Electronic Supplementary Material (ESI) for Materials Horizons.

Cancer-Microenvironment Triggered Self-Assembling Therapy by Molecular Blocks

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Material and methods

Materials

Tosylated multi-arm polyethylene glychols were purchased from creative PEG works (NC, USA). Sodium deoxycholate (DCA), methanesulfonyl chloride and trypsin were purchased from Fujifilm wako pure chemicals (Osaka, Japan). Dibenzylcyclooctyne (DBCO) was purchased from Click chemistry tools (AZ, USA). 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) was purchased from Watababe chemical (Hiroshima, Japan). Dulbecco's modified eagle medium (DMEM), RPMI-1640, antibiotic-antimycotic mixed solution and pyrene were purchased from Nacalai tesque (Kyoto, Japan). Apoptotic/necrotic/healthy cell detection kit was purchased from Promocell (Heidelberg, Germany). Normal human dermal fibroblast (NHDF), human umbilical vein endothelial cell (HUVEC) and endothelial cell growth medium 2 bullet kit (EGM2) was purchased from Lonza (Basel, Switzerland). Human pancreatic cancer cells (MiaPaCa-2), human colon cancer cell (HT29), HeLa and human lung carcinoma (A549) were purchased from ATCC (USA). Fatal bovine serum (FBS) was purchased from Thermofisher scientific (MA, USA). Dialysis membrane (MWCO: 5k-10k) was purchased from Repligen (MA, USA). Collagen I was provided from NH Foods Ltd (Osaka, Japan). Fluoresceinyl glycine amide was purchased from Setareh Biotech (OR, USA). Cyanine5.5-amine was purchased from Lumiprobe (MD, USA). Cell culture related instruments were purchased from AGC techno glass (Shizuoka, Japan). Histological stainings (hematoxylin and eosin (HE), Azan staining) and immunohistochemical stainings (CD3, F4/80) were performed by applied medical research (Osaka, Japan).

Methods

Synthesis of 3α-methylsulfonyldeoxycholic acid (DCA-Ms)

296 mg of DCA was dissolved in 5ml of pyridine on ice. 70 µl of methanesulfonyl chloride was added to dropwise and stirred for 30 min. The mixture was allowed to stir at room temperature and stirred for 3 h. The mixture was diluted with 100 ml of hydrochloric acid (4N) and extracted by 100 ml of ethyl acetate. The ethyl acetate was removed under vaccum and subjected to column chromatography (SiO_2, 2:1 ethylacetate-hexane).

Synthesis of 3β-azidodeoxycholic acid (DCA-N_2)

42 mg of 3α-methylsulfonyldeoxycholic acid was dissolved in 5 ml of N,N-dimethylformamide (DMF). 20 mg of sodium azide was added and stirred for 2 h at 70°C. The mixture was diluted by 100 ml of hydrochloric acid (2N). The product was collected by filtration.

Synthesis of multi-arm PEG-DBCO

6.5 μmol of tosylated PEG and 16 mg of DBCO were dissolved in pyridine. The mixture was refluxed for 24 h at 100 °C. The pyridine was removed under vaccum. The compounds were purified by dialysis (MWCO: 5-10k) against to water for 3 days and lyophilized.

Synthesis of multi-arm PEG-DCA

4.8 μmol of PEG-DBCO and 26 μmol of DCA azide were stirred in 1 ml of methanol for 24 h at room temperature. The product was purified by dialysis (MWCO: 5-10k) against to water for 3 days and lyophilized.

Characterization of molecular block (MB)

MBs were dissolved in phosphate buffer saline (PBS) at each concentration and purified 0.22 μm^2 pore filter. Static light scattering and dynamic light scattering measurement was performed by Zetasizer nano ZS (Malvern Panalytical, Worcs, UK). FT-IR spectra of MBs were measured by Spectrum 100 (Perkin Elmer, MA, USA) using attenuated total reflectance. ^{1}H-NMR measurement was measured by JNM-GSX400 (JEOL. Tokyo, Japan).

Evaluation of self-assemble property response to weak acidic condition

1 mg of MBs were dissolved in PBS at pH7.4. The MB solutions were adjusted at pH 6.2 by adding hydrochloric acid. Absorption spectra of each solutions were evaluated by V670 (JASCO, Tokyo, Japan). In addition, the size of MBs in each solution was evaluated by DLS measurement and microscopic images (IX71, Olympus, Tokyo, Japan). 2 mg of MBs were dissolved in PBS at pH 7.4 and pH6.2 made from D_2O and ^{1}H-NMR spectra of each solutions were recorded.

Fluorescence labeling of MBs

0.2 mg of fluoresceinyl glycine amide and 0.1 mg of DMT-MM were mixed in 0.5 ml of ethanol for 1 h at 0 °C. 6 mg of 4-MB20k was added and stirred for 1 h. The mixture was allowed to stir at room temperature and stirred for 24 h. Fluorecein labeled 4-MB20k (4-MB20k-F) was purified by dialysis against to water (MWCO: 5-10k) for 3 days and lyophilized.

5 mg of 4-MB20k and 0.08 mg of DMT-MM was dissolved in methanol on ice. The mixture was stirred for 1 h at 0°C with stepwise addition of sodium hydroxide to keep the solution at pH8.3. 0.06 mg of cyanine5.5 amine was added and stirred for 1 h. The mixture was

allowed to stir at room temperature and stirred for 24 h. Cy5.5 labeled 4-MB20k (4-MB20k-C) was purified by dialysis against to water (MWCO: 5-10k) for 3 days and lyophilized.

Evaluation of critical micelle concentration (CMC)

CMC was evaluated from fluorescence spectra of pyrene. 10 µl of pyrene solution in ethanol (1 x 10^{-4}M) was dried in glass tube by N_2 gas. MBs were dissolved in PBS at each concentration from 0.001 to 1 mg/ml. 1 ml of each MB solutions were added in glass tubes and incubated for 24h at room temperature. Fluorescence spectra of each solutions were evaluated by FP-8500 (JASCO, Tokyo, Japan).

Cell culture

NHDF, A549 MiaPaCa-2 and HeLa were maintained in DMEM supplemented with 10 % FBS and 1 % antibiotics. HT29 was maintained in RPMI-1640 supplemented with 10 % FBS and 1 % antibiotics. HUVEC was maintained in EGM-2. Cells were cultured in 5 % CO_2 and 95 % humidified air at 37°C and passage every 3-6 days.

Cell adhesion property of MBs

HT29 and NHDF were seeded in glass bottom dish at 1 x 10^5 cells / well. After 24h, 1 ml of fluorescence labeled MBs at 1 mg/ml in culture medium at pH7.4 or PH6.2 were added. Fluorescence images of MB treated cells were measured by confocal microscopy (FV10i, Olympus, Tokyo, Japan) every hour.

Evaluation of cytotoxicity response to weak acidic condition

HT29 was seeded on 6 well plate at 1 x10^5 cells /well. After 24 h, 1ml of MBs at each concentration in culture medium at pH7.4 or pH6.2 were added in each plate and incubated 24 h. The cells were trypsinized for 5 min and collected. Cell viability was evaluated by trypan blue solution and Countess II (Thermofisher scienrific, MA, USA).

NHDF, HeLa, A549 and MiaPaCa-2 were seeded on 6 well plate at 1 x10^5 cells /well. After 24 h, 1ml of MBs at 1 mg/ml in culture medium at pH7.4 or pH6.2 were added in each plate and incubated 24 h. The cells were trypsinized for 5 min and collected. Cell viability was evaluated by trypan blue solution and Countess II.

Detection of apoptosis/necrosis

HT29 was seeded on 6 well plate at 1 x10^5 cells /well. After 24 h, 1ml of MBs at 1 mg/ml in culture medium at pH7.4 or pH6.2 were added in each plate and incubated 24 h. Floating cells and trypsinized cells were collected by centrifugation. The cells were stained by

Apoptotic/Necrotic/Healthy cells detection kit. The stained cells were passed 40 μm pore cell strainer and evaluated by flowcytometer (Guava easycyte HT, Merck, Hessen, Germany).

