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Supplementary Materials for

Nanoparticle-enhanced chemo-immunotherapy to trigger robust antitumor immunity

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Supplementary Methods

1 Instruments

^{1}H and ^{13}C NMR spectra were collected on a Bruker Advance-III 400 MHz NMR and were analyzed with MestReNova software. Gel permeation chromatography (GPC) data were collected on a Wyatt GPC/SEC-MALS (Wyatt Technology Corporation, Santa Barbara, USA) system at 50ºC using chromatographically pure DMF containing 0.2 v/v% triethylamine and 50 mM LiBr as eluent at a flow rate of 0.80 mL/min. High-performance liquid chromatography (HPLC) was performed on an Agilent 1260 series instrument and data were recorded and processed with preinstalled ChemStation software. The hydrodynamic diameters and zeta potentials of prepared samples were measured on a Zetasizer Nano-ZS (Malvern Instruments, UK) at 25ºC with 632.8 nm laser light set at a scattering angle of 173º. Transmission electron microscopy (TEM) was performed with a Hitachi HT-7700 instrument at a voltage of 120 kV.

2 Synthesis of monomer DTMA-SN38 (fig. S1A)

2.1 3-((3-(2-(methacryloyloxy)ethoxy)-3-oxopropyl)disulfanyl)propanoic acid (DTMA)

HEMA (13.6 g, 0.1 mol), DTDPA (20 g, 0.095 mol) and DMAP (1.74 g, 0.014 mol) were mixed in 100 ml anhydrous DCM and then the reaction solution was cooled to 0 ℃. A solution of EDC·HCl (20 g, 0.1 mol) in 100 ml anhydrous DCM was added dropwise into the reaction solution under vigorous stirring. Then the cloudy reaction solution was stirred at room temperature (RT) overnight. After that, the reaction solution was successively washed with purified water (100 ml, 3 times), 1N HCl (100 ml, 3 times), and saturated NaCl aqueous solution (100 ml, 1 time). Then, the reaction solution was dried with anhydrous $NaSO_4$ and the solvent was removed by rotary evaporation to form a crude product of DTDPA-HEMA (named as DTMA). The pure DTMA was further purified by column chromatography and dried in a vacuum oven to afford a colorless liquid (12.6 g, 41% of yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 6.14 (s, 1H), 5.65-5.58 (s, 1H), 4.37 (s, 4H), 3.00-2.84 (m, 4H), 2.88-2.61 (m, 4H), 1.95 (s, 3H).

2.2 Synthesis of monomer DTMA-SN38

DTMA (1.6 g, 5.2 mmol), SN38 (1 g, 2.5 mmol), pyridine (10 ml) were blended in 50 mL anhydrous DCM and cooled to 0 ℃. A solution of EDC·HCl (1 g, 5.2 mmol) in 10 ml anhydrous DCM was added dropwise into the reaction under vigorous stirring. Then the reaction solution was stirred at RT overnight to dissolve SN38. After that, the reaction solution was successively washed with saturated $NaHCO_3$ aqueous solution (100 ml, 3 times), 1N HCl (100 ml, 3 times) and saturated NaCl aqueous solution (100 ml, 1 time). After that, the DCM solution was dried with anhydrous $NaSO_4$ and the solvent was removed by rotary evaporation to form a crude product of DTMA-SN38. The pure DTMASN38 was further purified by column chromatography as a white powder (1.45 g, 82% of yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.28 (d, 1H), 7.86 (s, 1H), 7.73 (s, 1H), 7.58 (d, 1H), 6.14 (s, 1H), 5.75 (d, 1H), 5.61 (s, 1H), 5.30 (d, 3H), 4.37 (s, 4H), 3.93 (br, 1H), 3.32-3.03 (m, 6H), 3.00 (t, 2H), 2.82 (t, 2H), 2.01-1.77 (m, 5H), 1.41 (t, 3H), 1.04 (t, 3H).

3 Synthesis of poly(ethylene glycol)-PETTC Macro-CTA (fig. S1B)

3.1 Synthesis of 4-Cyano-4-(2-phenylethanesulfanylthiocarbonyl) sulfanylpentanoic acid (PETTC)

2-Phenylethanethiol (10.5 g, 76 mmol) was gradually added into diethyl ether (150 ml) solution containing sodium hydride (60% in oil) (3.15 g, 79 mmol). After stirring for 1 h, the reaction mixture was cooled to 0 °C and then CS_2 (6.0 g, 79 mmol) was gradually added to generate a yellow precipitate, which was filtrated 0.5 h later, and then was rinsed with diethyl ether, and dried for the product. Solid iodine (7.5 g, 29 mmol) was gradually added to a suspension of the product (7.5 g) from the previous step in diethyl ether. After stirring at RT for 1 h, the insoluble NaI was removed by filtration to gain a yellow−brown filtrate, which was subsequently washed with sodium thiosulfate aqueous solution (200 ml, 3 times), saturated NaCl aqueous solution (200 ml, 1 time), dried over anhydrous $NaSO_4$, and then concentrated by rotary evaporation. A solution of dry

bis(2-phenylethanesulfanylthiocarbonyl) disulfide (11.3 g, 26 mmol) and 4,4′-azobis(4-cyanopentanoicacid) (ACVA) (11.3 g, 40 mmol) in ethyl acetate (100 ml) was bubbled with dry N_2 for 30 min and subsequently was heated reflux at 80 ℃ for 18 h. After that, the reaction solution was successively washed with water (200 ml, 5 times), saturated NaCl aqueous solution (200 ml, 1 time). After that, the mixture solution was dried with anhydrous $NaSO_4$ and the solvent was removed by rotary evaporation to a form crude product of PETTC. The pure PETTC was further purified by column chromatography as a yellow powder (13.0 g, 50.6% of yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.34 – 7.21 (m, 5H), 3.57 (t, 2H), 3.01 – 2.96 (t, 2H), 2.71 – 2.65 (t, 2H), 2.57 – 2.49 (m, 1H), 2.44 – 2.35 (m, 1H), 1.88 (s, 3H).

