Supporting Information

Bioresponsive Chimaeric Nanopolymersomes Enable Targeted and Efficacious Protein Therapy for Human Lung Cancers *in Vivo*

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Materials. Poly(ethylene glycol) monomethyl ether (PEG, M_n = 5.0 kg/mol, Fluka) was dried by azeotropic distillation from anhydrous toluene before use. 4-Cyanopentanoic acid dithionaphthalenoate (CPADN) and PEG-CPADN were synthesized according to previously described procedure.[1] Azobisisobutyrontrile (AIBN, 98%, J&K) was recrystallized twice from methanol. N-2-hydroxypropyl methacrylamide (HPMA, 97.5%, Suzhou NuoHang trade Co., Ltd.) and acrylic acid (AA, 99%, Shanghai Titan Chemical Co., Ltd.) were purified by passing through a basic alumina column before polymerization. Dichloromethane (DCM) and tetrahydrofuran (THF) were dried by refluxing over $CaCH_2$ and sodium wire, respectively. N, N-dimethyl formamide (DMF) was dried over anhydrous magnesium sulfate and distilled prior to use. Dimethyl sulfoxide (DMSO) was dried over CaH_2 and distilled prior to use. α-Amino-ω-hydroxyl poly(ethylene glycol) hydrochloride (HO-PEG-NH_2·HCl, M_n = 7.5 kg/mol, ≥ 95%, Beijing Jenkem Technology Co. Ltd.), triethylamine (99%, Alfa Aesar), *p*-anisic acid (99%, J&K), haloperidol (98%, J&K), lipoic acid (98%, Acros), 4-dimethylamino pyridine (DMAP, 99%, Alfa Aesar), dicyclohexyl carbodiimide

(DCC, 99%, Alfa Aesar), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (EDC·HCl, > 97%, Shanghai Titan Chemical Co., Ltd.) N-hydroxysuccinimide (NHS, 98%, Alfa Aesar), 1, 4-dithio-D, L-threitol (DTT, 99%, Merck), glutathione (GSH, 99%, Roche), fluorescein isothiocyanate (FITC, 95%, Fluka), cyanine5 amine (Cy5-NH_2, 98%, Lumiprobe Corp., USA), cyanine5 NHS ester (Cy5-NHS, 98%, Lumiprobe Corp., USA), 4′ 6-diamidino-2-phenyl-indole (DAPI, Merck), cytochrome C from equine heart (Sigma), recombinant human granzyme B (GrB, Biovision) were used as received. Lysotracker-red (Invitrogen), rat anti-mouse CD31 primary MAb (BD Pharmingen), Alexa fluor 488 goat anti-rat secondary antibody (Molecular Probes), mouse anti-human sigma receptor (B-5) (Santa Cruz Biotechnology, Inc.), Alexa Fluor ® 488 donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.), and MitoCapture™ mitochondrial apoptosis kit (Biovision) were used according to manufacturer's protocol. Regenerated cellulose dialysis membranes were purchased from Union Carbide Corp.. Human large cell lung cancer cells (NCI-H460, H460) were obtained from China Center for Type Culture Collection (CCTCC).

Characterization. ^{1}H NMR spectra were recorded on a Unity Inova 400 spectrometer operating at 400 MHz or an Agilent DD2 operating at 600 MHz using deuterated dimethyl sulfoxide (DMSO-d_6). The chemical shifts were calibrated against solvent signal of DMSO-d_6. The molecular weight and polydispersity of copolymers were determined by a Waters 1515 gel permeation chromatography (GPC) instrument equipped with three ultra-hydrogel columns following an INLINE precolumn and a differential refractive-index detector. PEG-PHPMA and Anis-PEG-PHPMA copolymers were esterified with trifluoroacetic anhydride (TFAA) and then analyzed by GPC using DMF with 0.05 mol/L LiBr as an eluent at 30 ^{o}C and a flow rate of 1.0 mL/min (standards: polystyrene). PEG-PHPMA-PAA and Anis-PEG-PHPMA-PAA copolymers were analyzed by GPC using aqueous solution of 0.003 M NaN_3 as an eluent at 30 ^{o}C and a flow rate of 1.0 mL/min (standards: polyethylene glycol). The size and size distribution of polymersomes were determined using dynamic light scattering (DLS) at 25 ^{o}C by a Zetasizer

Nano-ZS (Malvern Instruments) equipped with a 633 nm He-Ne laser using back-scattering detection. The zeta potential was determined with a Zetasizer Nano-ZS equipped with a standard capillary electrophoresis cell. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV. The samples were prepared by dropping 10 μL of 0.1 mg/mL polymersomes suspension on the copper grid followed by staining with 1wt.% phosphotungstic acid. The loading efficiency (PLE) of FITC-labeled proteins was determined by UV-vis (U-3900, Hitachi) at 492 nm. The released FITC-labeled proteins were measured by fluorometry (Eclipse, Agilent Technologies). The images of polymersomes and cellular uptake were taken on a confocal laser scanning microscope (CLSM, Leica TCS SP5). The *in vivo* fluorescence images were acquired using a near-infrared fluorescence imaging system (Caliper IVIS Lumina II, Ex 643 nm, Em 668 nm).