3D tissue model

50 mg of collagen type I was homogenized for 5 min in 5 ml 10x PBS. The collagen dispersion was centrifuged at 10000 rpm for 5 min and redispersed in PBS twice. 0.3 mg of collagen, $5x10^4$ cells of HT29, $5x10^4$ cells of NHDF and $5x10^4$ cells of HUVEC were mixed and seeded in low adhesion surface microplate and cultured for 7 days. 1 mg/ml of 4-MB20k in culture medium at pH7.4 or pH6.2 were added in the microplate and incubated for 24 h. The amount of DNS in 3D tissue was evaluated by DNeasy Blood & tissue kit.

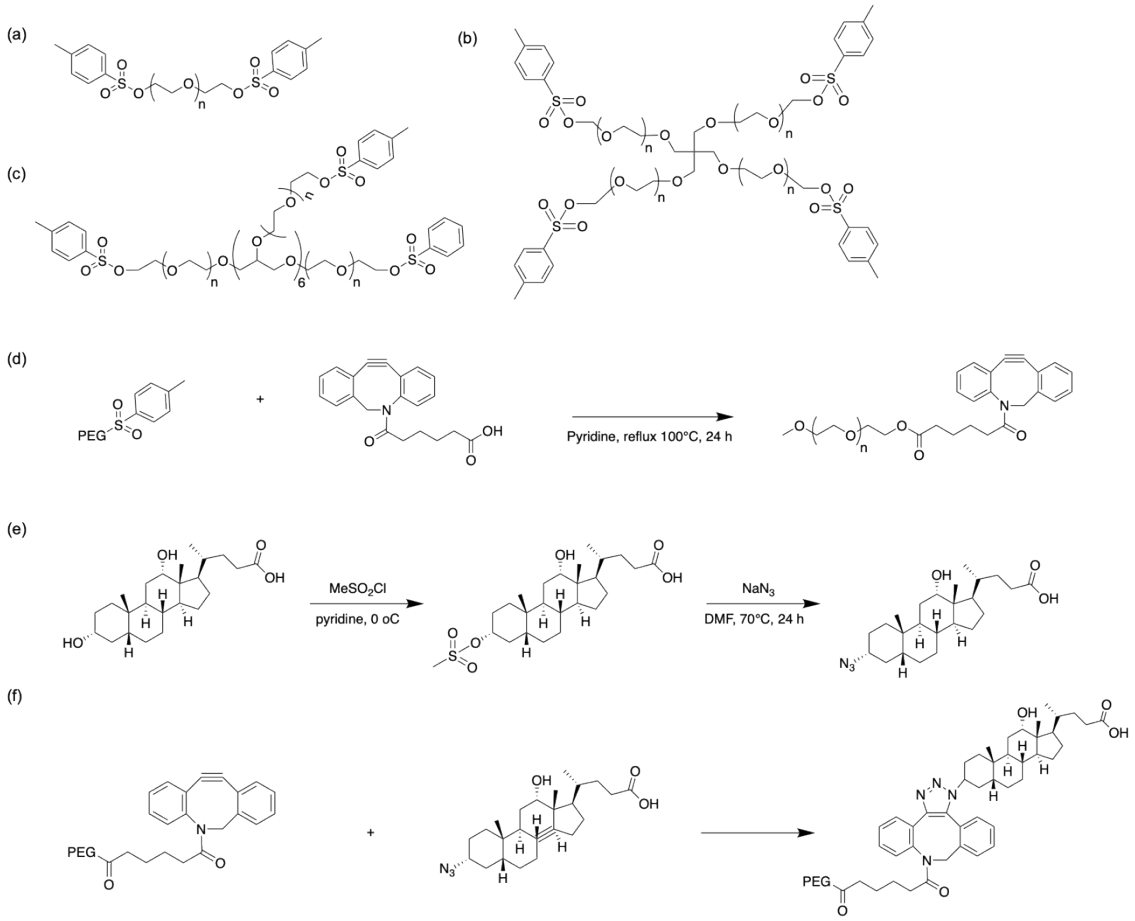
Animal experiments

Female mice (BALB/cSlc-nu/nu, Japan SLC Inc., Shizuoka, Japan) were inoculated with $1x10^6$ cells of colon26 (mouse colon cancer) intradermally on the back. 200 μl/20g b.w. of 4-MB20k-C solution at 0.075 mg/ml or PBS were injected intravenously per day until 7 days. 4-MB20k-C injected mice were observed by in vivo fluorescence imaging system (Maestro EX, Perkin Elmer, MA, USA). Tumor size were evaluated by caliper. The amount of 4-Mb20k-C in cancer tissue was evaluated by fluorescence image and extracted 4-MB20k-F. The cancer tissues were homogenized for 5 min and centrifuged at 10000 rpm for 5 min. The residue was redispersed by 1 ml of methanol and centrifuged at 10000 rpm for 5 min. The fluorescence spectra of supernatant were evaluated by V-670.

The isolated organs were frozen at -80°C. The frozen sections of them were analyzed by HE staining and immunohistochemical staining of CD3, CD31 and F4/80. All experimental procedures related to animals and their care were approved by the Animal Ethics Committee of the National Institutes for Quantum and Radiologic Science and Technology. National Institutes, (31 Environmental Preservasion ordiance, #21, updated on March 27th, 2020, Chiba city, Chiba prefecture, Japan).

Statistical analysis

All data are expressed as the means ± standard deviation. Statistical comparisons between groups were analyzed using two-tailed Student's T-test.



Scheme 1. (a) Structure of 2-arm PEG-tosylate (b) 4-arm PEG-tosylate (c) 8-arm PEG-tosylate (d) Synthesis of multi-arm PEG-DBCO (e) Synthesis of DCA-N_3 (f) Synthesis of multi-arm PEG-DCA by Cu free click reaction.

Table 1. Characterization of synthesized MBs.

	PEG Mw	Repeat unit	Yield[a]	Calcd. Mw[b]	Measd. Mw[c]	N agg[d]	Size (nm)[e]	PDI[f]	CMC[g]
2-MB10k	10	110	66	11	660	61	110 ± 10	0.24 ± 0.01	0.01
4-MB10k	11	55	69	13	140	11	210 ± 140	0.77 ± 0.29	0.01
4-MB20k	21	110	70	23	200	9	50 ± 5	0.22 ± 0.00	0.02
8-MB40k	38	100	67	40	53	1	120 ± 10	0.08 ± 0.03	0.03

[a]The total yield is calculated from yields of multi-PEG-DBCO and multi-PEG-DCA. [b]The M_w of multi-PEG-DCA was calculated from ^{1}H-NMR spectra. [c]The M_w of multi-PEG-DCA was measured in phosphate buffer saline (PBS) at pH7.4 by static light scattering (SLS). [d]The number of multi-PEG-DCA aggregates (N_{agg}) was calculated by the measured M_w / calculated M_w. [e]The particle diameter was measured in 1mg/mL PBS at pH7.4 by dynamic light scattering (DLS). [f]PDI represents polydispersity index. [g]Critical micelle concentration (mg/mL)

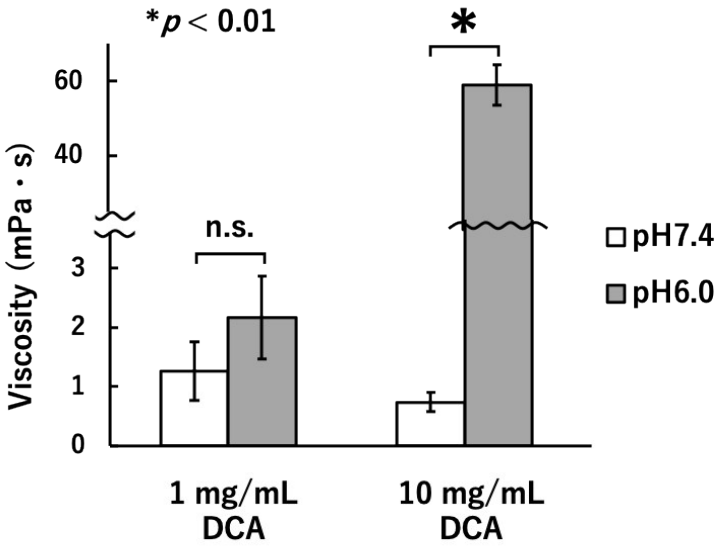


Fig. S1 Viscosity of 1 mg/ml and 10 mg/ml of DCA in PBS at pH7.4 or 6.0.