3.2 Synthesis of poly(ethylene glycol)-PETTC (PETTC)

Poly(ethylene glycol) methyl ether (M_n = 5,000 Da) (5 g, 1 mmol) was removed water by refluxing with toluene at 130℃. The flask containing PEG was cooled down prior to addition of anhydrous 100 mL DCM with PETTC (1.4 g, 4 mmol) and DMAP (0.2 g, 1.6 mmol). A flask was charged with DCM (50 ml) and DCC (1.65 g, 8 mmol) then transferred into the reaction solution via a constant voltage funnel under an ice bath. After addition, the reaction solution was stirred at RT for 3 days. The reaction solution was filtered to remove the insoluble dicyclohexylurea (DCU) by-product and concentrated via rotary evaporation under vacuum to yield a dark-yellow solution. The crude product PEG-PETTC was further purified by reprecipitation in cold anhydrous ethanol for 4 times and cold ethyl ether for 2 times. The purified PEG-PETTC was dried in a vacuum oven to afford a faint yellow powder (4.7 g, 89% of yield)

4 Synthesis of diblock copolymer poly(ethylene glycol)-block-poly(2-(diethylamino)ethyl methacrylate) (PEG-b-PDEA) and poly(ethylene glycol)-block-poly(DTMASN38) (PEG-b-PSN38) (fig. S1C)

The typical reaction of Reversible Addition-Fragmentation Chain Transfer (RAFT) Polymerization was as follows: PEG-PETTC (200 mg, 0.04 mmol), redistilled DEAEMA (200 mg, 1.08 mmol) or monomer DTMASN38 (200 mg, 0.29 mmol), and AIBN (2 mg, 0.012 mmol) were dissolved in anhydrous 1,4-dioxane (2 ml) and bubbled with dry N_2 for 30 min. Then the reaction solution was carried out at 75 ℃ for 12 h, then the reaction mixture was immersed to liquid nitrogen to stop the radical polymerization. The reaction mixture was further purified by reprecipitation in cold ethyl ether for 3 times, or reprecipitation in cold anhydrous ethanol for 3 times and cold ethyl ether for 1 time, respectively. The purified polymer was dried in a vacuum oven to afford a faint yellow powder. The DEAEMA or DTMASN38 content in polymer conjugate was analyzed by ^{1}H-NMR.

5 Synthesis of triblock copolymer poly(ethylene glycol)-block-poly(2-(diethylamino)ethyl methacrylate)-block--poly(DTMASN38) (PEG-b-PSN38-b-PDEA) (fig. S1C)

Triblock copolymer PEG-b-PSN38-b-PDEA were synthesized by a two-step RAFT polymerization procedure. PEG-PETTC (200 mg, 0.04 mmol), a certain amount of monomer DTMASN38, and AIBN (2 mg, 0.012 mmol) were dissolved in anhydrous 1,4-dioxane (2 ml) and bubbled with dry N_2 for 30 min. Later the reaction solution was polymerized for 12 hr and then the resulting reaction mixture was immersed to liquid nitrogen to stop the radical polymerization. Next, a certain amount of DEAEMA and AIBN were added to the above mixture for the second polymerization following a similar procedure. After reaction heating for another 12 h, the reaction mixture was stopped by liquid nitrogen. The reaction mixture was further purified by reprecipitation in cold anhydrous ethanol for 3 times and cold ethyl ether once, respectively. GPC measurements of three triblock copolymers were shown in **fig. S3**.

6 Transmission Electron Microscope (TEM) observation

PS3D1@DMXAA nanoparticles was dropped onto a carbon film on 200 mesh copper grids. The excess solution was wicked off and exsiccated for 30 min. Then the sample was stained with phosphotungstic acid for 60 s and exsiccated before imaging. Transmission electron microscopy (TEM) was performed with a Hitachi HT-7700 instrument at a voltage of 120 kV.

7 Molecular dynamics (MD) simulation

Topologies and parameters of PSN38 and PSN38-PDEA and DMXAA were generated using the generalized Amber force field (GAFF)(*16*). PSN38 was composed of 7 DTMASN38 monomers, while PSN38-PDEA polymer was composed of 6 DTMASN38 monomers and 8 DEAEMA monomers. The 9^{th} and 12^{th} monomer of PSN38-PDEA were protonated DEAEMA monomers. Four PSN38-PDEA polymers and 28 DMXAA molecules were solvated in an 11.8×9.6×12.6 nm^3 water box with a TIP3P water model. To neutralize the system and achieve a physiological ionic strength of 150 mM, appropriate number of sodium and chloride ions were added. A similar procedure was adopted to build another system with four PSN38 polymers and 28 DMXAA molecules. For comparison, a system of four PSN38 polymers was constructed in a way similar to the previous procedure. MD simulations were performed with GROMACS 5.1.4. The simulations were performed for 200 ns with a time step of 2 fs.

8 *In vitro* drug release of PS3D1@DMXAA nanoparticles in NaCl solution and DTT solution

PS3D1@DMXAA nanoparticles was fabricated as described above and concentrated at a DMXAA concentration of 0.8 mg/ml. 2 ml nanoparticles solution was loaded into a dialysis bag (MWCO, 3.5 kDa). Then, the bag was immersed into 20 ml deionized water with/without 0.01 M or 0.1 M of NaCl and shaken at 100 rpm. For reduction triggered drug release, 2 ml nanoparticles solution was loaded into a dialysis bag (MWCO, 3.5 kDa) against 20 ml of PBS (pH=7.4) with/without 10 mM of 1,4-Dithiothreitol (DTT) and shaken at 37℃ at 100 rpm. 0.1 ml of solution was taken out for HPLC measurement at different time intervals. The releases of DMXAA and SN38 were calculated according to the standard curve, respectively. The drug release experiments were repeated for three times.