Synthesis of PEG-P(HPMA-LA)-PAA Triblock Copolymers. PEG-P(HPMA-LA)-PAA triblock copolymers were prepared by sequential RAFT polymerization of HPMA and AA using PEG-CPADN (M_n = 5.0 kg/mol) as a macro-RAFT agent and AIBN as a radical source, followed by esterification reaction with lipoic acid anhydride (LAA).

Using the procedure reported earlier,[2] PEG-PHPMA was synthesized. Typically, to a Schlenk bottle equipped with a magnetic stir bar were charged HPMA (240 mg, 1.68 mmol), PEG-CPADN (M_n = 5.0 kg/mol,100 mg, 20 μmol) and AIBN (0.49 mg, 3 μmol) in 4.0 mL of THF. After 30 min degassing with nitrogen flow, the reaction vessel was sealed and immersed in an oil bath thermostated at 70 °C. The polymerization was allowed to proceed for 48 h. The product PEG-PHPMA was isolated by precipitation in cold diethyl ether, filtration and drying in vacuo. Yield: 84%. ^{1}H NMR (400 MHz, DMSO-d_6): PEG: δ 3.23 and 3.51; PHPMA: δ 0.8−1.02, 2.89, 3.68 and 4.71. The M_n of PHPMA block was determined to be 9.0 kg/mol (DP = 63) by comparing the intensities of signals at δ 3.68 and 3.51. For GPC measurements, PEG-PHPMA was esterified with trifluoroacetic acid anhydride (TFAA). M_n (GPC) = 14.2 kg/mol, M_w/M_n = 1.10.

Subsequently, PEG-PHPMA (9.0 kg/mol, 100 mg, 7.14 μmol), AA (27.2 μL, 0.40 mmol) and AIBN (0.18 mg, 1.07 μmol) in DMF (1.0 mL) were added into a Schlenk bottle equipped with a magnetic stir bar. The polymerization proceeded at 70 °C for 48 h. PEG-PHPMA-PAA was isolated by precipitation in cold diethyl ether, filtration and drying in vacuo. Yield: 54.4%. ^{1}H NMR (400 MHz, DMSO-d_6): PEG: δ 3.23 and 3.51; PHPMA: δ 0.8−1.02, 2.89, 3.68 and 4.71; PAA: δ 1.53-1.74, 2.20. The M_n of PAA block was calculated to be 2.4 kg/mol (DP = 33) by comparing the intensities of signals at δ 2.20 and 3.68.

Then, PEG-P(HPMA-LA)-PAA was synthesized by grafting lipoic acid anhydride (LAA) onto PEG-PHPMA-PAA via esterification reaction similar to an earlier report.[3] Briefly, under a nitrogen atmosphere, to a stirred solution of lipoic acid (0.16 g, 0.77 mmol) in CH_2Cl_2 (2 mL) was added dropwise a solution of DCC (0.095 g, 0.46 mmol) in CH_2Cl_2 (1 mL). The reaction was allowed to proceed under magnetic stirring for 12 h at room temperature (rt) in the dark. The precipitate generated during the reaction was removed by filtration. The filtrate LAA solution was concentrated to about 0.5 mL and added to a stirred solution of PEG-PHPMA-PAA (100 mg, 6.10 μmol, 384 μmol OH eq.) in DMSO (1.35 mL), followed by addition of DMAP (53.7 mg, 0.39 mmol) in DMSO (2 mL). The flask was sealed and placed into an oil bath thermostated at 30 °C for 48 h in the dark. PEG-P(HPMA-LA)-PAA was purified by precipitation in cold diethyl ether, filtration and drying in vacuo. Yield: 49.7%. ^{1}H NMR (400 MHz, DMSO-d_6): PEG: δ 3.23 and 3.51; PHPMA: δ 0.8-1.13, 3.14, 3.67, 4.71 and 4.83; PAA: δ 1.53-1.74, 2.20; lipoic acid moiety: δ 1.40, 1.58-1.69, 2.29, 1.89/2.43, 3.02, and 3.74. The degree of substitution (DS, defined as number of LA units per 100 hydroxyl groups of PHPMA) of LA was determined to be 75 by comparing the integrals of signals at δ 1.40 (γ-methylene protons of LA moieties) and 3.51 (PEG methylene protons).

Synthesis of Anis-PEG-PHPMA-PAA and Anis-PEG-P(HPMA-LA)-PAA. The two copolymers were synthesized using the same method as above, except that Anis-PEG-CPADN (M_n = 7.5 kg/mol) was used as a macro-RAFT agent. ^{1}H NMR of Anis-PEG-P(HPMA-LA)-PAA (600 MHz,

$CDCl_3$): δ 7.78 (Ar*H*-CO-), 6.90 (Ar*H*-OCH_3), 6.03 (aromatic protons), 5.83 (Ar-*CH*-), 4.17 (-COO*CH*$_2$C-), 3.89 (-O*CH*$_2$*CCH*$_2$O-), 3.75 (Ar-O*CH*$_3$), 3.65 (PEG), PHPMA: δ 0.8-1.13, 3.14, 3.67, 4.71, and 4.83; PAA: δ 1.53-1.74, 2.20; lipoic acid moiety: δ 1.40, 1.58-1.69, 2.29, 1.89/2.43, 3.02, and 3.74. The DS of LA was determined to be 67.