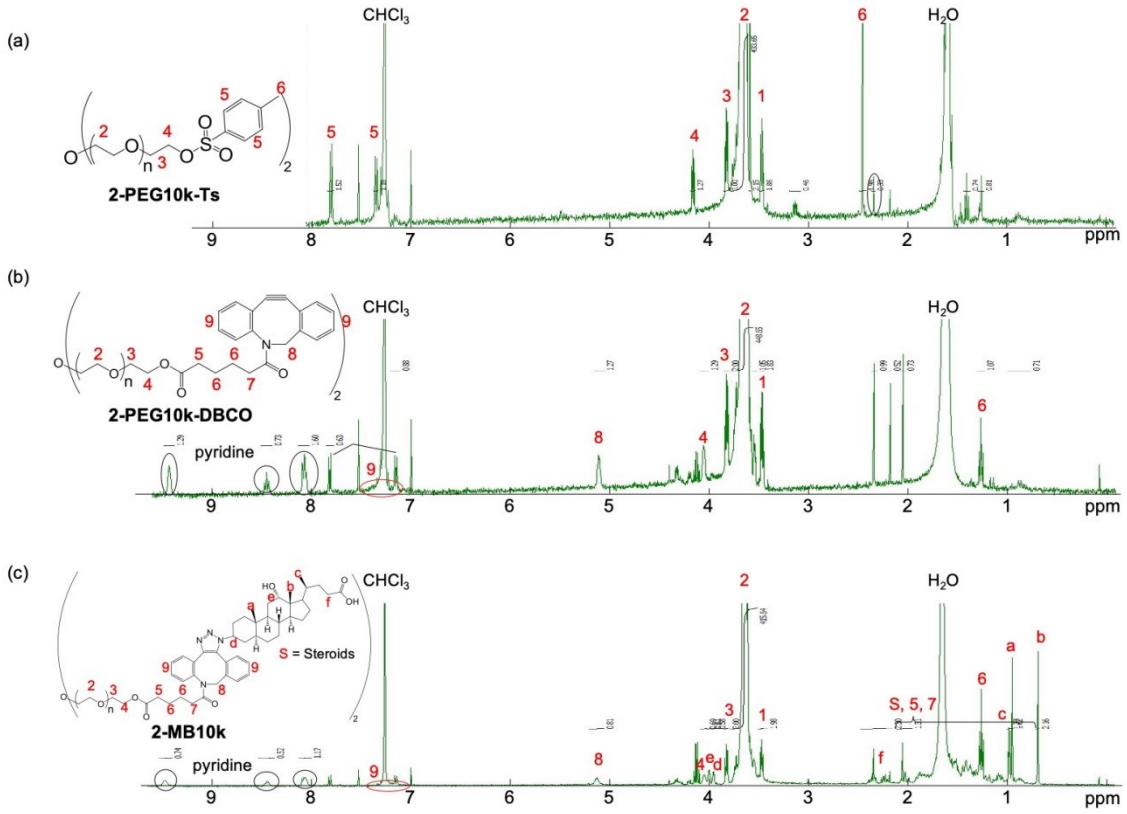


Fig. S2 ^{1}HNMR spectra ($CDCl_3$, 400 MHz) (a) 2-PEG10k-Ts, (b) 2-PEG10k-DBCO, (c) 2-MB10k

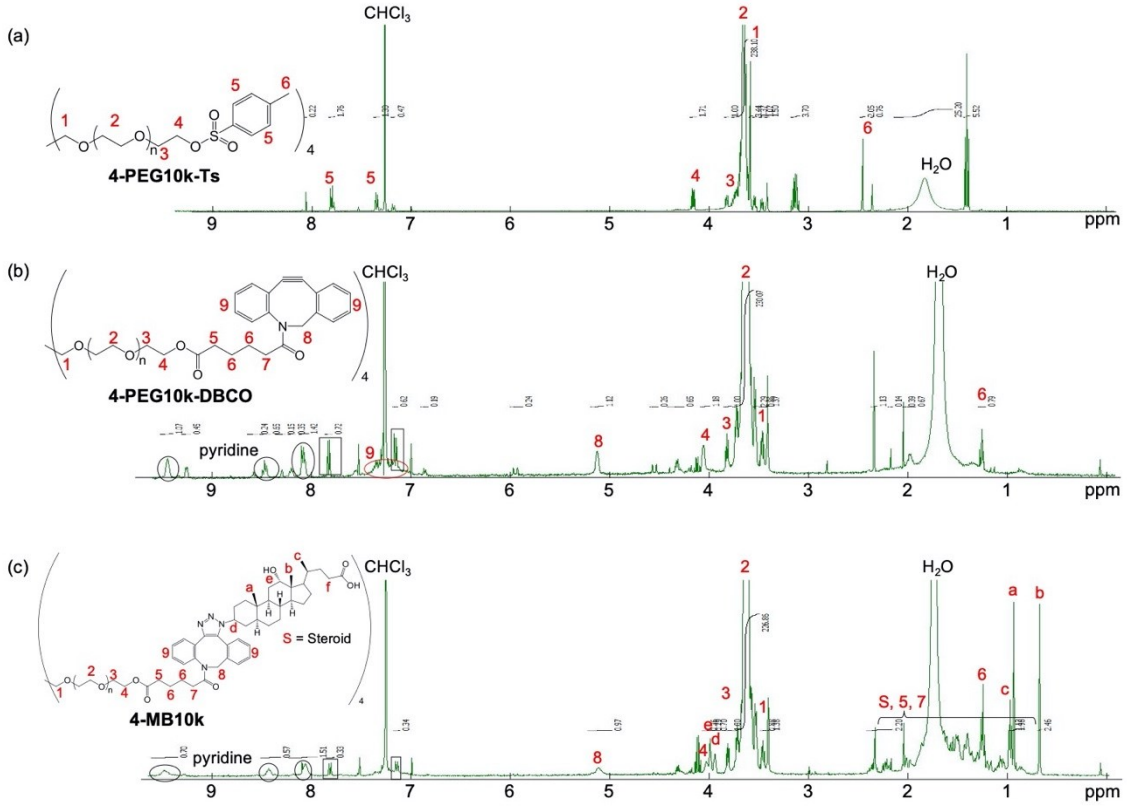


Figure. S3 ^{1}HNMR spectra ($CDCl_3$, 400 MHz) of (a) 4-PEG10k-Ts, (b) 4-PEG10k-DBCO, (c) 4-MB10k

