylhydrazone (HPAH-DOX).

HPAH (485 mg) was dissolved in 15 mL of dry *N*,*N*-dimethylformamide (DMF), and doxorubicin (DOX, 38.6 mg) was added. The mixed solution was stirred at room temperature for 24 h under N_2

atmosphere. After removal of solvent by vacuum, the product was purified by dialysis against distilled water for 4 days using dialysis tubing (MWCO, 1 kDa). HPAH-DOX was obtained by freeze-drying.

5. Synthesis and characterization of HPAH

HPAH with pH-sensitivity was synthesized from BD (A_2 monomer) and AEP-$NHNH_2$ (B_3 monomer) by one-pot polymerization with different feeding ratios. The systems with 1.2/1 and 1.5/1 feeding mole ratios of BD and AEP-$NHNH_2$ were cosslinked after 24 h reaction and the products could not be dissolved in any solvents. Here, HPAH (with 1/1 feeding mole ratio of A_2 and B_3) with plentiful acylhydrazine terminals was used as drug carrier. B_3 monomer was prepared by two steps. Firstly, AEP-ester was synthesized by Michael addition of AEP and MA. Then, the reactant B_3 monomer AEP-$NHNH_2$ was obtained from further hydrazinolysis of AEP-ester. Fig. S1A shows that the C=O stretching vibration of AEP-ester is located at 1742 cm^{-1}. After the complete hydrazinolysis of AEP-ester, the $\nu_{C=O}$ band of AEP-$NHNH_2$ shifts to 1643 cm^{-1} as seen in Fig. S1B. In the meantime, two new bands at 3420 and 3312 cm^{-1} appear, which correspond to asymmetric and symmetric NH_2 stretching vibration respectively. By polycondensation of BD and AEP-$NHNH_2$ with a mole ratio of 1:1, HPAH was prepared. The correspondent FTIR spectrum in Fig. S1C exhibits an obvious methyl deformation vibration at 1380 cm^{-1}. Moreover, a strong band at 1670 cm^{-1} can be attributed to imine (C=N) stretching vibration and C=O stretching vibration.

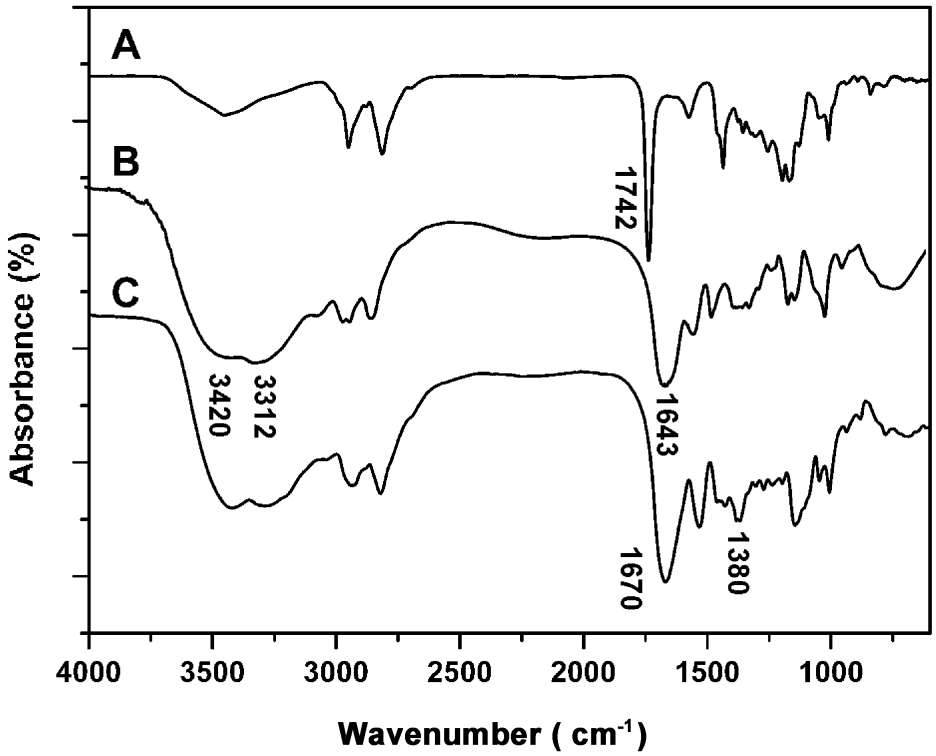


Fig. S1 FTIR spectra of (A) AEP-ester; (B) AEP-$NHNH_2$; and (C) HPAH.

