Overcoming multidrug resistance using folate receptor-targeted and pH-responsive polymeric nanogels containing covalently entrapped doxorubicin

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Synthesis of *N*-(2-azidoethyl)methacrylamide AzEMAm

First, 2-azidoethanamine was synthesized. In brief, sodium azide (48.75 g, 750 mmol) and 2-bromoethylamine hydrobromide (51.25 g, 250 mmol) were dissolved in 250 mL deionized water. The reaction mixture was refluxed for 24 hours at 80 °C. Next, the mixture was cooled to 0 °C in an ice bath followed by the addition of 250 mL ethyl acetate and 16 g potassium hydroxide and thorough mixing. Next, the organic and aqueous phases were separated and the aqueous phase was extracted with ethyl acetate (3 × 500 mL). The organic layers were combined and dried using anhydrous $MgSO_4$. The salt was removed by filtration and the product was obtained after evaporation of ethyl acetate under reduced pressure. Then, the obtained 2-azidoethanamine (7.7 g, 88.55 mmol) and triethylamine (14.63 mL, 103.95 mmol) were dissolved in DCM (200 mL) and cooled in an ice-water bath. Methacryloyl chloride (10 g, 96.25 mmol) dissolved in 75 mL of DCM was slowly added. The reaction mixture was stirred overnight at room temperature. Next, the mixture was washed with 200 mL saturated sodium chloride solution. The organic phase was dried over anhydrous $MgSO_4$ and concentrated using a rotovap. The final product (yield 6.2 g, 44.1%) was obtained after purification by flash chromatography using a GraceResolv^{TM} silica cartridge on a VersaFlash chromatography system (ethyl acetate/hexane 7/3, R_f = 0.5).

Characterizations of p(HEMAm-co-AzEMAm)

FT-IR analysis of the polymer was carried out with a BIO-RAD FTS6000 FT-IR (BIO-RAD,

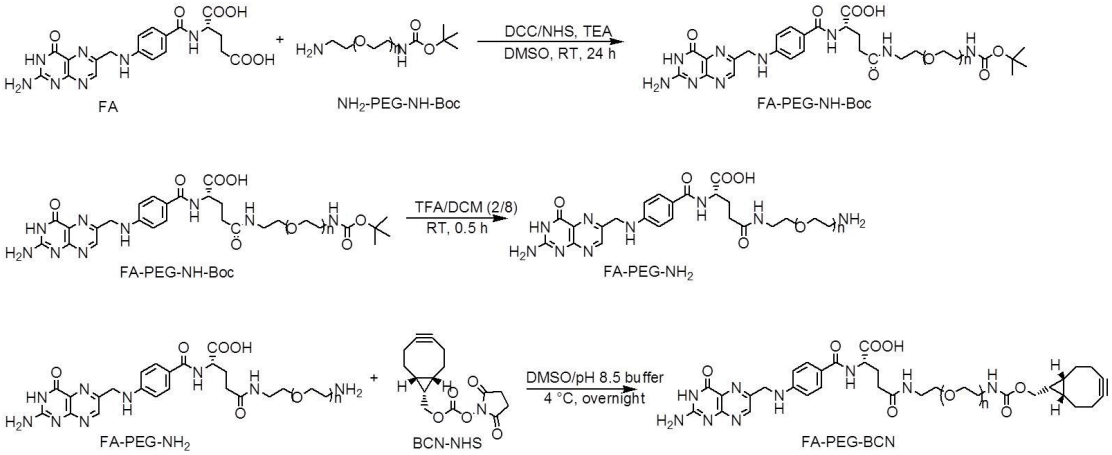
Cambridge, MA, USA) instrument by accumulating 32 scans per spectrum at a data point resolution of 2 cm^{-1}. Solid state spectra of the polymer were acquired using KBr pellets.