Synthesis of Cy5-NH_2 Labled PEG-P(HPMA-LA)-PAA (PEG-P(HPMA-LA)-PAA-Cy5). In brief, 1 mL PEG-P(HPMA-LA)-PAA (50 mg, 1.92 μmol) dissolved in DMSO was mixed with EDC·HCl (0.88 mg, 4.61 μmol) and NHS (0.53 mg, 4.61 μmol) in 1 mL DMSO and stirred for 30 min. 0.2 mL Cy5-NH_2 (3.05 mg, 4.61 μmol) in DMSO was then added at r.t.. After reaction for 24 h under constant stirring, the product was dialyzed in DMSO for 24 h and in DI water for 24 h, followed by lyophilization. Cy5 content per PEG-P(HPMA-LA)-PAA chain determined by UV-vis was ca. 2.

Preparation of FITC or Cy5 Labeled Cytochrome C (FITC-CC and Cy5-CC). For FITC labeling, 0.2 mL FITC (1.8 mg, 4.63 μmol) in DMSO was dropwise added into 2 mL CC solution (50 mg, 3.85 μmol) in sodium carbonate and sodium bicarbonate buffer (pH 9.4, 50 mM) at 0 ^oC. After reaction for 24 h at 30 ^oC, the product was extensively dialyzed against PB buffer (pH 8.0, 10 mM) for 24 h and DI water for 24 h, followed by lyophilization. FITC content per CC molecule determined by UV-vis was ca. 1.

For Cy5-NHS labeling, 2 mL CC (50 mg, 3.85 μmol) solution in PB buffer (pH 8.0, 10 mM), 0.2 mL Cy5-NHS (3.05 mg, 4.63 μmol) in DMSO was added at 30 ^oC. After reaction under stirring for 24 h, the product was dialyzed against PB buffer (pH 7.4, 10 mM) for 24 h and DI water for 24 h, followed by lyophilization. Cy5 content per CC molecule determined by UV-vis was ca. 1.

The Loading and Reduction-Triggered Release of FITC-CC from Polymersomes. FITC-CC was used as a model protein at varying theoretical loading contents from 1 to 50 wt.%. For determination of protein loading content (PLC) and protein loading efficiency (PLE), FITC-CC-Anis-BCPs were diluted and analyzed with UV-vis (U-3900) at 492 nm. PLC and PLE

were calculated according to the following formula based on a calibration curve obtained with FITC-CC in PB of known FITC-CC concentrations:

PLC (*wt*.%) = (weight of loaded protein/total weight of protein and polymer) × 100 (1)

PLE (%) = (weight of loaded protein/weight of protein in feed) × 100 (2)

The *in vitro* FITC-CC release from FITC-CC-Anis-BCPs was studied at a low polymersome concentration of 80 μg/L using a dialysis tube (MWCO 300,000) in a shaking bath (200 rpm) at 37 ^{o}C in two different media, i.e. phosphate buffered saline (PBS, 5 mM, pH 7.4, 150 mM NaCl) and PBS containing 10 mM GSH. In order to acquire sink conditions, protein release was performed using 0.5 mL of FITC-CC-Anis-BCPs to dialyze against 25 mL of release media. At desired time intervals, 5.0 mL of release media was taken out and replenished with an equal volume of fresh media. The FITC-CC released was quantified using fluorescence measurements (FLS 920, ex. 492 nm, em. 525 nm). The release experiments were conducted in triplicate and the results presented were the average data with standard deviations.