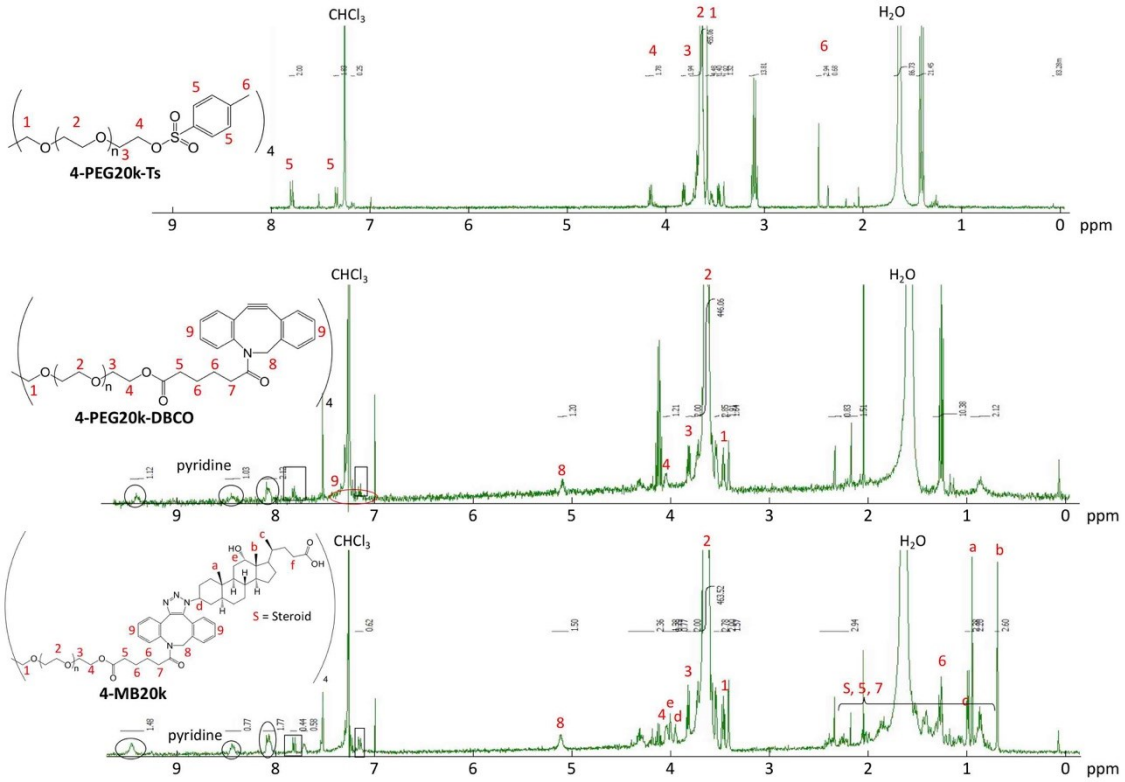


Fig. S4 ^{1}HNMR spectra ($CDCl_3$, 400 MHz) of (a) 4-PEG20k-Ts, (b) 4-PEG20k-DBCO, (c) 4-MB20k

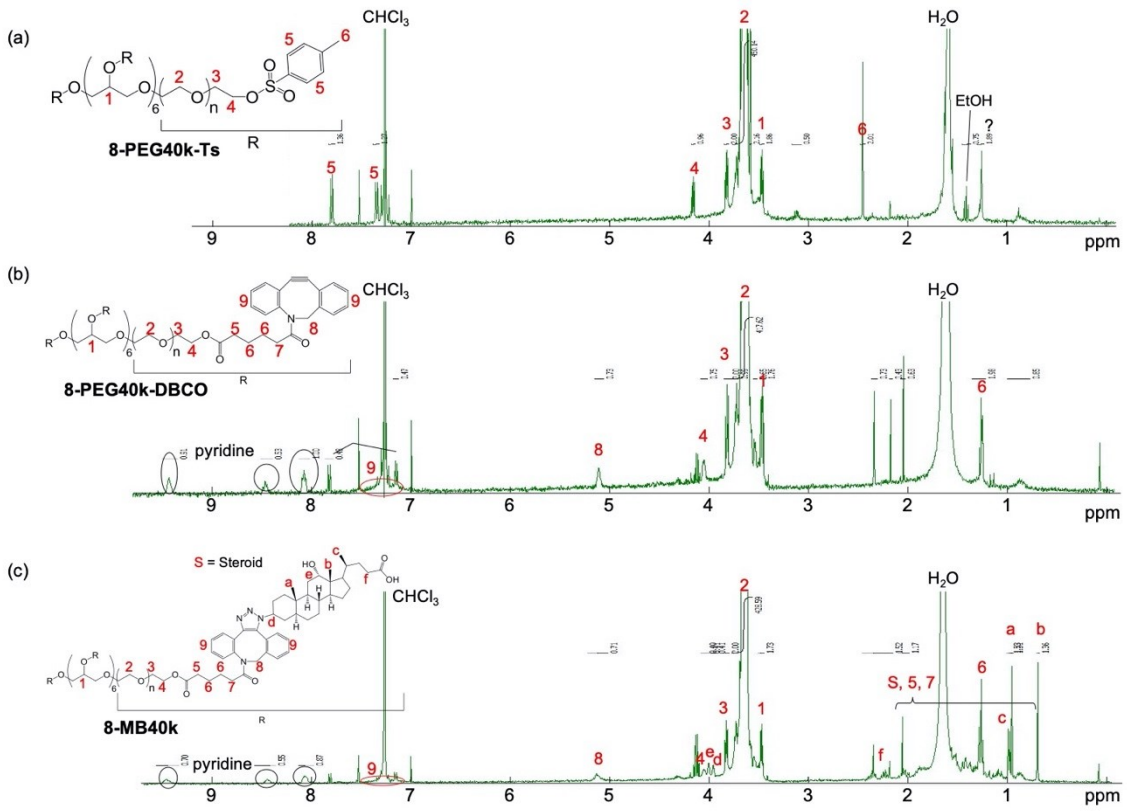


Fig. S5 ^{1}HNMR spectra ($CDCl_3$, 400 MHz) of (a) 8-PEG20k-Ts, (b) 8-PEG20k-DBCO, (c) 8-MB20k