The mole percentage AzEMAm in the formed copolymer was determined from 1H-NMR analysis (deuterium oxide as the solvent) using integral intensities $I_{3.46}$ and $I_{3.65}$ of protons at 3.46 ppm (AzEMAm) and 3.65 ppm (HEMAm) (Eq (1)).

$$Mole\%_{AzEMAm} = \frac{I_{3.46}}{I_{3.65} + I_{3.46}} \times 100\% \quad (1)$$

The amount of unreacted monomers in the reaction mixture after polymerization was determined by UPLC (Section 2.2) and the conversions of HEMAm and AzEMAm were calculated according to Eq (2).

$$Conversion\ (\%) = \frac{amount\ of\ unreacted\ monomer}{amount\ of\ added\ monomer} \times 100\% \quad (2)$$



Scheme S1 Synthesis of folic acid-polyethylene glycol-bicyclo[6.1.0]nonyne (FA-PEG-BCN)

Synthesis of folic acid-polyethylene glycol-bicyclo[6.1.0]nonyne (FA-PEG-BCN)

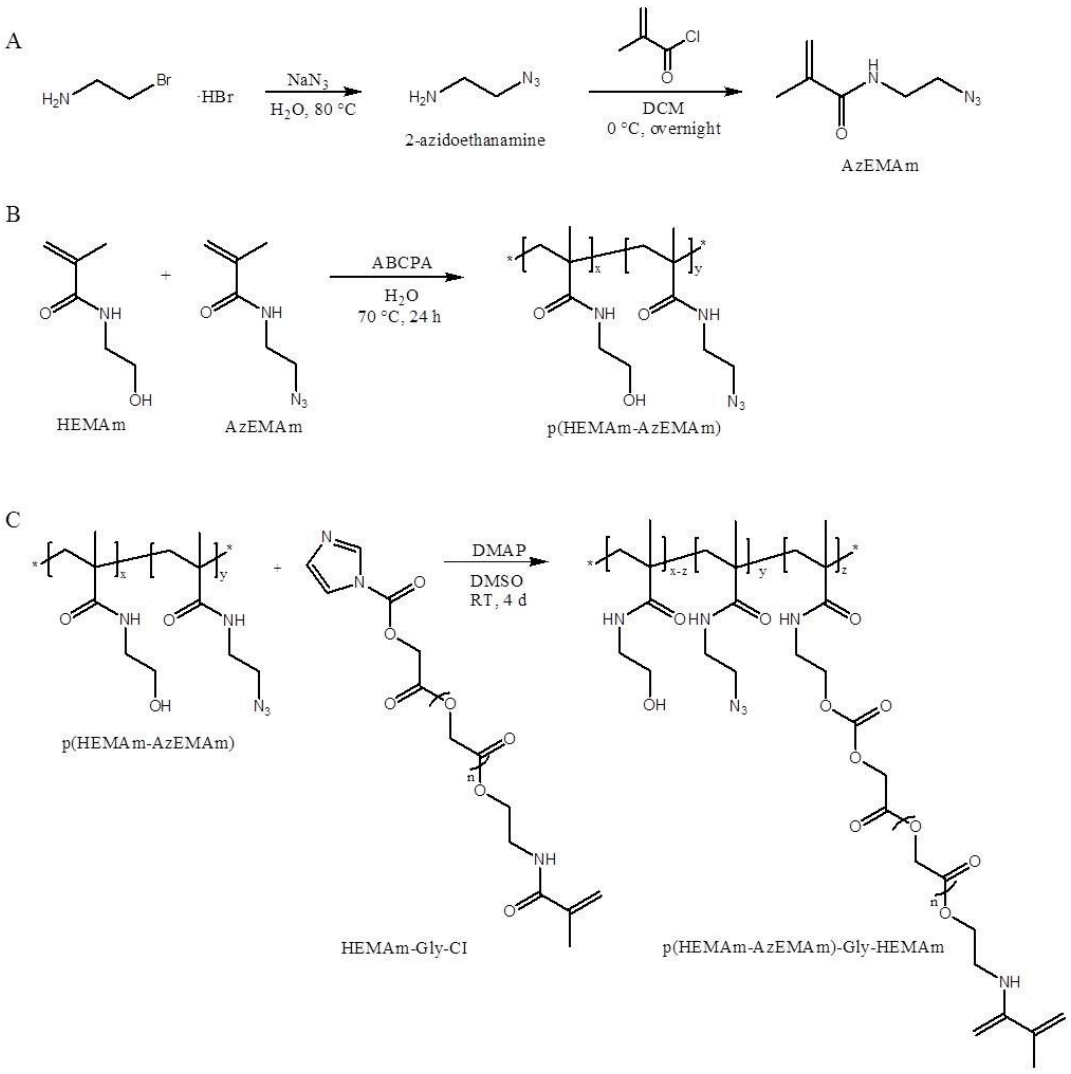
FA-PEG-BCN was synthesized in three steps.