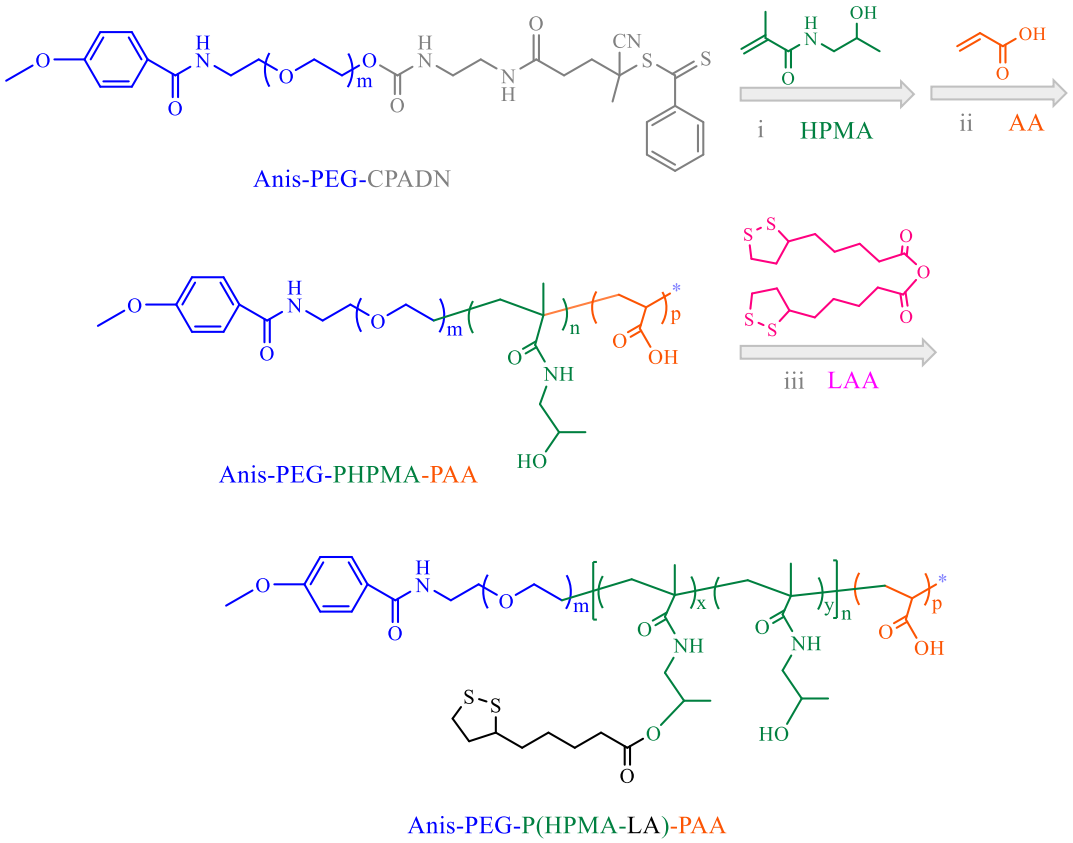
Determination of sigma receptor expression on the tumor cells. Human large cell lung cancer H460 cells, human lung carcinoma A549 and human breast adenocarcinoma MCF-7 cells were cultured in 6-well plates (1×10^6 cells/well) in 1 mL RPMI 1640 medium, which was supplemented with 80 units/mL penicillin G, 100 μg/Ml streptomycin, and 10% FBS. After culturing for 24 h the cells were digested by 0.25w/v% trypsin/0.03 w/v% EDTA were centrifuged (1000 ×g, 3 min), washed with PBS (×3), and treated with mouse sigma receptor antibody (4 μg/mL) for 1 h at r.t.. After PBS washing (×3) and centrifugation (×3), Alexa Fluor ® 488 donkey anti-mouse secondary antibody (10 μg/mL) were added and incubated for 1 h at r.t.. Following PBS washing (×3) and centrifugation (×3), the cells in 0.5 mL PBS were measured immediately using a BD FACS Calibur flow cytometer and analyzed using Cell Quest (10,000 gated events).

Blood Circulation of Anis-BCPs-Cy5. Cy5-labeled polymersomes Anis-BCPs-Cy5 were prepared from the co-self-assembly from PEG-P(HPMA-LA)-PAA, PEG-P(HPMA-LA)-PAA-Cy5 and

Anis-PEG-P(HPMA-LA)-PAA at molar ratio of ca. 1/7.0/7.7. And BCPs-Cy5 was similarly from PEG-P(HPMA-LA)-PAA and PEG-P(HPMA-LA)-PAA-Cy5 at molar ratio of ca. 1.3:1.

Nude mice were weighted and randomly grouped. Cy5-labeled polymersomes, Anis-BCPs-Cy5 or BCPs-Cy5 (0.40 μmol Cy5 equiv./kg), in 200 μL of PB were intravenously injected into nude mice (18 ~ 22 g) via the tail vein (n = 3). At prescribed time points post injection, ca. ~10 μL of blood was withdrawn from the tail vein of nude mice. The blood samples upon withdrawing were immediately dissolved in 0.1 mL of lysis buffer (1% triton X-100) with brief sonification. Cy5 polymers were extracted by incubating blood samples in 0.6 mL of extraction solution (DMSO containing 20 mM DTT) in a shaking bath (37 ^{o}C, 200 rpm) overnight followed by centrifugation (14.8 krpm, 30 min). Cy5 levels in the supernatant were determined by fluorometry. The blood circulation curve of the polymersomes followed a two compartment model: a rapid decline in distribution phase and long period elimination phase. The half-lives of two phases ($t_{1/2,\alpha}$ and $t_{1/2,\beta}$) were calculated by fitting the experimental data using Origin 8 exponential decay 2 model: $y = A_1 \times \exp(-x/t_1) + A_2 \times \exp(-x/t_2) + y_0$, taking $t_{1/2,\alpha} = 0.693\times t_1$ and $t_{1/2,\beta} = 0.693\times t_2$.

Preliminary In Vivo Antitumor Experiments of GrB-Anis-BCPs. The H460 tumor bearing mice were weighed and randomly grouped (n = 3). GrB-Anis-BCPs or GrB-BCPs (1.56 nmol GrB equiv./kg) were *i.v.* injected via the tail vein on day 0, 4, 8 and 12. Empty Anis-BCPs and PBS were used as controls. A single dose of GrB-Anis-BCPs at 1.56 nmol GrB equiv./kg was injected on day 0. The tumor size was measured using calipers every 2 days and tumor volume (V) was calculated according to the formula $V = 0.5 \times L \times W^2$, wherein L and W are the tumor dimension at the longest and widest point, respectively. The body weight of mice was measured every 2 days and the relative body weight was normalized to their initial weights.