9 *In vitro* drug release of PS3D1@DMXAA nanoparticles in whole blood

PS3D1@DMXAA nanoparticles was fabricated as described above and concentrated at a DMXAA concentration of 1.6 mg/ml. For released DMXAA measurement, 1 ml nanoparticles solution and 4 ml heparinized whole blood were loaded into a dialysis bag (MWCO, 3.5 kDa). Then, the bag was immersed into 20 ml of heparinized PBS (pH=7.4) and shaken at 37℃ at 100 rpm. 0.1 ml of solution was taken out for HPLC analysis. The cumulative DMXAA release was normalized by the unbound fraction of DMXAA in whole blood. For released SN38 measurement, 0.2 ml nanoparticles solution and 2 ml heparinized whole blood were directly mixed and shaken at 37℃ at 100 rpm. At timed intervals, 0.05 ml mixture solution was taken out and precipitated by methanol (0.2 ml). After centrifugation, the supernatant was immediately detected via HPLC.

10 *In vitro* cytotoxicity assays

Cytotoxicity of the nanoparticles and contrastive formulations was determined by (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) assay using B16.F10 and 4T1 cells. Cells were seeded in 96-well plates at a density of 4,000 cells per well for 12 h and the cells were exposed with different formulations. 24 h later, cells were incubated with MTT (0.5 mg ml^{-1}) for an additional 4 h. Then the cell medium was replaced with 100 μl of DMSO to dissolve the formed formazan. The absorbance of each well was determined at 562 nm by a microplate spectrophotometer (Molecular Devices, SpectraMax M2e).

11 *In vivo* blood clearance measurement

Female ICR mice (≈25 g) were treated with PS3D1@DMXAA or free DMXAA through intravenous injection at an injection dose of 10 mg kg^{-1} SN38 and 8 mg kg^{-1} DMXAA. At different time intervals, 50 μl blood samples were collected and precipitated by acetonitrile (0.2 ml). The blood samples were ultrasonic for 30 min and then were centrifuged. The concentration of DMXAA in the supernatant was determined via HPLC.

12 Tumor accumulation of PS3D1@DMXAA nanoparticles

BALB/c mice bearing 4T1 tumors (~200 mm^3) were treated with PS3D1@DMXAA at an injection dose of 10 mg kg^{-1} SN38 and 8 mg kg^{-1} DMXAA. The mice were sacrificed at indicated time intervals after the administrations. The whole tumor tissues were harvested and homogenized in a double volume of PBS. 50 μl tissue

homogenate was taken out into a centrifugal tube containing 50 μl NaOH (0.1 N) and incubated at 65 °C for 6 hours. The samples were precipitated by methanol (0.4 ml) and acidized with HCl (0.1 N). The mixture was ultrasonic for 30 min and centrifuged. The concentrations of SN38 and DMXAA in the supernatants were determined via HPLC.

13 Primers for qPCR

Gene primer name	Primer sequences(5'-3')
m*Ifnb*	F: CAGCTCCAAGAAAGGACGAAC
	R: GGCAGTGTAACTCTTCTGCAT
m*IRF7*	F: ATGCACAGATCTTCAAGGCCTGGGC
	R: GTGCTGTGGAGTGCACAGCGGAAGT
m*CXCL9*	F: TGGGCAGAAGTTCCGTCTTG
	R: ATTACCGAAGGGAGGTGGACAACG
m*CXCL10*	F: GCCGTCATTTTCTGCCTCA
	R: CGTCCTTGCGAGAGGGATC
m*Ctnnb1*	F: GTTCGCCTTCATTATGGACTGCC
	R: ATAGCACCCTGTTCCCGCAAAG
m*ATF3*	F: GCTGGAGTCAGTTACCGTCAA
	R: CGCCTCCTTTTCCTCTCAT
m*Ccl4*	F: GCCCTCTCTCTCCTCTTGCT
	R: GGAGGGTCAGAGCCCATT
m*Ccl5*	F: GTGCCCACGTCAAGGAGTAT
	R: TTCTCTGGGTTGGCACACAC
m*Ifng*	F: ATGAACGCTACACACTGCATC
	R: CCATCCTTTTGCCAGTTCCTC
m*Tnfa*	F: CCTGTAGCCCACGTCGTAG
	R: GGGAGTAGACAAGGTACAACCC