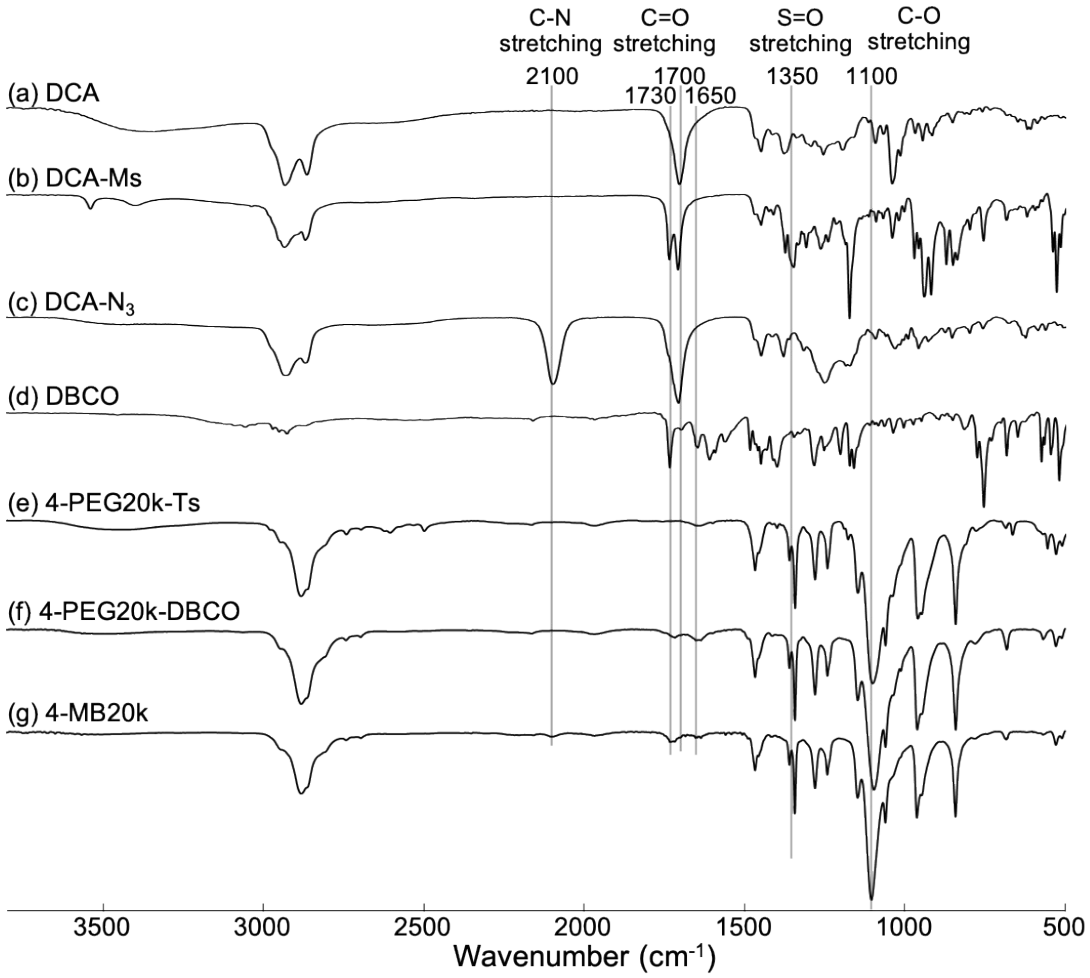


Fig. S6 IR spectra of (a) DCA (b) DCA-Ms (c) DCA-N_3 (d) DBCO (e) 4-PEG-20k-Ts (f) 4-PEG-20k-DBCO (g) 4-MB20k

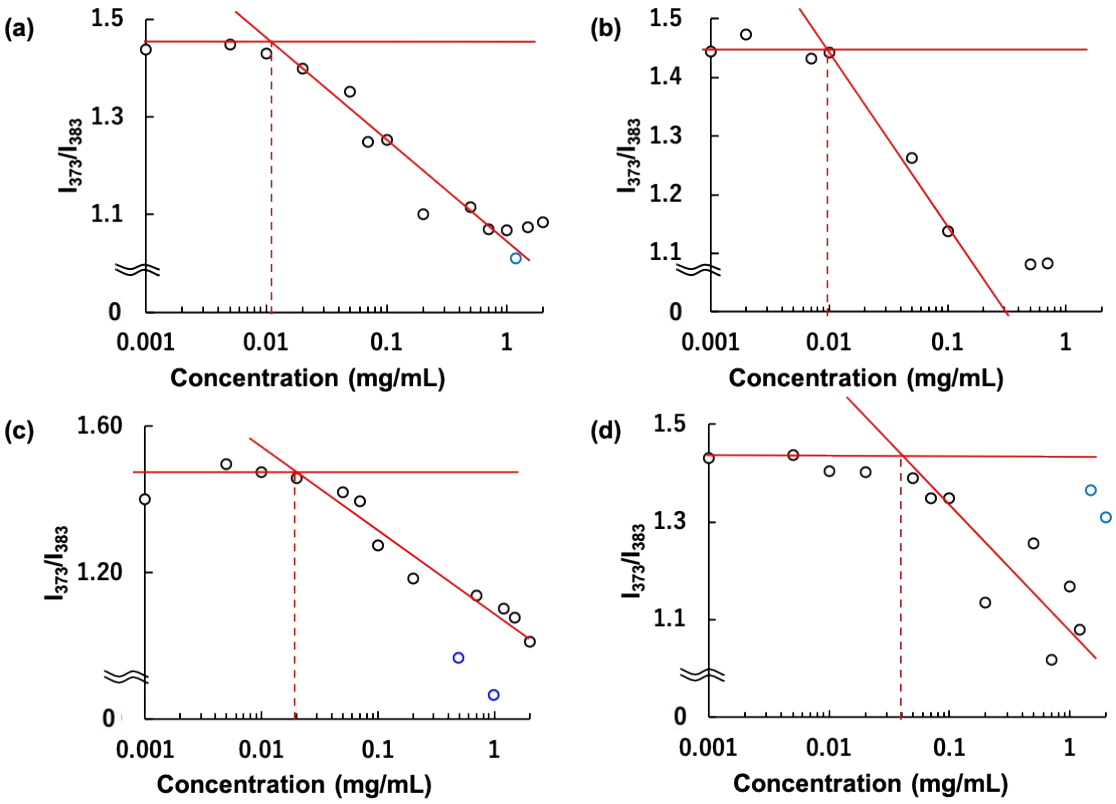


Fig. S7 Concentration dependent change of fluorescence intensity ratio $I_{373}/I_{383.}$ (a) 2-MB10k (b) 4-MB10k (c) 4-MB20k (d) 8-MB40k. The change of the fluorescence behavior indicate CMC.

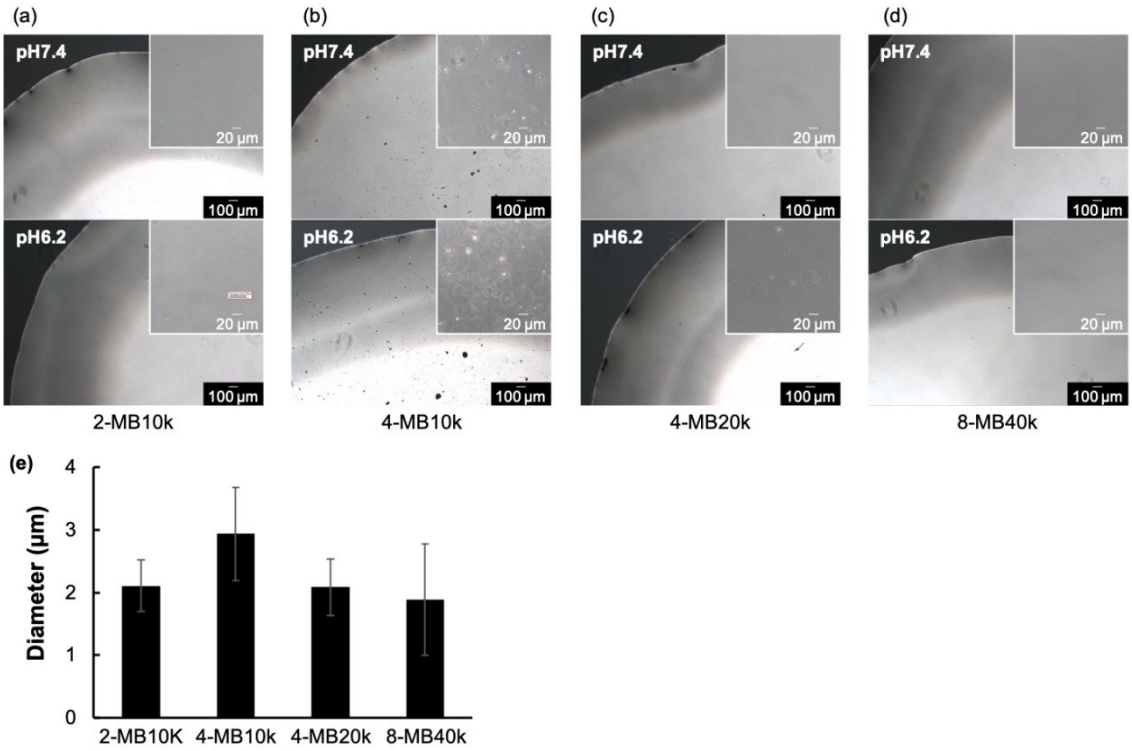


Fig. S8 Phase contrast microscopic images of (a) 2-MB10k (b) 4-MB10k (c) 4-MB20k (d) 8-MB40k at pH7.4 or pH 6.2. (b) The average size of MB aggregations in PBS at pH 6.2 (n=100).