Scheme S1. Synthetic route of Anis-PEG-P(HPMA-LA)-PAA. Conditions: (i) AIBN, THF, 70 °C, 2d; (ii) AIBN, DMF, 70 °C, 2d; (iii) DMAP, DMSO, 30°C, 2 d.

Table S1. Characteristics of PEG-PHPMA-PAA and Anis-PEG-PHPMA-PAA before and after lipoylation

Entry	Copolymers	M_n (kg/mol)		GPC[b]		M_n[a] (kg/mol)
		Design	^{1}H NMR[a]	M_n (kg/mol)	$Đ$	lipoylated
1	PEG-PHPMA-PAA	5.0-12.0-2.4	5.0-9.0-2.4	14.5	1.07	5.0-18.7-2.4
2	Anis-PEG-PHPMA-PAA	7.5-14.0-2.4	7.5-9.2-2.4	17.0	1.14	7.5-18.1-2.4

[a] Calculated from ^{1}H NMR by comparing the integrals of signals at δ 3.67, 2.20 and 3.51, respectively.

[b] From GPC measurements.

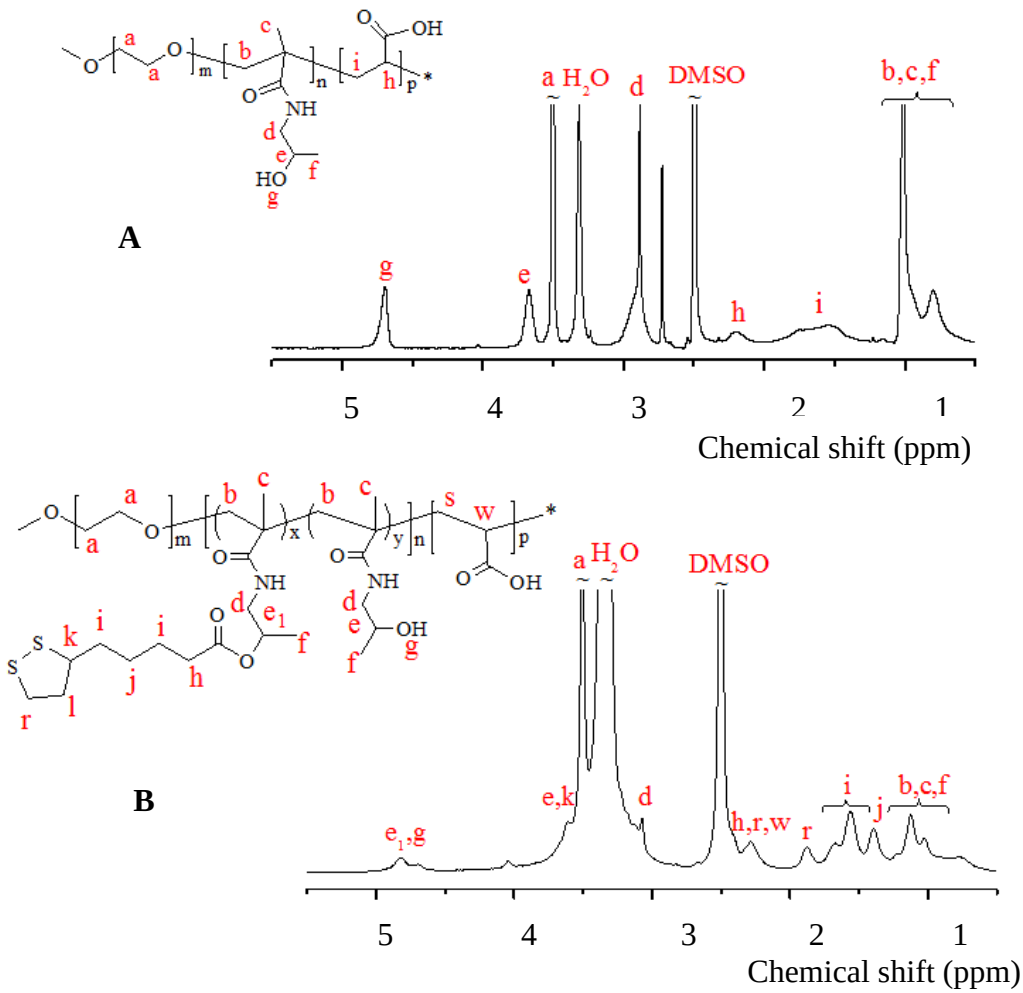
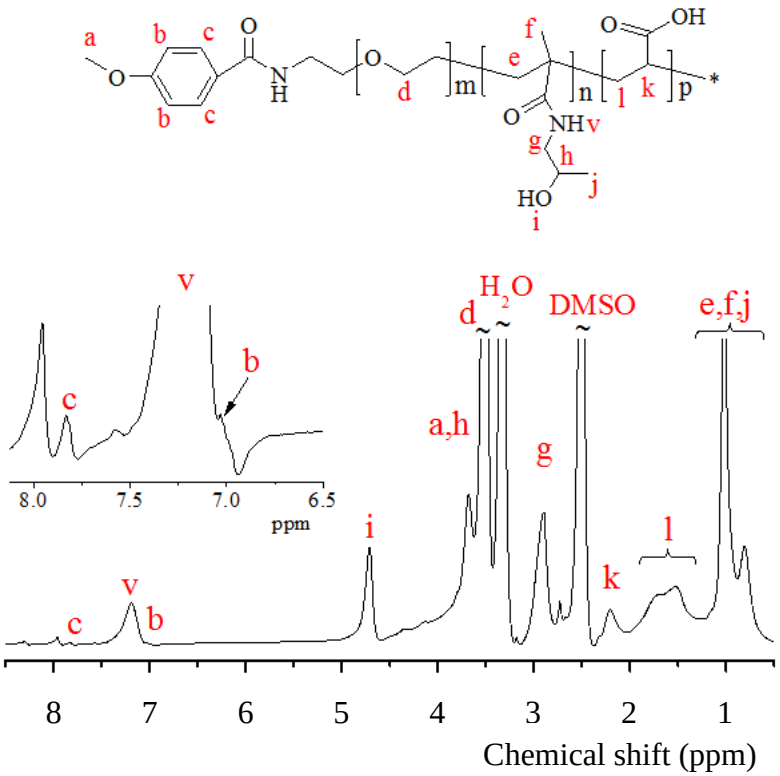