In detail, folic acid was activated by NHS. Folic acid (25 mg, 0.05 mmol) was dissolved in 1.2 mL of DMSO. Subsequently, 5.8 mg of NHS (0.05 mmol), 10.3 mg of DCC (0.05 mmol) and 32.4 µL of triethylamine were added. The reaction was performed at room temperature for 24 h under stirring. Subsequently, this mixture was added to 200 mg of NH_2-PEG-NH-Boc (0.04 mmol) dissolved in 1 mL DMSO and stirred at room temperature in the dark for 24 h. Next, the mixture was centrifuged to remove formed dicyclohexylurea (DCU). The supernatant was dialyzed against sodium bicarbonate buffer (100 mM, pH 8.5) and deionized water before freeze drying.

In the next step, FA-PEG-NH-Boc (100 mg) was dissolved in 0.5 mL of TFA/DCM (2/8, v/v). The solution was stirred at room temperature for 0.5 h. The formed product FA-PEG-NH_2 was purified by dissolution in methanol and precipitation in diethyl ether for three times and dried in vacuo.

Finally, FA-PEG-NH_2 (30 mg, 0.006 mmol) was dissolved in 6 mL DMSO and mixed with 14 µL of trimethylamine (100 µmol). Next, 1 mL of (1R, 8S, 9s)-bicyclo[6.1.0]non-4-yn-9-ylmethyl N-

succinimidyl carbonate (BCN-NHS) solution (14 mg/ml in DMSO, 0.048 mmol) was added to the reaction mixture which was subsequently stirred at room temperature overnight. The reaction mixture was purified by dialysis against DMSO for 24 h, followed by a gradual exchange of the dialysis medium to deionized water. The product was obtained after freeze drying.



Scheme S2 Synthesis of p(HEMAm-co-AzEMAm)-Gly-HEMAm

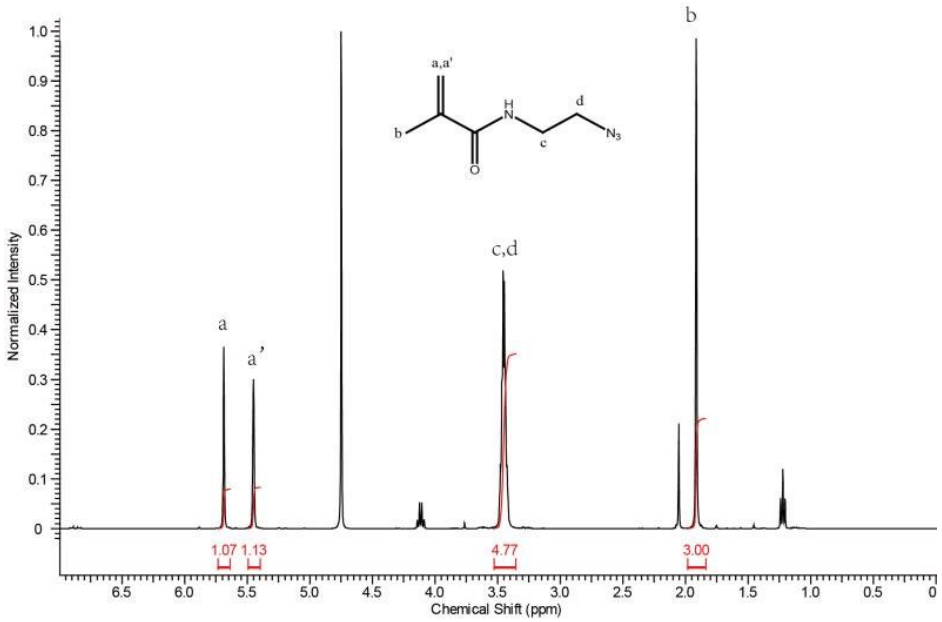


Figure S1 1H-NMR spectrum of AzEMAm.