Supplementary figures

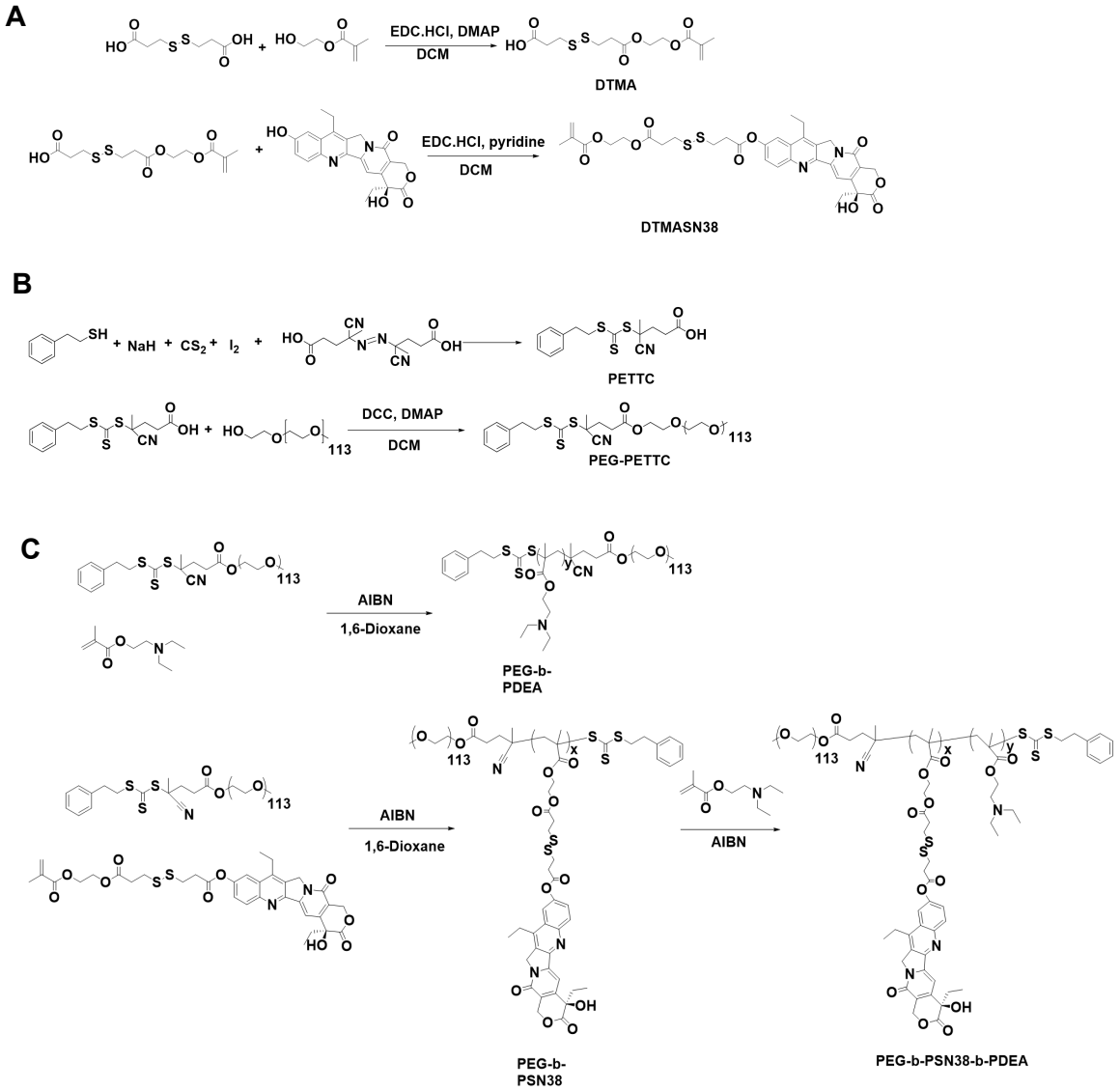

fig. S1. Synthesis of PEG-b-PDEA and PEG-b-PSN38-b-PDEA. (A) Synthesis of monomer DTMASN38. **(B)** Synthesis of macro-CTA agent (PEG-PETTC). **(C)** Synthesis of diblock copolymer PEG-b-PDEA, PEG-b-PSN38 and triblock copolymer PEG-b-PSN38-b-PDEA.