The formation of HPAH was also confirmed by the ^{1}H NMR measurements. Fig. S2A gives the ^{1}H NMR spectrum of AEP-ester. The signals at 3.64 and 3.65 ppm are ascribed to the methyl protons, which account for the fine differences in local chemical environment. For AEP-$NHNH_2$ (Fig. S2B), the double peaks at 8.98 and 8.96 ppm and the broad peak at 4.09 ppm are ascribed to the protons of NH and NH_2 in acylhydrazine, respectively. The ^{1}H NMR spectrum of HPAH in Fig. S2C shows that the NH signal in acylhydrazone group shifts to 10.89-10.16 ppm. Furthermore, the proton signals at 9.29 and 8.98 ppm indicate the existence of acylhydrazine end-groups. Chemical shifts at 1.21 and 1.14 ppm are ascribed to the methyl signal of butanedione after polymerization.

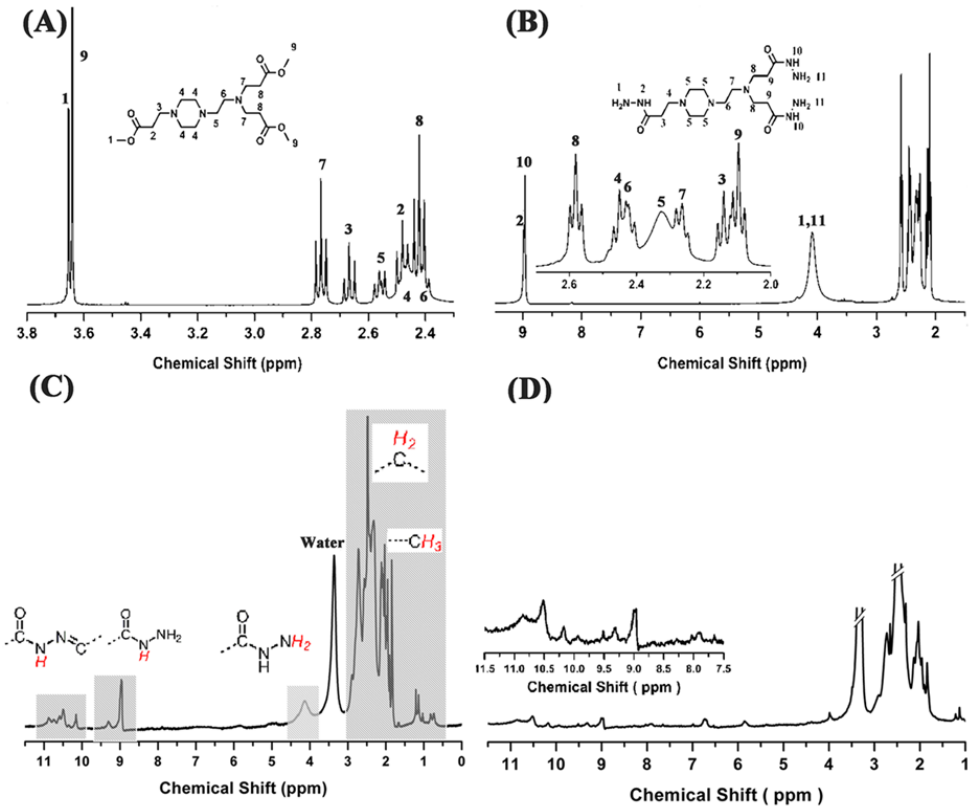


Fig. S2 ^{1}H NMR spectra of: (A) AEP-ester in $CDCl_3$; (B) AEP-$NHNH_2$ in DMSO-d_6; (C) HPAH in DMSO-d_6; and (D) HPAH-DOX in and DMSO-d_6 (400 MHz, 298 K).