Table S1 Characteristics of p(HEMAm-co-AzEMAm) as determined by ^{1}H-NMR, UPLC and GPC.

HEMAm/AzEMAm mol/mol in the feed	Yield (%)	Copolymer composition[a)]	Conversion (%)[b)]		M_n (kDa) [c)]	PDI [c)]
			HEMAm	AzEMAm		
80/20	95.6	79/21	98.8	99.1	14.6	3.0

[a)]Determined by ^{1}H-NMR. [b)]Determined by UPLC. [c)]Determined by GPC.

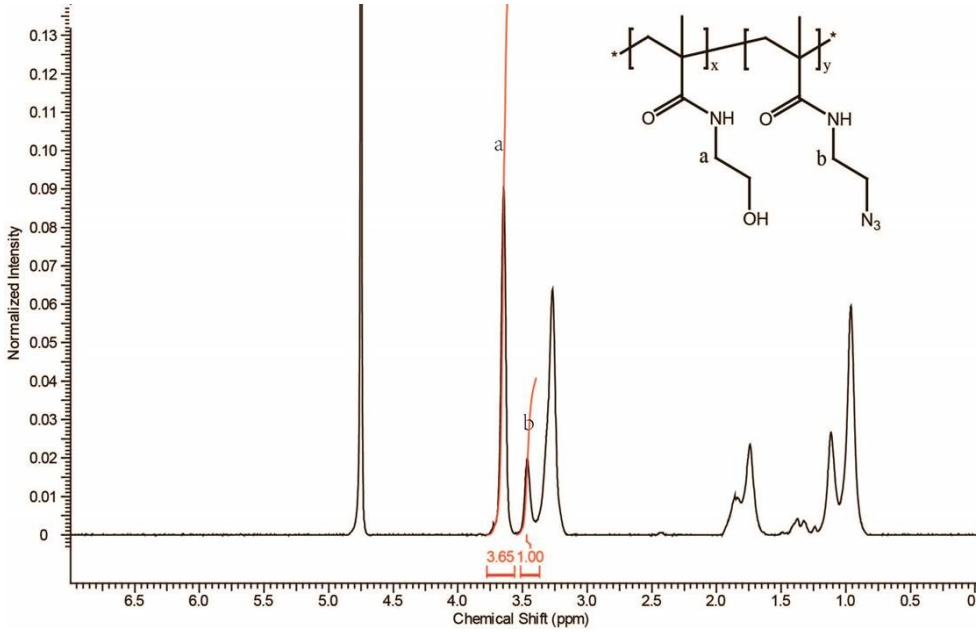


Figure S2 1H-NMR spectrum of p(HEMAm-co-AzEMAm).

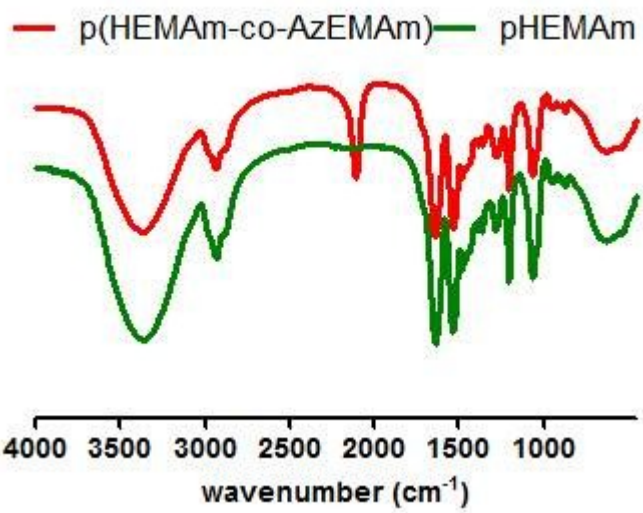


Figure S3 IR spectra of p(HEMAm-co-AzEMAm) and pHEMAm.