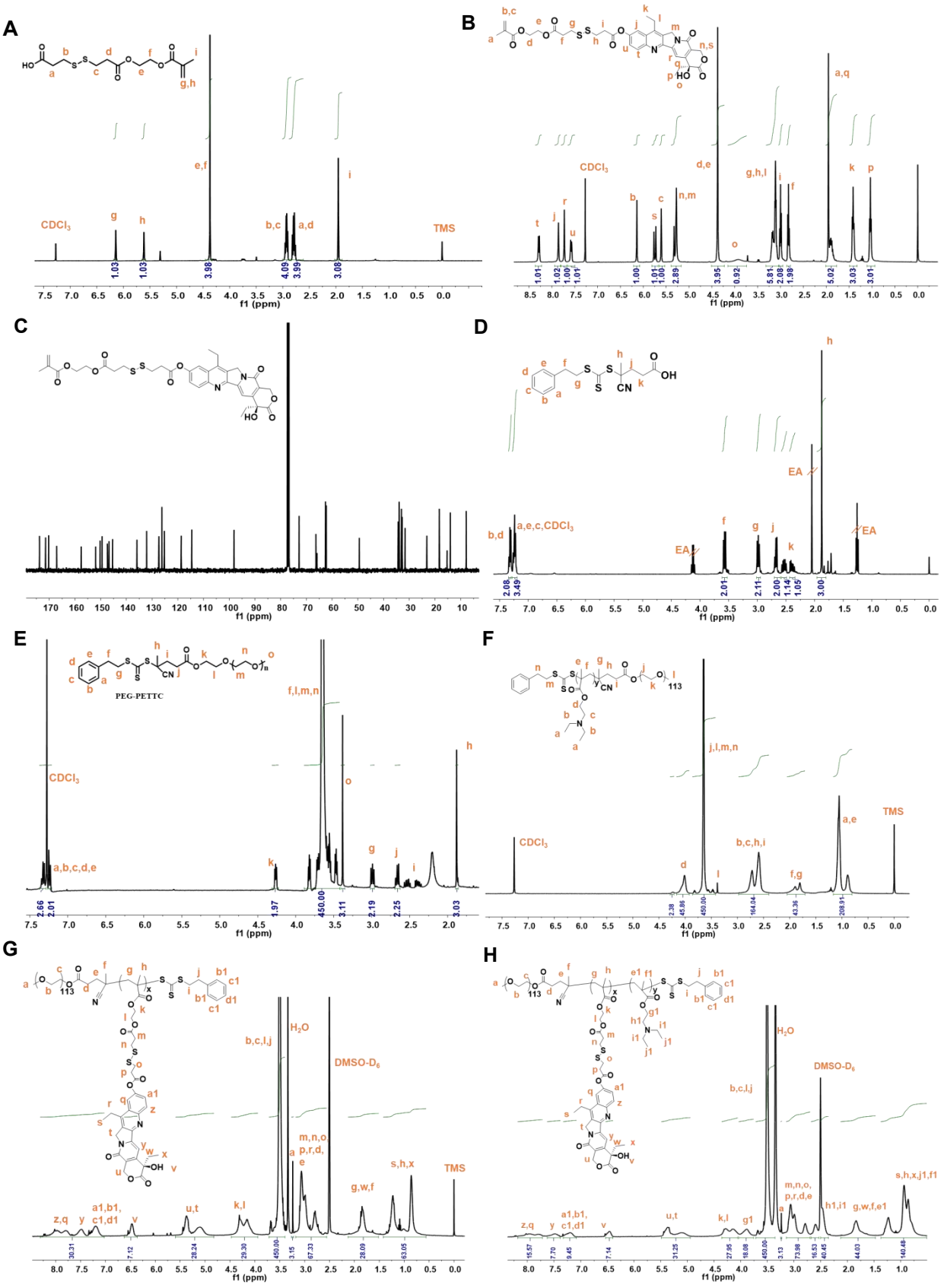

fig. S2. ^{1}H-NMR and ^{13}C-NMR spectra of various compounds synthesized. (A) ^{1}H-NMR spectra of DTMA in chloroform-d. **(B)** ^{1}H-NMR spectra of DTMASN38 in chloroform-d. **(C)** ^{13}C-NMR spectra of DTMASN38 in chloroform-d. **(D)** ^{1}H-NMR spectra of PETTC in chloroform-d. **(E)** ^{1}H-NMR spectra of PEG-PETTC in

chloroform-d. **(F)** ^{1}H-NMR spectra of PEG-PDEA in chloroform-d. **(G)** ^{1}H-NMR spectra of PEG-PSN38 in DMSO-d6. **(H)** ^{1}H-NMR spectra of PEG-PSN38-PDEA in DMSO-d6.

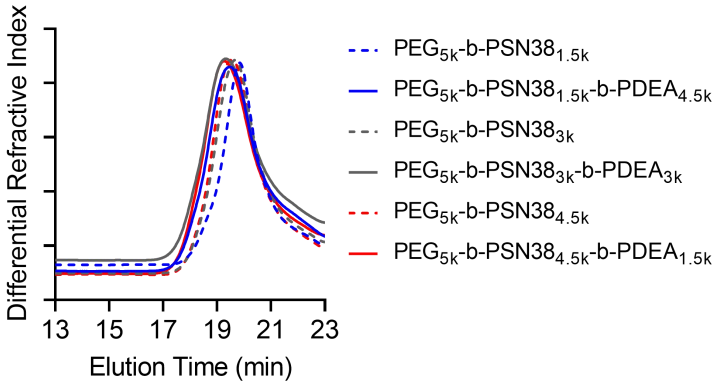

fig. S3. Gel Permeation Chromatograms (GPC) of three triblock copolymer PEG-PSN38-PDEA.

table. S1. Characterization of copolymers properties.

Polymer	Short name	% of ingredient in feed			M_w/M_n [a]
		PEG-PETTC	DTMASN38	DEAEMA	
PEG_{5k}-b-$PSN38_{5k}$	PS	50	50	0	1.22
PEG_{5k}-b-$PDEA_{5k}$	PD	50	0	50	1.17
PEG_{5k}-b-$PSN38_{1.5k}$-b-$PDEA_{4.5k}$	PS1D3	45.5	13.5	41	1.3
PEG_{5k}-b-$PSN38_{3k}$-b-$PDEA_{3k}$	PS2D2	45.5	27.2	27.2	1.27
PEG_{5k}-b-$PSN38_{4.5k}$-b-$PDEA_{1.5k}$	PS3D1	45.5	41	13.5	1.34

a Determined by GPC

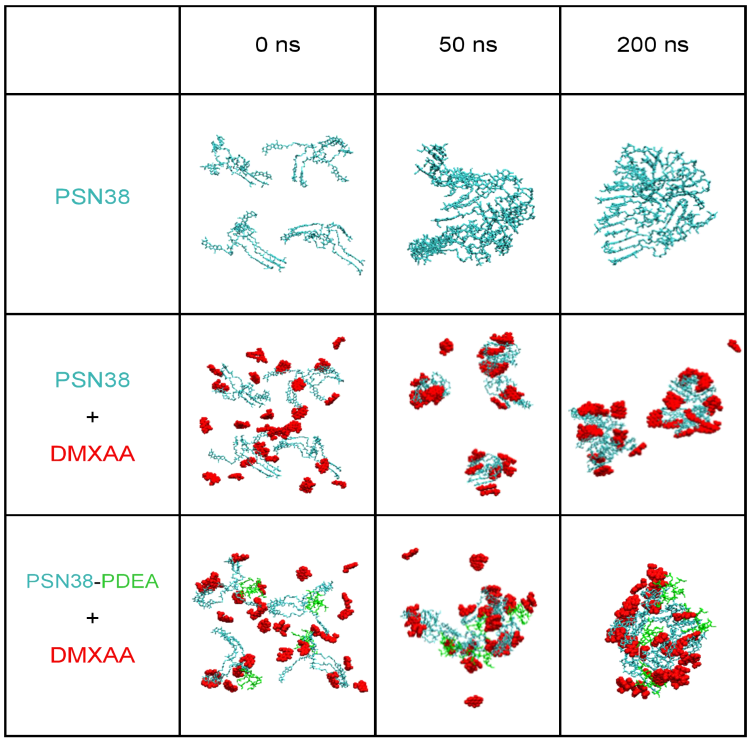

fig. S4. Molecular dynamics (MD) simulation. Snapshot of distinct polymer chains with/without DMXAA after molecular dynamic simulation. PEG content was not included in the MD simulation.