For HPAH, the degree of branching (DB) was analyzed by quantitative ^{13}C NMR technique. All possible structural units of HPAH are displayed in Fig. S3. The chemical shifts at 174.87-174.35 ppm in Fig. S4 come from the carbonyl of terminal acylhydrazine. The absence of carbonyl of ketone indicates that all the terminal groups of HPAH are acylhydrazine. The chemical shifts in the region of 171.63-171.23 ppm and 169.07-168.39 ppm are related to the carbon signal of carbonyl in the acylhydrazone, which only exits in the linear units. The carbon signals at 56.10-50.05 and 31.05-29.79 ppm in Fig. S4 indicates the existence of methylene groups. According to the integral area of NMR pattern, the DB of HPAH can be calculated from the following equation:

$$DB = (D + T) / (D + T + L)$$

where D, T and L represent the fractions of the dendritic, terminal, and linear units, respectively. It is found that the DB of HPAH is about 0.60, confirming the formation of highly branched products.

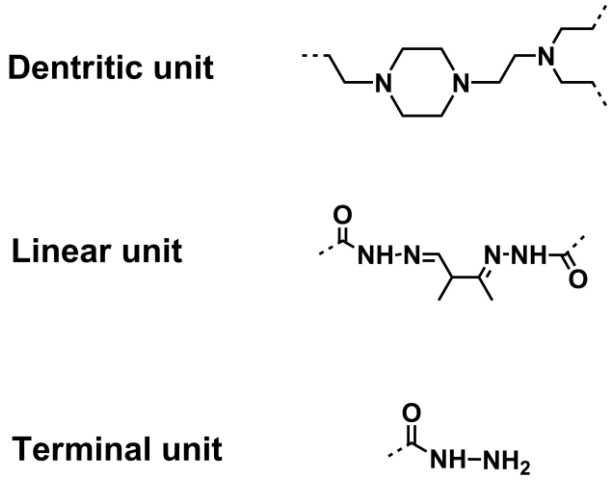


Fig. S3 Chemical structure of branched unit, linear unit and terminal unit of HPAH.

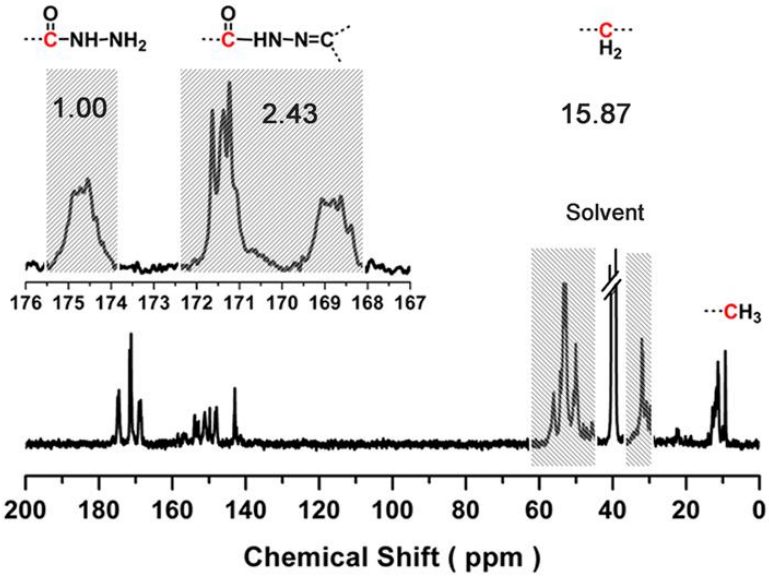


Fig. S4 Quantitative ^{13}C NMR of HPAH.

SEC-MALLS measurement suggests that the weight-average molecular weight (Mw) of end-capped HPAH is around 4.0×10^3, with a polydipersity index (PDI) of 1.6 and dn/dc value of 0.07. Before SEC-MALLS measurement, HPAH was end-capped with benzaldehyde to decrease the interaction between the column and acylhydrazine terminals. HPAH (200 mg) was dissolved in 5 mL dry DMF, then the solution was bubbled with N_2 for 20 min, followed by injecting benzaldehyde (80 μL, 0.79 mmol). The mixture was vigorously stirred for 12 h at 60 °C. After the reaction, the solution was poured into excess amount of cold diethyl ether to precipitate. The obtained yellow product was dried in vacuum at 30 °C for 24 h.

DLS measurements were used to analyze the size distribution of HPAH in aqueous solution. Fig. S5 shows that the average hydrodynamic diameter is less than 7 nm, suggesting the absence of micro-gels in the resultant product.