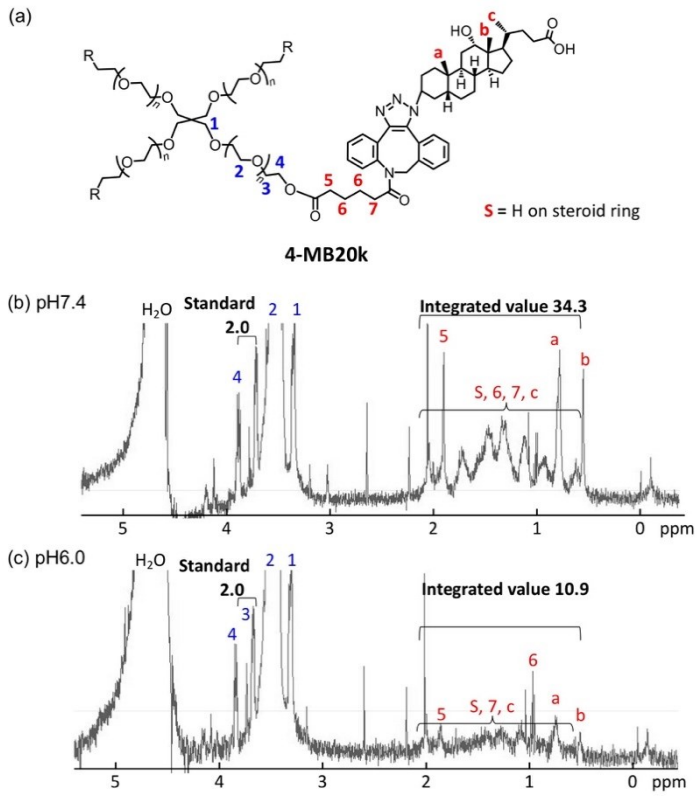


Fig. S9 (a) Structure of 4-MB20k. ^{1}H-NMR (D_2O, 400 mHz) spectra of 4-MB20k at (a) pH 7.4 and (b) pH 6.0.

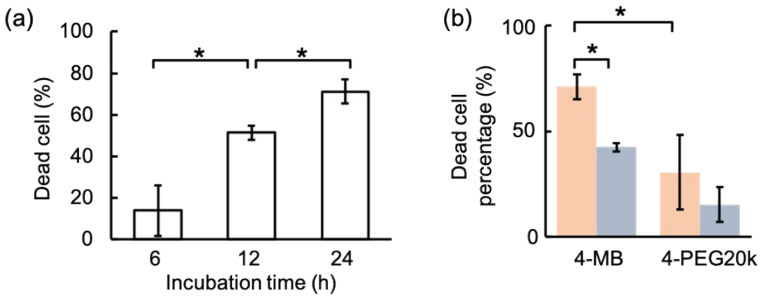


Fig. S10 (a) Time dependent change of dead cell percentage of HT29 treated with 4-MB20k at pH 6.2 (n=3). (b) Cytotoxicity of 1mg/ml of 4-MB and 4-PEG20k-Ts at pH6.2 for HT29. *P*-value was evaluated by student's T-test. *p<0.05

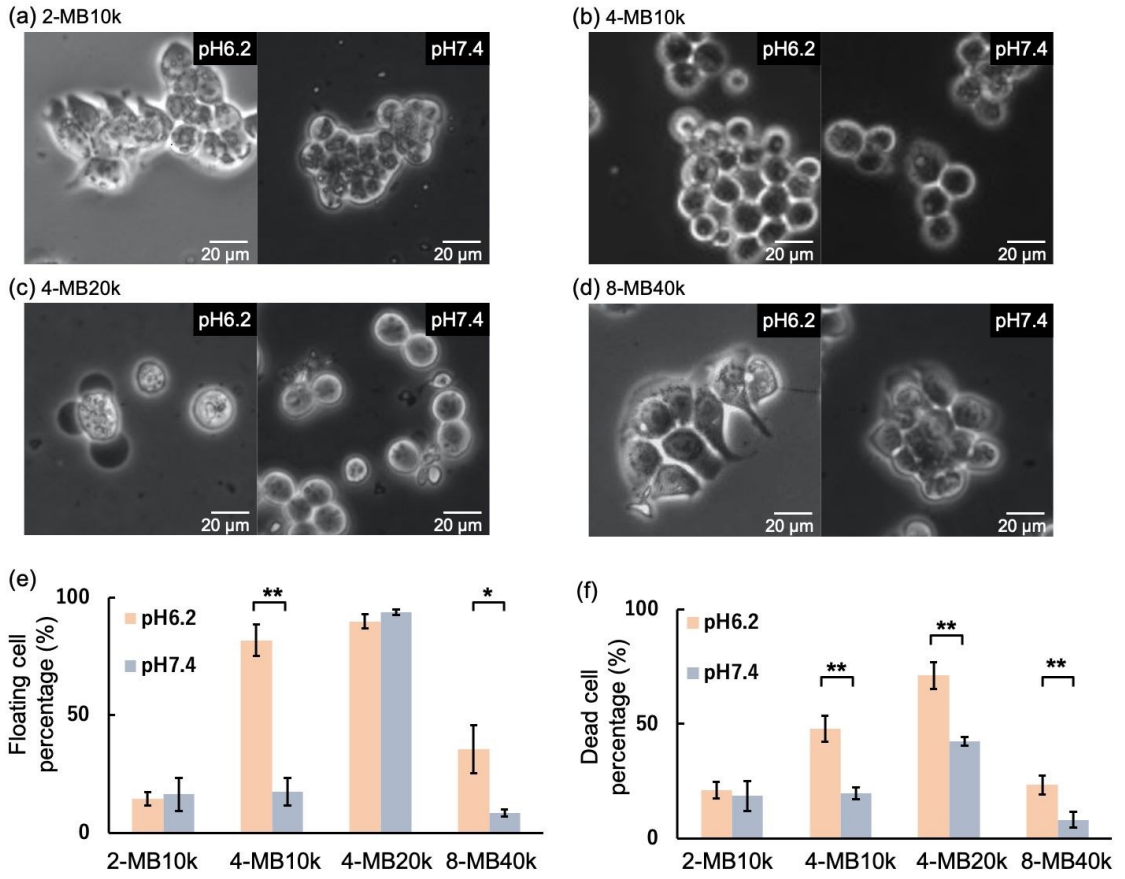


Fig. S11 Phase contrst images of HT29 treated with 1mg/ml of (a) 2-MB10k (b) 4-MB10k (c) 4-MB20k (d) 8-MB20k at pH6.2 and pH7.4. (e) Floating cell percentage and (f) dead cell percentage of HT29 treated with 1mg/ml of MBs for 24 h (n=3). *P*-value was evaluated by student's T-test. *p<0.05, **p<0.01