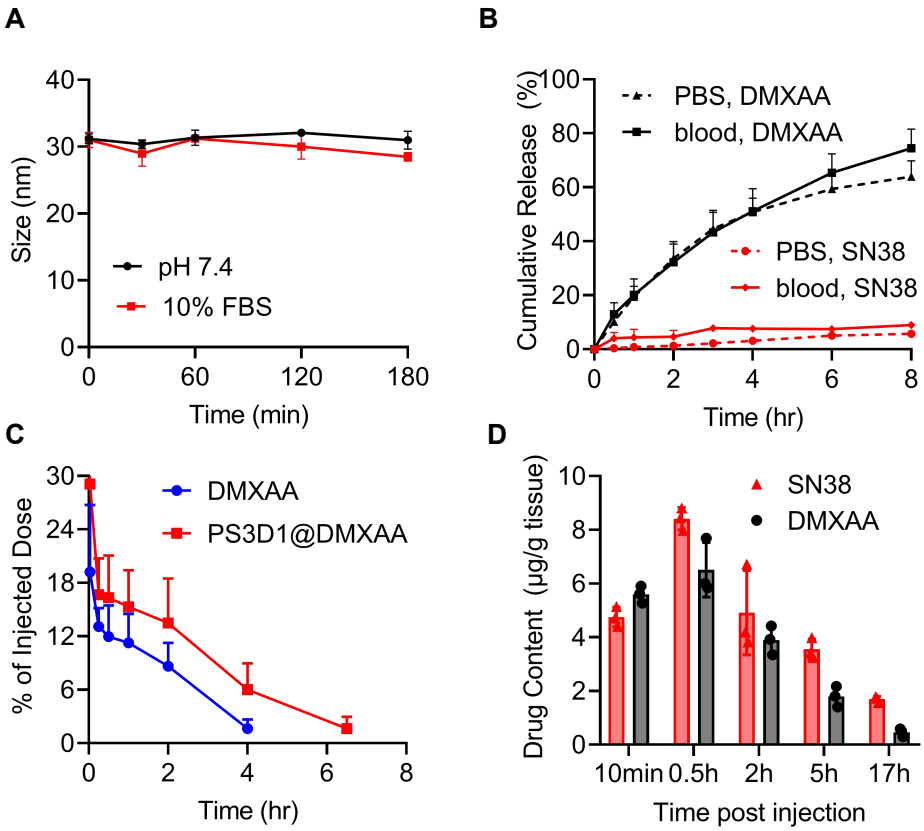

fig. S5. Stability, pharmacokinetics profiles and tumor accumulation of PS3D1@DMXAA. (A) Stability of PS3D1@DMXAA nanoparticle in PBS and culture medium containing 10% of fetal bovine serum. **(B)** *In vitro*

SN38 and DMXAA release profiles from PS3D1@DMXAA in the presence or absence of heparinized whole blood, respectively (n=3, mean ± s.d.). **(C)** *In vivo* pharmacokinetics study of PS3D1@DMXAA in ICR mice**.** (n=3 biologically independent mice per group). **(D)** Corresponding SN38 and DMXAA contents in tumor tissues at different time points post-injection. (n=3 biologically independent mice). Data are shown as mean ± SD.