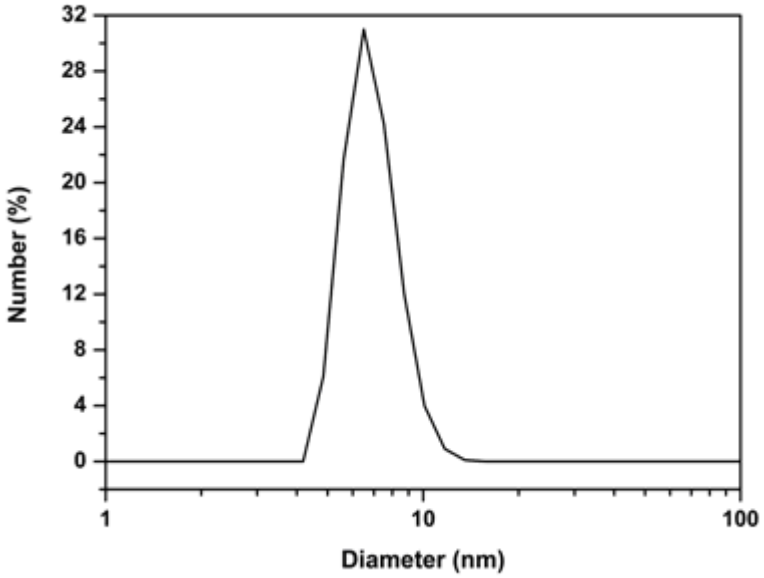


Fig. S5 Dynamic light scattering (DLS) measurements of the hydrodynamic diameter of HPAH.

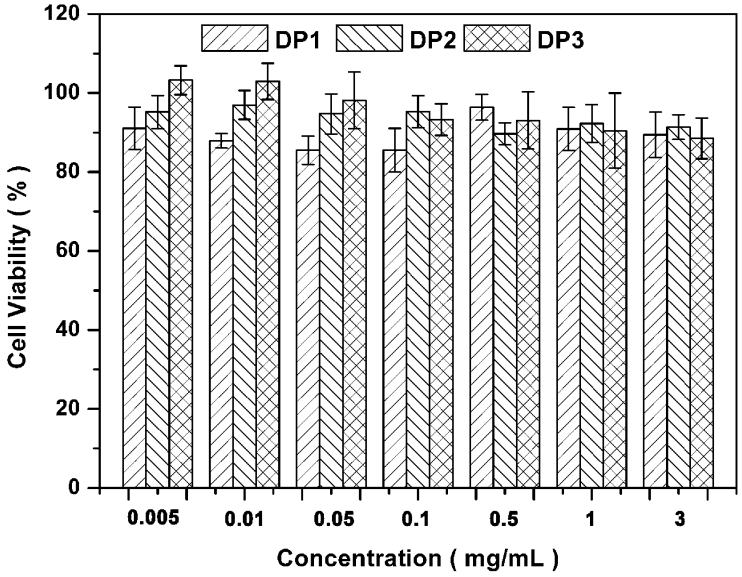


Fig. S6 Cytotoxicity of the degradation products of HPAH at different times (DP1: 5 d, DP2: 13 d, DP3: 19 d).