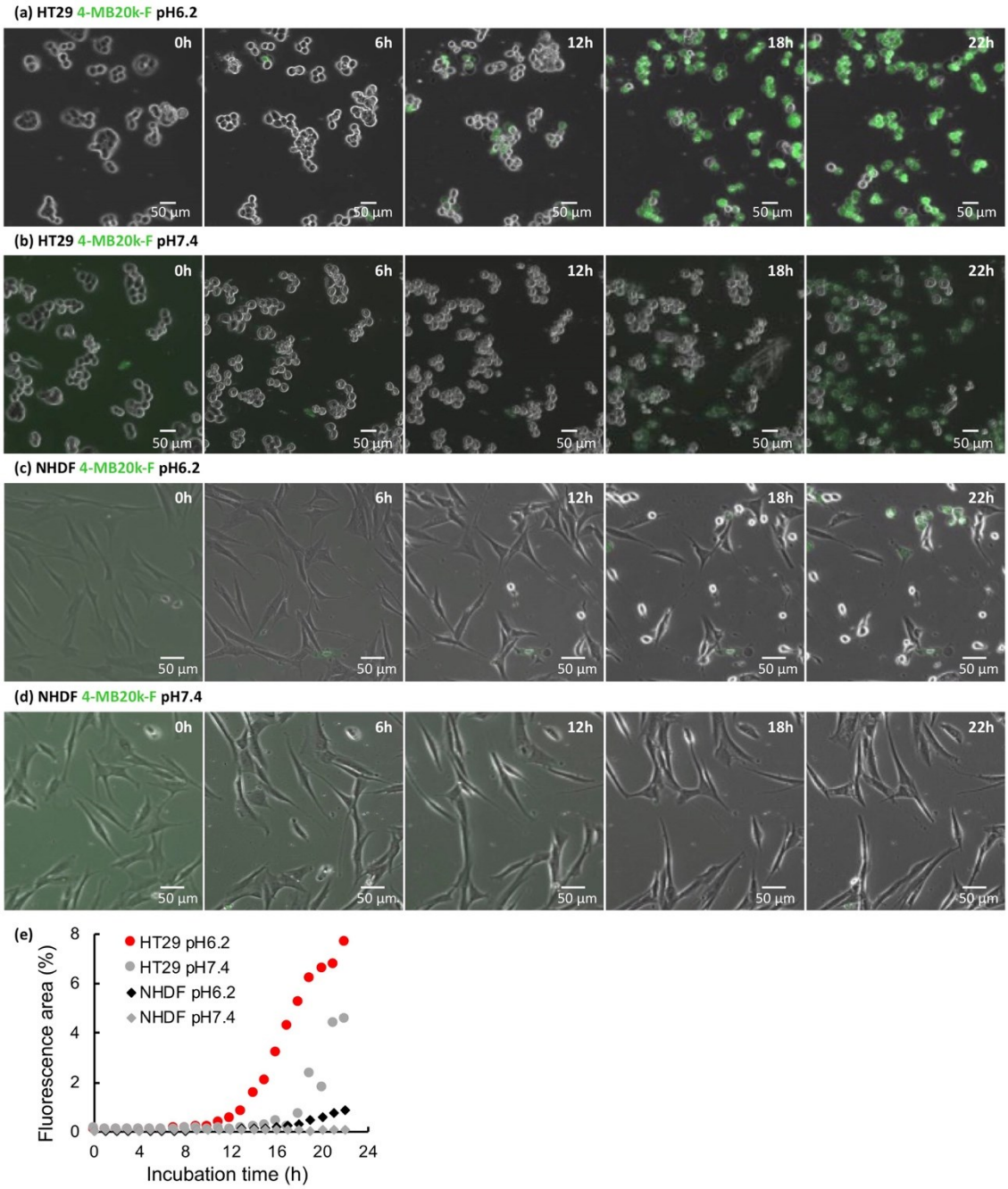


Fig. S12 Time-lapse merged images of phase contrast images and fluorescence images of (a) HT29 at pH6.2, (b) pH 7.4 and (c) NHDF at pH6.2, (d) pH 7.4 treated with 1mg/ml of 4-MB20k-F. (e) Time course of fluorescence area of 4-MB20k-F in each images.

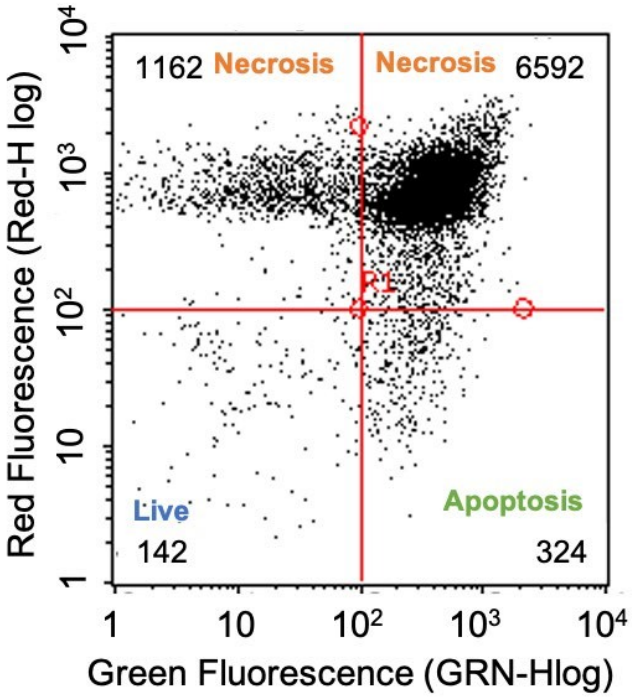


Fig. S13 Flow cytometry analysis of necrotic/apoptotic/living cell in HT29 treated with 4-MB20k at pH6.2 for 24 h.

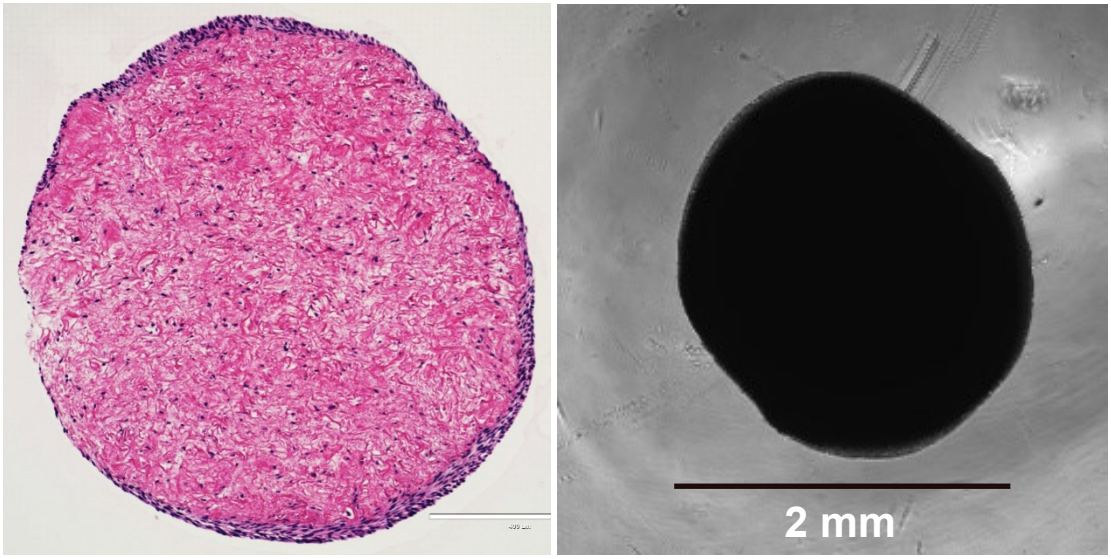


Fig. S14 HE staining histological image (left) and phase contrast microscope images (right) of constructed 3D tumor models.

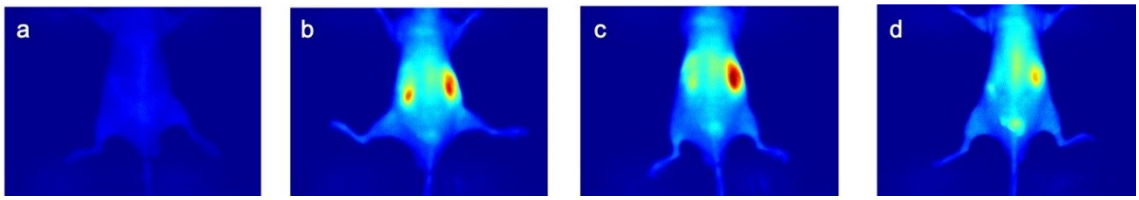


Fig. S15 Fluorescence *in vivo* images of tumor-bearing mice. (a) untreated (b) immediately after administration of 200 μl 4-MB20k-C at 20 mg/ml (c) 1h after (d) 2h after (e) 24h after.