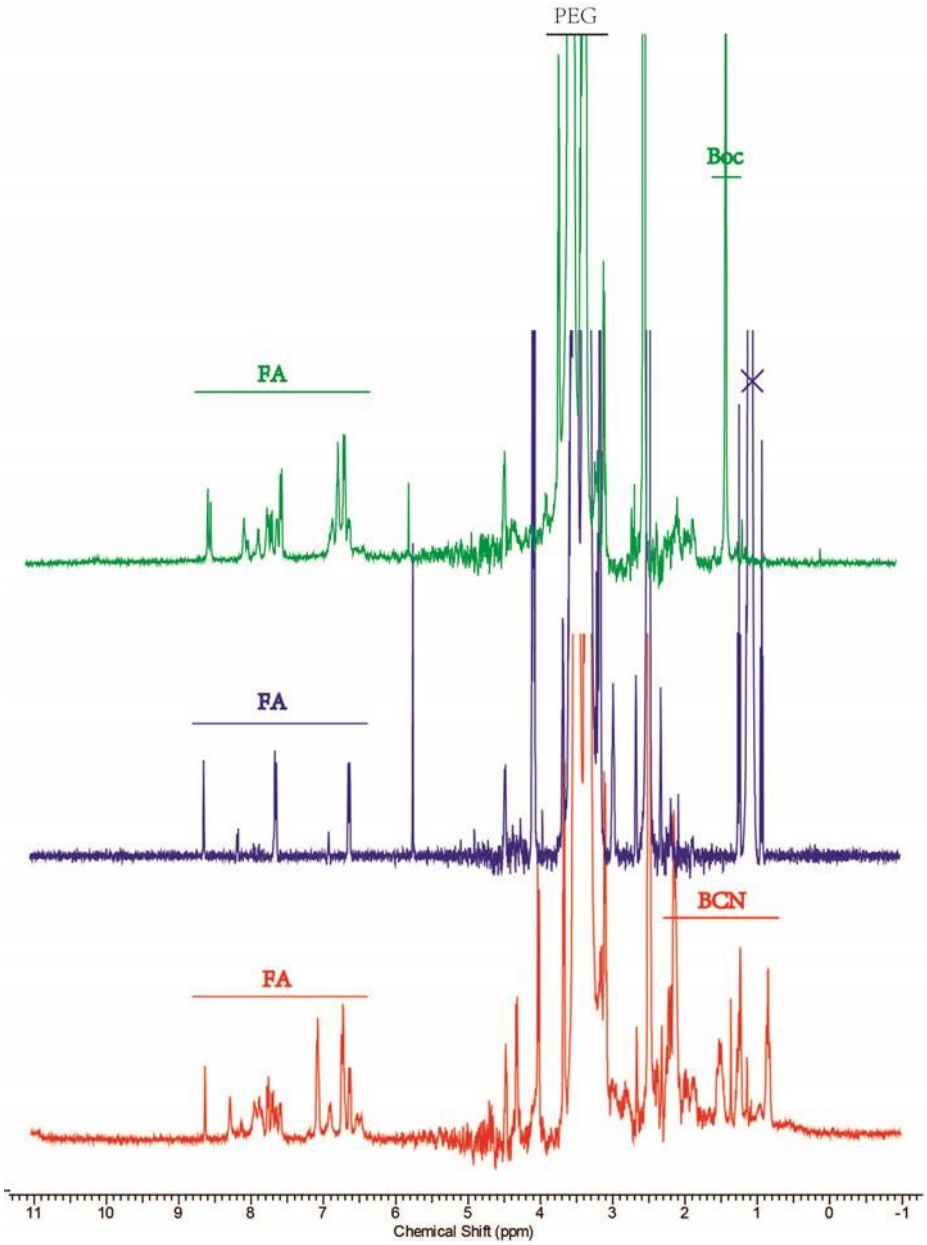


Figure S4 1H-NMR spectra of FA-PEG-NH-Boc (green), FA-PEG-NH2 (blue) and FA-PEG-BCN (red).