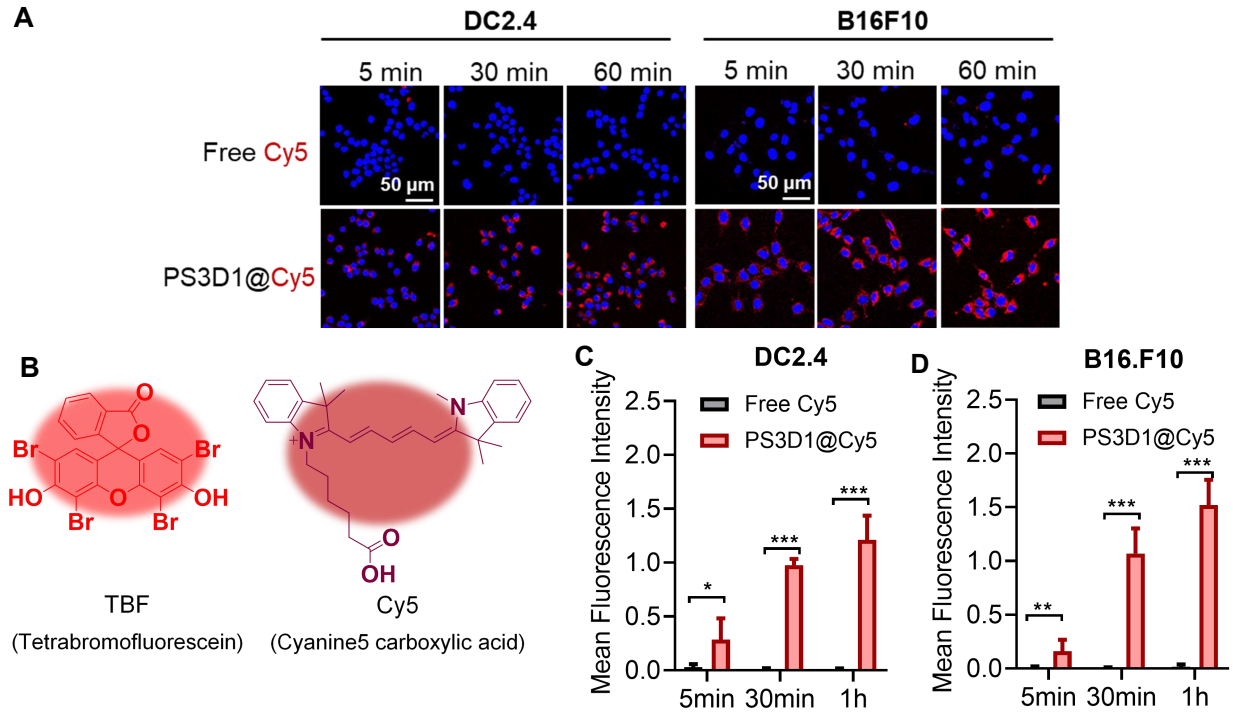

fig. S6. Nanoparticle encapsulation enhances the endocytosis of encapsulated drug. (A) Confocal laser scanning microscopy (CLSM) images of DC2.4 and B16F10 cells upon incubating with free cy5 and PS3D1@cy5 for varying time intervals. **(B)** Structure of TBF and cy5. **(C-D)** Mean fluorescence intensity analysis of PS3D1@cy5 uptake in DC2.4 **(C)** and B16F10 cells **(D)**. (n = 3 biologically independent samples). Data are shown as means ± SD. Statistical significance was calculated by Student's t-test: ***P < 0.001, **P<0.01, *P < 0.05.

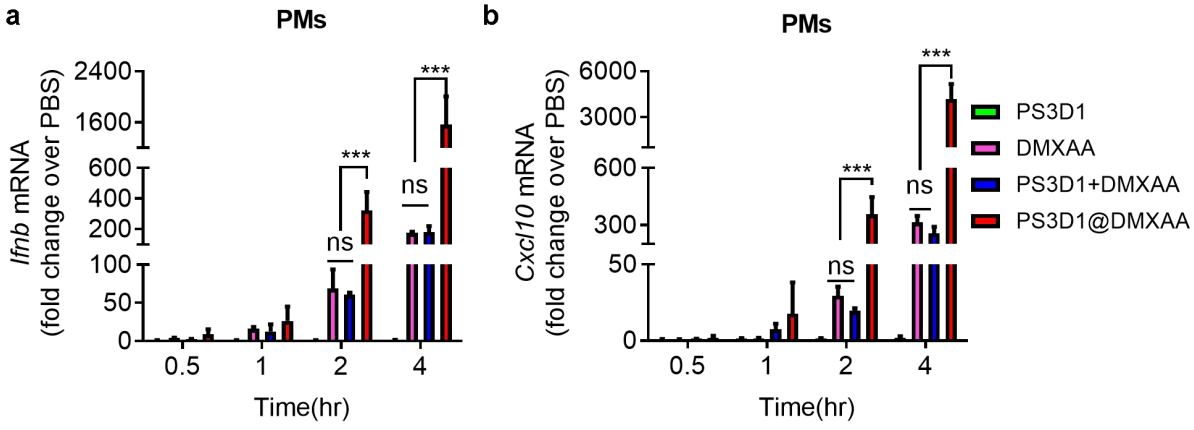

fig. S7. qPCR analysis of *Ifnb* and *Cxcl10* gene expression. qPCR analysis of *Ifnb* **(a)** and *Cxcl10* **(b)** gene expression in PMs after treatment with different formulations for varying time intervals. (n = 3 biologically independent samples). Data are shown as means ± SD. Student's T test. ***P < 0.001, ns, not significant.

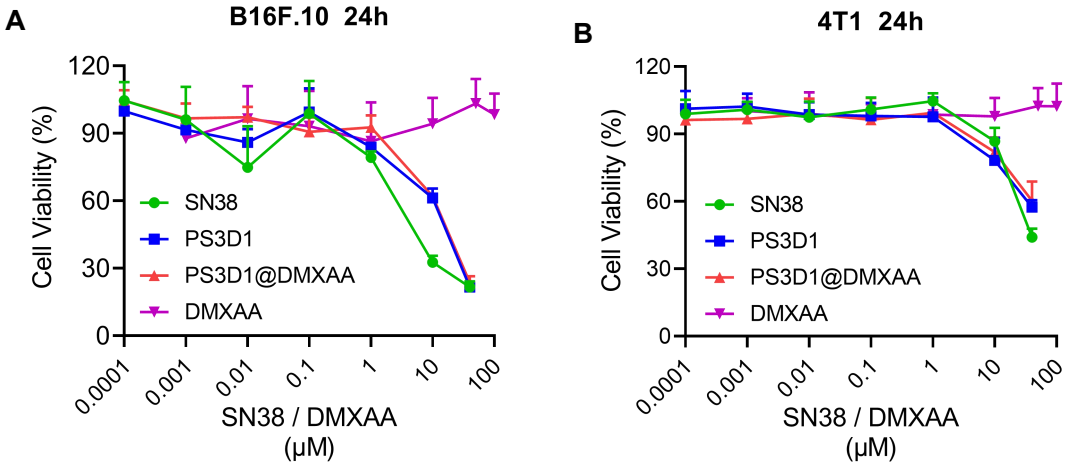

fig. S8. ***In vitro*** **cytotoxicity evaluation. (A-B)** *In vitro* cytotoxicity evaluation of different formulations in B16.F10 **(A)** and 4T1 **(B)** for 24 h. Data are shown as means ± SD.