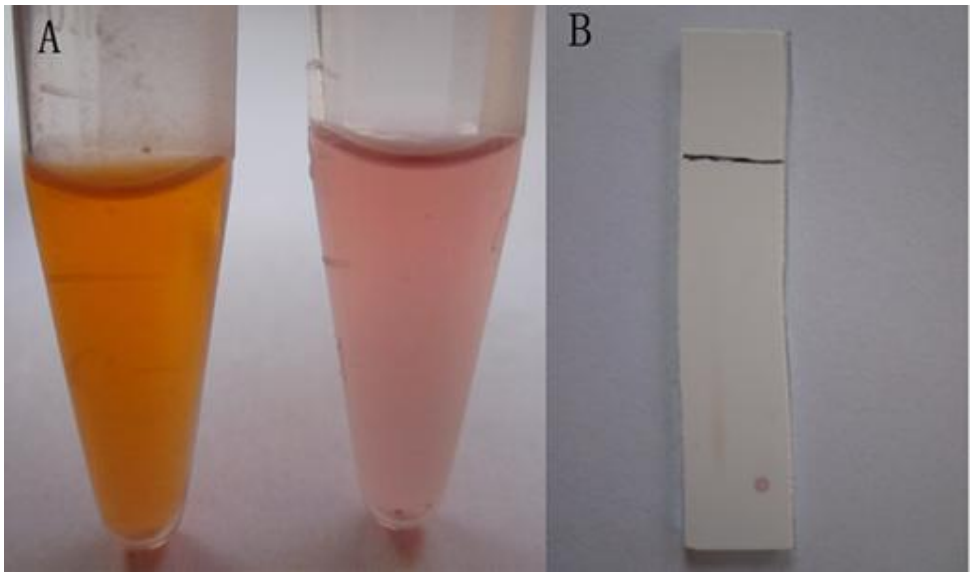
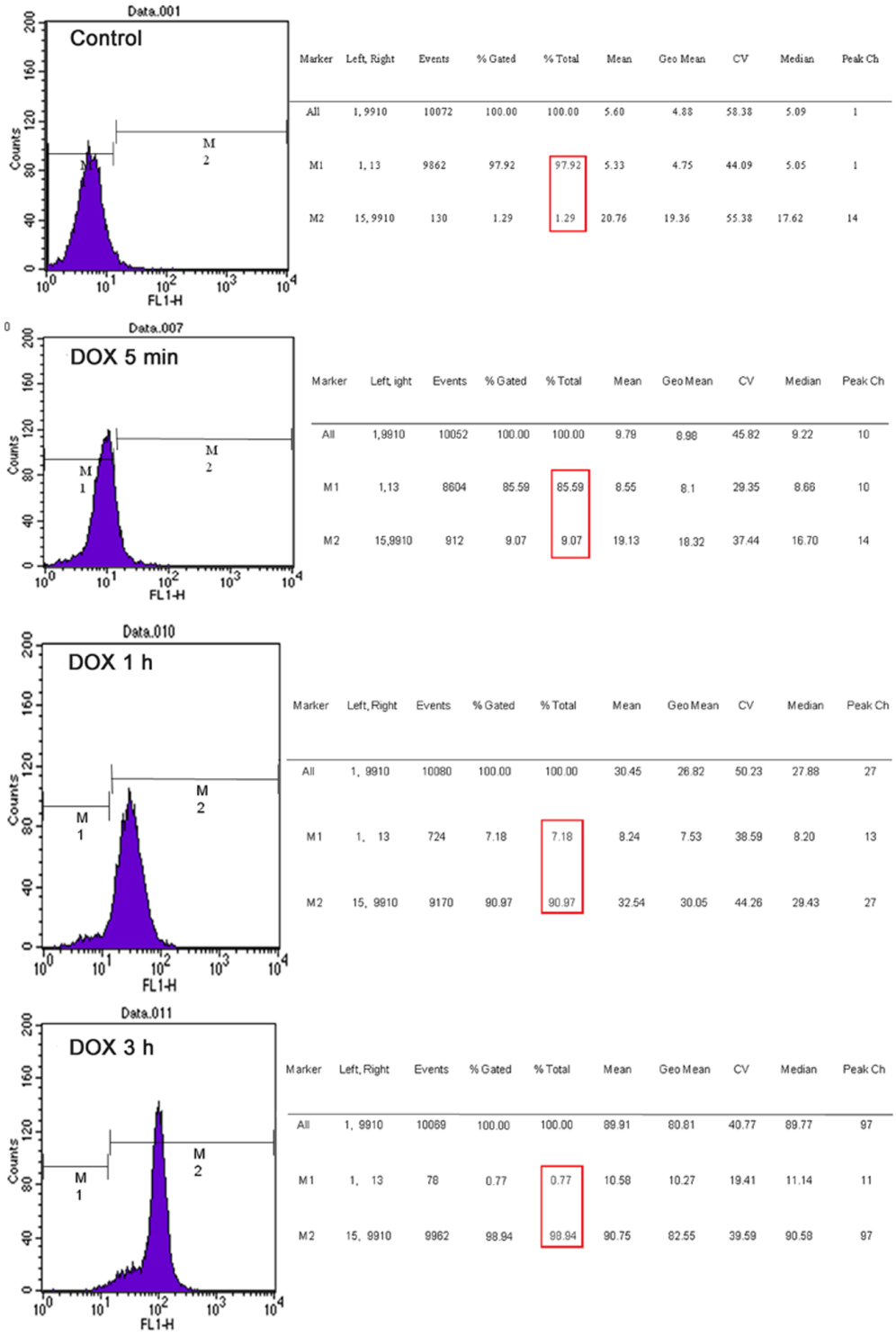


Fig. S7 (A) Methanol solution of free DOX (left) and HPAH-DOX (right); (B) Thin layer chromatography (TLC) of free DOX (left) and HPAH-DOX (right).