Figure S1. ^{1}H NMR spectra (400 MHz, DMSO-d_6) of PEG-PHPM-PAA (A) and PEG-P(HPMA-LA)-PAA (B).

A



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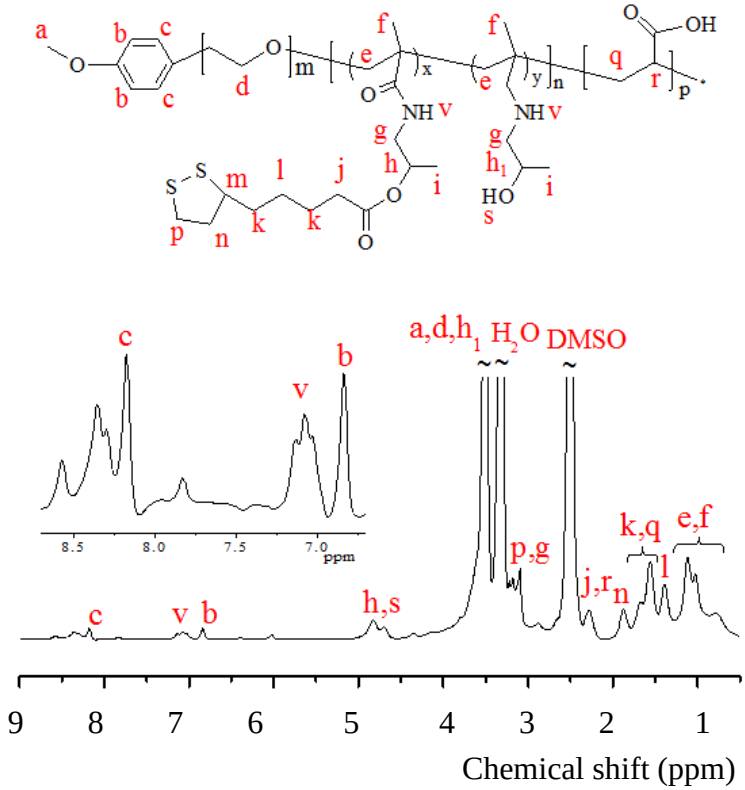

Figure S2. ^{1}H NMR spectra (600 MHz, DMSO-d_6) of Anis-PEG-PHPMA-PAA (A) and Anis-PEG-P(HPMA-LA)-PAA (B).