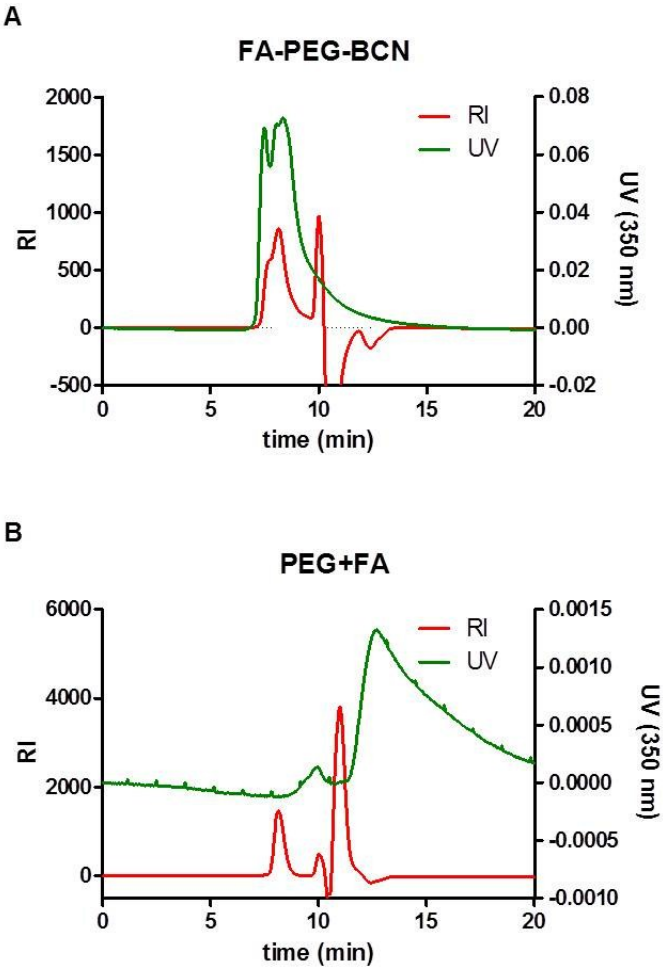


Figure S5 GPC analysis with dual RI and UV (350 nm) detection of (A) FA-PEG-BCN and (B) physical mixture of FA and NH2-PEG-NH-Boc.

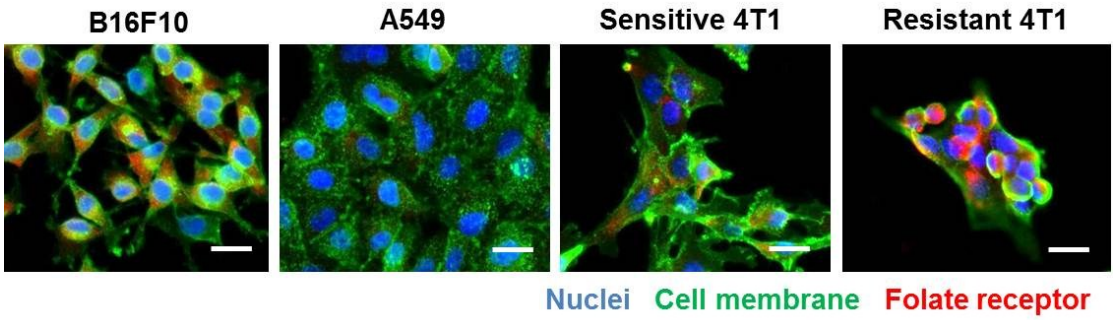


Figure S6 Immunostaining of the folate receptor on B16F10, A549, DOX-sensitive and resistant 4T1 cells; nuclei are stained in blue with DAPI, whereas cell membrane is stained in green with WGA and IgG-PE0 is stained in red in red. Bars, 50 µm.