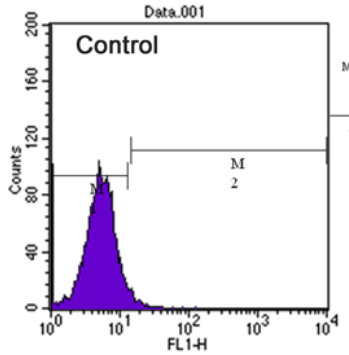
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	10072	100.00	100.00	5.60	4.88	58.38	5.09	1
M1	1, 13	9862	97.92	97.92	5.33	4.75	44.09	5.05	1
M2	15, 9910	130	1.29	1.29	20.76	19.36	55.38	17.62	14

Marker	Left, ight	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1,9910	10052	100.00	100.00	9.79	8.98	45.82	9.22	10
M1	1,13	8604	85.59	85.59	8.55	8.1	29.35	8.66	10
M2	15,9910	912	9.07	9.07	19.13	18.32	37.44	16.70	14

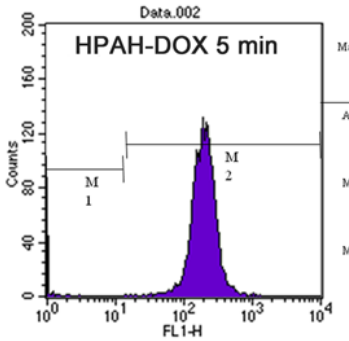
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	10080	100.00	100.00	30.45	26.82	50.23	27.88	27
M1	1, 13	724	7.18	7.18	8.24	7.53	38.59	8.20	13
M2	15, 9910	9170	90.97	90.97	32.54	30.05	44.26	29.43	27

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	10069	100.00	100.00	89.91	80.81	40.77	89.77	97
M1	1, 13	78	0.77	0.77	10.58	10.27	19.41	11.14	11
M2	15, 9910	9962	98.94	98.94	90.75	82.55	39.59	90.59	97

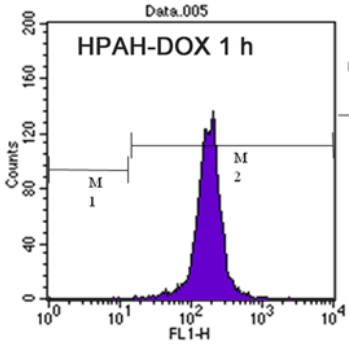
Fig. S8 Flow cytometry histogram profiles of HeLa cells incubated with free DOX at different time.



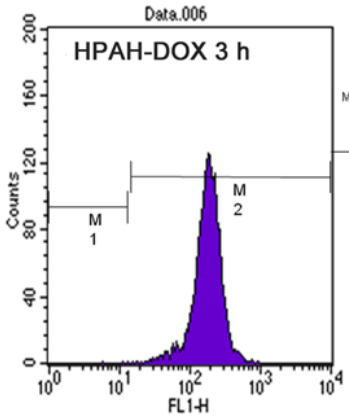
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	10072	100.00	100.00	5.60	4.88	58.38	5.09	1
M1	1, 13	9862	97.92	97.92	5.33	4.75	44.09	5.05	1
M2	15, 9910	130	1.29	1.29	20.76	19.36	55.38	17.62	14



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	10078	100.00	100.00	197.47	177.41	38.84	187.69	186
M1	1, 13	106	1.05	1.05	1.10	1.07	26.48	1.00	1
M2	15, 9910	9970	98.93	98.93	199.60	187.41	37.24	189.38	186



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	10053	100.00	100.00	178.47	166.35	40.98	168.49	191
M1	1, 13	3	0.03	0.03	5.95	4.13	73.05	7.70	1
M2	15, 9910	10050	99.97	99.97	178.52	166.53	40.94	168.49	191



Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	10051	100.00	100.00	181.64	168.22	39.21	173.09	174
M1	1, 13	3	0.03	0.03	9.75	9.22	37.23	10.94	5
M2	15, 9910	10048	99.97	99.97	181.70	168.36	39.17	173.09	174

Fig. S9 Flow cytometry histogram profiles of HeLa cells incubated with HPAH-DOX at different time.