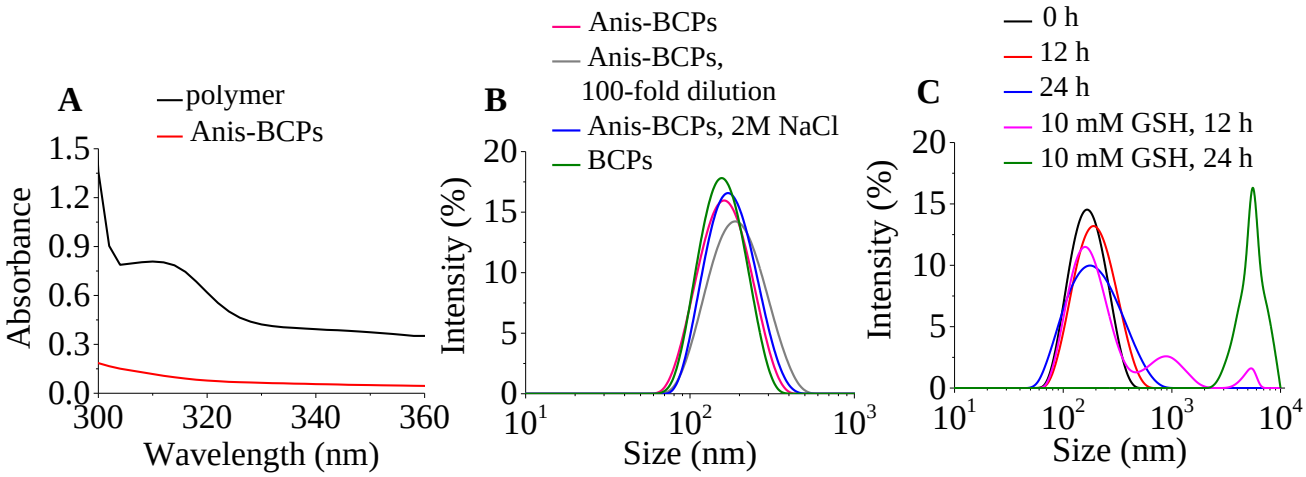

Figure S3. Characterization of Anis-BCPs. (A) UV spectra of the copolymer solution and polymersome dispersion at 1.0 mg/mL; (B) The size and size distribution measured by DLS, colloidal stability of Anis-BCPs against 100-fold dilution and 2 M NaCl. (C) The size change of Anis-BCPs in response to 10 mM glutathione (GSH).

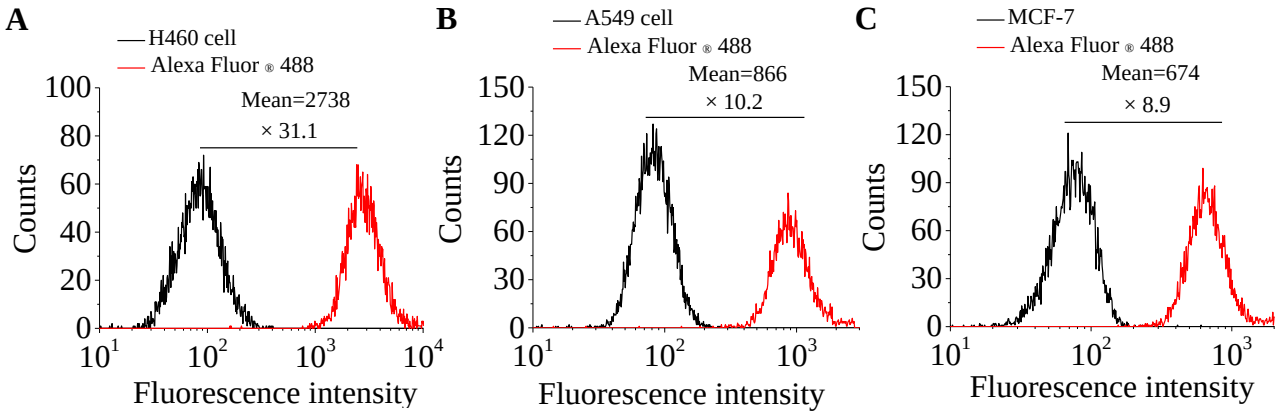

Figure S4. Flow cytometric analyses of the expression of sigma receptor on the surface of H460 cell (A), A549 cell (B) and MCF-7 cell (C). All the cells were treated with mouse sigma receptor antibody followed by Alexa Fluor 488 donkey anti-mouse secondary antibody.

Table S2. Characteristics of BCPs with different Anis molar contents

Entry	Anis content (mol.%)	Size [a] (nm)	PDI[a]	ζ[b] (mV)
1	0	156±1	0.16±0.04	-10.3
2	18.5	158±2	0.18±0.02	-9.8
3	28.0	160±2	0.15±0.01	-9.2
4	47.6	161±1	0.12±0.03	-8.7
5	67.9	167±3	0.19±0.05	-8.3

[a] Determined by DLS in PB (pH 7.4, 5 mM);

[b] Determined by zeta potential measurements in PB (pH 7.4, 5 mM).

Table S3. Characteristics of FITC-CC-Anis-BCPs and FITC-CC-BCPs

Polymersomes	PLC[c] (wt.%)		Size[a] (nm)	PDI[a]	ζ[b] (mV)	PLE[c] (%)
	Theory	Deter.				
FITC-CC Anis-BCPs	1	1.0	154±2	0.15±0.02	-10.3	~100
	2	1.9	156±1	0.12±0.06	-9.8	98.9
	5	4.9	159±4	0.16±0.03	-8.5	98.0
	10	8.8	162±3	0.18±0.05	-7.4	86.4
	50	38.6	166±6	0.17±0.03	-10.2	62.9
FITC-CC -BCPs	1	1.0	153±1	0.14±0.01	-11.2	~100
	2	1.9	154±6	0.15±0.03	-10.4	98.5
	5	4.8	158±2	0.13±0.04	-9.3	96.0
	10	9.3	161±3	0.17±0.03	-8.8	91.4
	50	36.5	165±3	0.13±0.01	-10.9	57.5

[a] Determined by DLS in PB (pH 7.4, 5 mM);

[b] Determined by zeta potential measurements in PB (pH 7.4, 5 mM). [c] Determined by fluorometry.