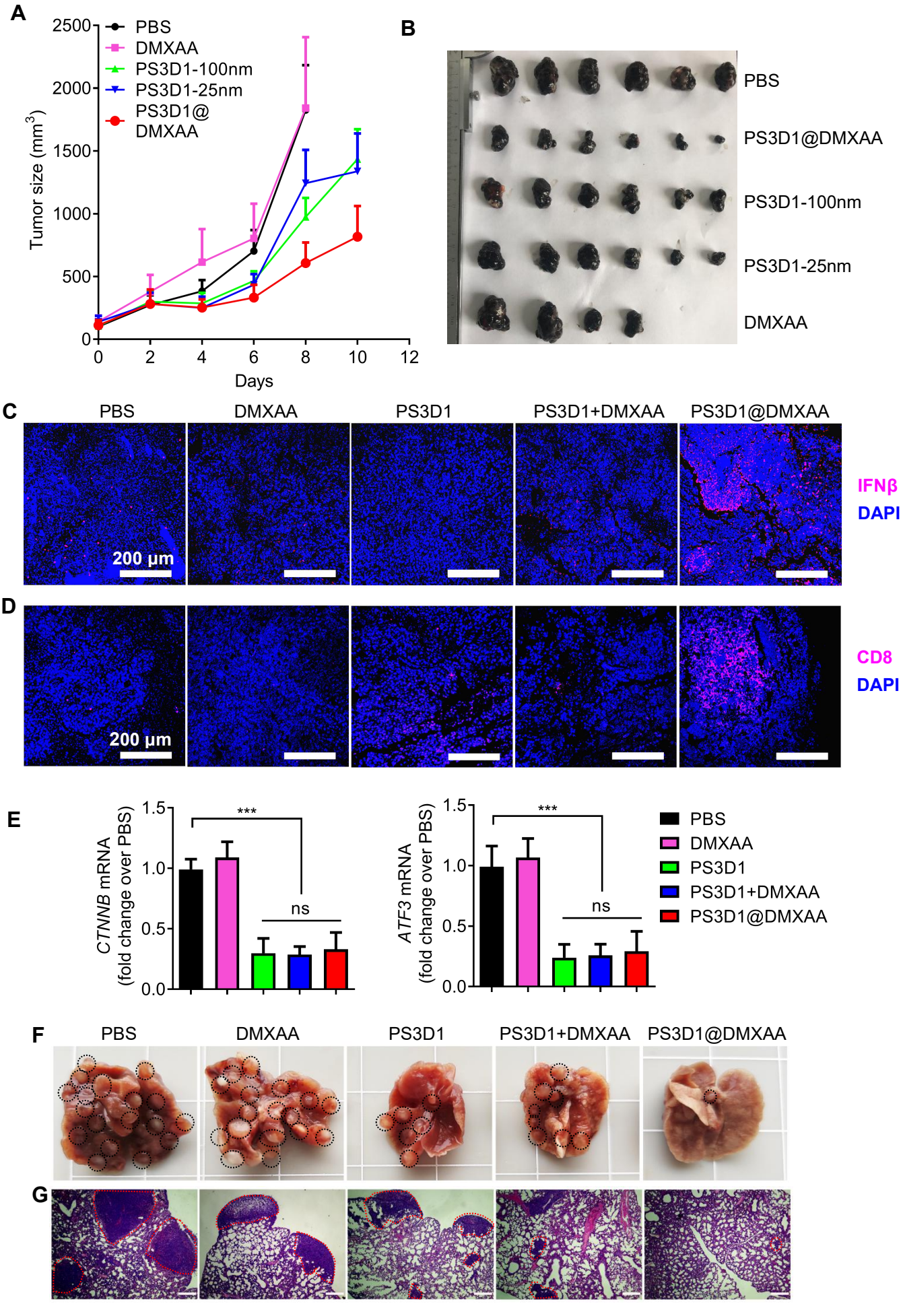
A
Tumor size (mm3)
Days
PBS
DMXAA
PS3D1-100nm
PS3D1-25nm
PS3D1@
DMXAA
B
PBS
PS3D1@DMXAA
PS3D1-100nm
PS3D1-25nm
DMXAA
C
PBS
DMXAA
PS3D1
PS3D1+DMXAA
PS3D1@DMXAA
200 μm
IFNβ
DAPI
D
200 μm
CD8
DAPI
E
CTNNB mRNA
(fold change over PBS)
ATF3 mRNA
(fold change over PBS)
***
ns
PBS
DMXAA
PS3D1
PS3D1+DMXAA
PS3D1@DMXAA
F
PBS
DMXAA
PS3D1
PS3D1+DMXAA
PS3D1@DMXAA
G

fig. S9. Anti-tumor effect of PS3D1@DMXAA in different tumor models. (A) Mice with subcutaneous B16-F10 melanoma tumors were administered with PBS, free DMXAA, PS3D1-100nm, PS3D1-25nm or PS3D1@ DMXAA intravenously for five times, 1 d apart. Tumor growth are shown (n = 6 biologically independent samples, respectively). Data are shown as means ± SEM. **(B)** *Ex vivo* tumor image represented from each treatment group on day 10. **(C)** Representative immuno-fluorescence staining of IFNβ within B16 tumor regions of mice treated with different administration on day 14. IFNβ staining is shown as red, DAPI (blue) in merged image. Scale bars, 200 μm. **(D)** Representative immuno-fluorescence staining of $CD8^+$ T cells within tumor regions of mice treated with different administration on day 14. CD8 staining is shown as red, DAPI (blue) in merged image. Scale bars, 200 μm. **(E)** qPCR analysis of CTNNB and ATF3 gene expression in B16.F10 tumors of mice treated as in Fig. 4a on day 14 (n=6 biologically independent mice per group). Data are shown as means ± SD. ***P < 0.001, ns, not significant, Student's T test. **(F-G)** Inhibiting effects of various treatments on the spontaneous lung metastatic tumor formation. The photographs **(F)**, H&E (**G**) of 4T1-Luci lung metastatic tumors of mice treated with different administration on day 25. Scale bars, 200 μm.