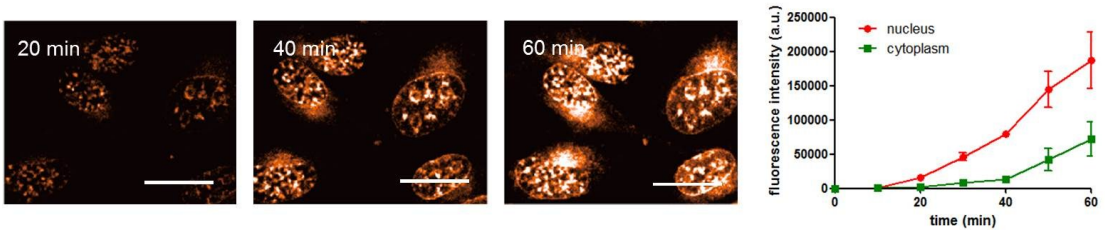


Figure S7 Confocal microscopy images (DOX is depicted as red) and quantification of fluorescence intensity of DOX (λex. 488 nm, λem. 600 nm) of uptake kinetics of free DOX (25000 nM equivalent DOX) in B16F10 cells in 1 h. Bars, 20 µm.

Table S2. IC_{50} (nM) of B16F10 and A549 cells after incubation with free DOX, DOX-MA and DOX-MA loaded nanogel formulations after 72 h incubation (n = 3).

	Free DOX	*DOX-MA*	*DOX-NGs*	*PEG-DOX-NGs*	*FA-DOX-NGs*
B16F10	44±4	370±30	180±30	200±27	120±20
A549	370±70	2,400±300	1,700±200	1,800±400	1,800±400

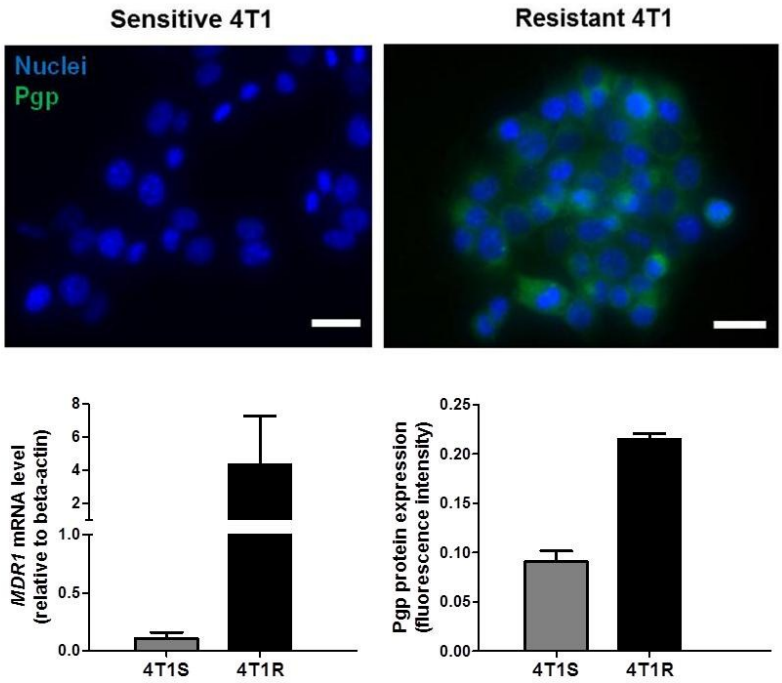


Figure S8 Immunostaining and quantification of Pgp on DOX-sensitive and resistant 4T1 cells; nuclei are stained in blue with DAPI and Pgp is in green. Relative MDR1 mRNA level was measured by RT-PCR and fluorescence intensity of Pgp is calculated by the software ImageJ. Bars, 50 µm.

Table S3. IC_{50} (nM) and resistance index (RI) of DOX-sensitive and resistant 4T1 cells induced by free DOX, DOX-MA and DOX-MA loaded nanogel formulations after 72 h incubation

Formulations	*IC_{50} (nM)*		*RI*
	Sensitive 4T1	Resistant 4T1	
Free DOX	1,000±200	13,900±2,900	13.4±2.4
DOX-MA	4,500±600	98,900±16,800	22.1±3.3
DOX-NGs	1,100±100	11,900±2,000	11.2±1.6
PEG-DOX-NGs	1,500±300	27,400±7,700	18.3±4.5
FA-DOX-NGs	900±200	3,300±70	3.7±0.8