Table S4. Characteristics of Granzyme-B loaded crosslinked polymersome at feed ratio of 2.4 %

Entry	Polymersomes	Size[a] (nm)	PDI[a]	ζ[b] (mV)
1	GrB-Anis-BCPs	160±1	0.18±0.02	-7.8
2	GrB-BCPs	155±1	0.17±0.01	-9.3

[a] Determined by DLS in PB (pH 7.4, 5 mM);

[b] Determined by zeta potential measurements in PB (pH 7.4, 5 mM).

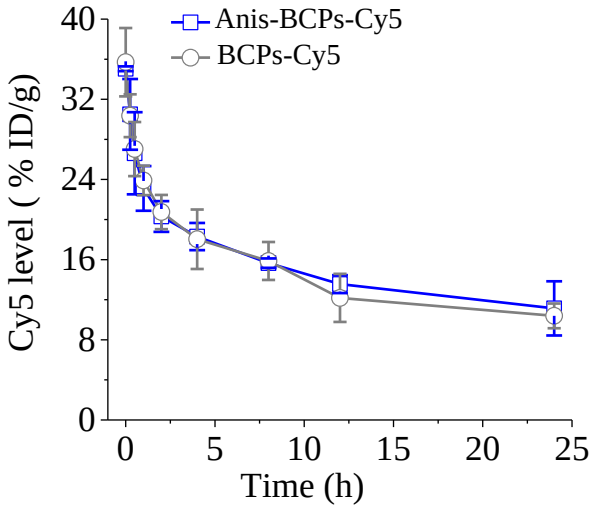

Figure S5. The blood circulation of Anis-BCPs-Cy5 and BCPs-Cy5 in nude mice (n = 3, 0.4 μmol Cy5/kg).

Table S5. Tumor-to-normal tissue (T/N) distribution ratios of Cy5-CC formulations

Formulation	Heart	Liver	Spleen	Lung	Kidney
Free Cy5-CC	4.05±2.45	0.53±1.66	1.89±2.12	1.19±1.67	1.35±2.06
Cy5-CC-BCPs	6.10±0.96	0.37±0.93	1.26±0.84	3.13±0.93	1.21±1.81
Cy5-CC-Anis- BCPs	17.80±2.19	1.73±1.05	3.58±0.84	6.06±1.22	2.84±1.41

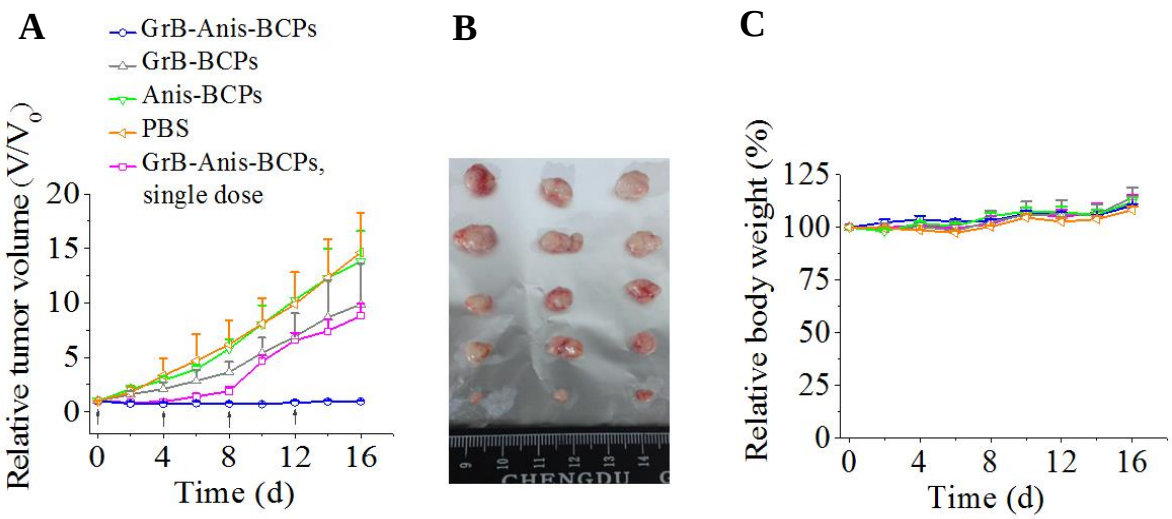

Figure S6. The preliminary *in vivo* antitumor performance of GrB-Anis-BCPs and GrB- BCPs in H460 tumor-bearing nude mice (dosage: 1.56 nmol GrB/kg, in 0.2 mL PBS). The drug was given on day 0, 4, 8 and 12. (A) Tumor volume changes in 16 d; (B) Photographs of typical tumor blocks on day 16; and (C) Body weight changes of mice in 16 d. Data are presented as mean ± SD (n = 3).

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[2] W. J. Yang, Y. Zou, F. H. Meng, J. Zhang, R. Cheng, C. Deng, Z. Y. Zhong, *Adv. Mater.* **2016**, *28*, 8234.

[3] Y. L. Li, L. Zhu, Z. Z. Liu, R. Cheng, F. H. Meng, J. H. Cui, S. J. Ji, Z. Y. Zhong, *Angew. Chem. Int. Ed.* **2009**, *48*, 9914.