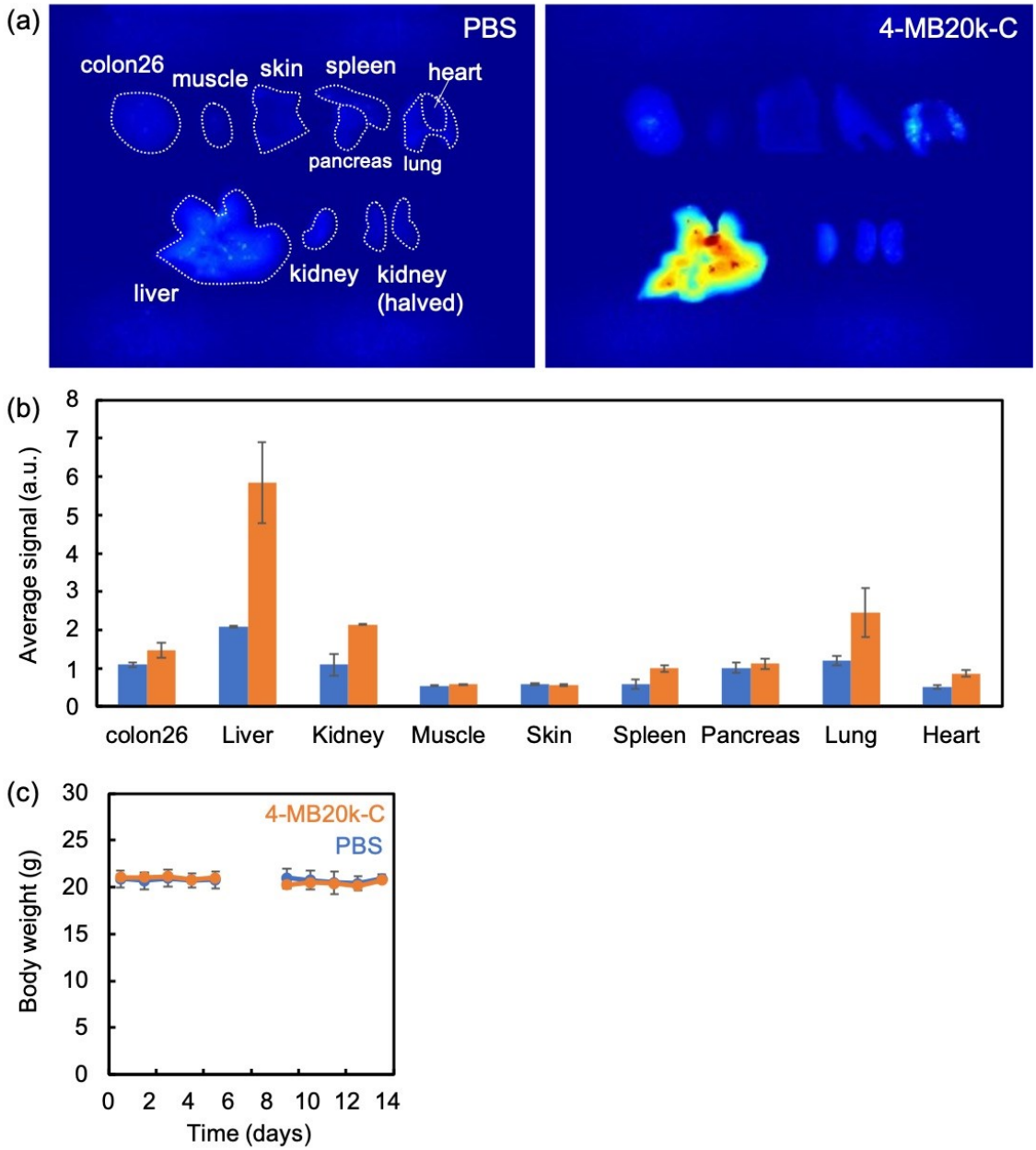


Fig. S16 (a) Fluorescence *ex vivo* images of organs from tumor-bearing mice 18 days after treatment of PBS and 4-MB20k-C. (b) Average fluorescence signals from each organ (n = 2-3). Blue: PBS treated mice. Orange: 4-MB20k-c treated mice. (c) Time dependent change of body weight of tumor-bearing mouse treated with PBS and 4 -MB20k-C. The mice are same as used in figure 4d and 4e.

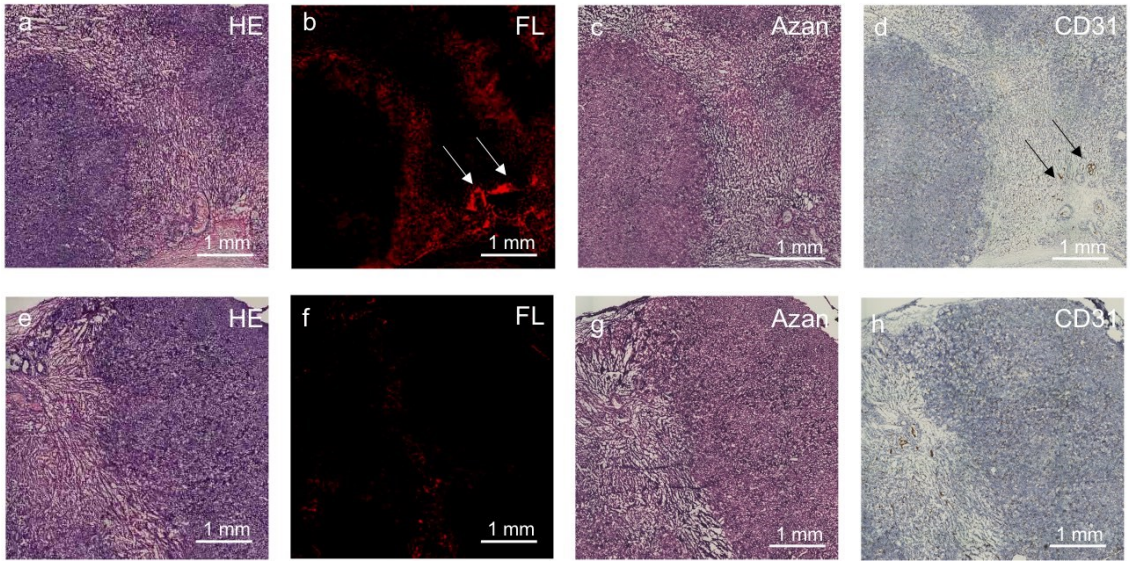


Fig. S17 Histological analyses with HE staining (a, e), fluorescence (b, f) azan staining (c, g) and immunohistochemical staining of CD31 (d, h) of the extracted tumor from the 4-MB20k-C (top, a-d) and the PBS injected mice (bottom, e-h). The mice were same as used in figure 4. Arrows indicate blood vessels.

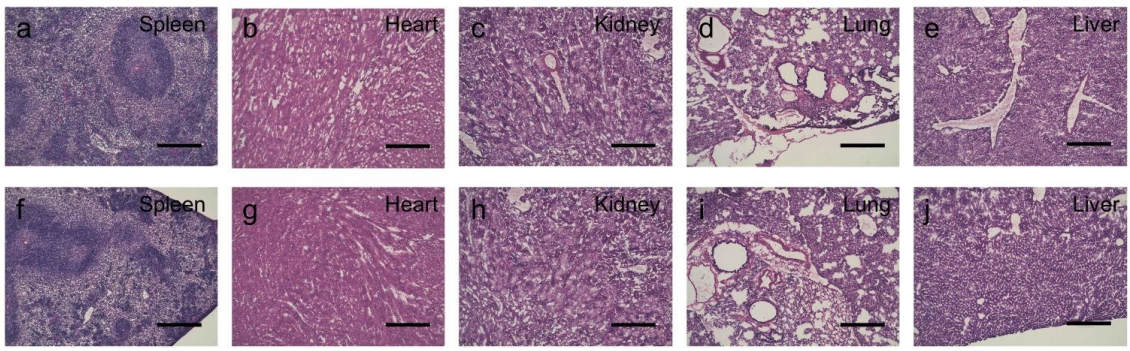


Fig. S18 Histological analyses of the extracted (a, f) spleen, (b, g) heart, (c, h) kidney, (d, i) lung and (e, J) liver from the 4-MB20k-C (top, a-e) and PBS (bottom, f-j) injected mice stained with HE staining (scale bar: 400 μm). The mice are same as used in figure 4.

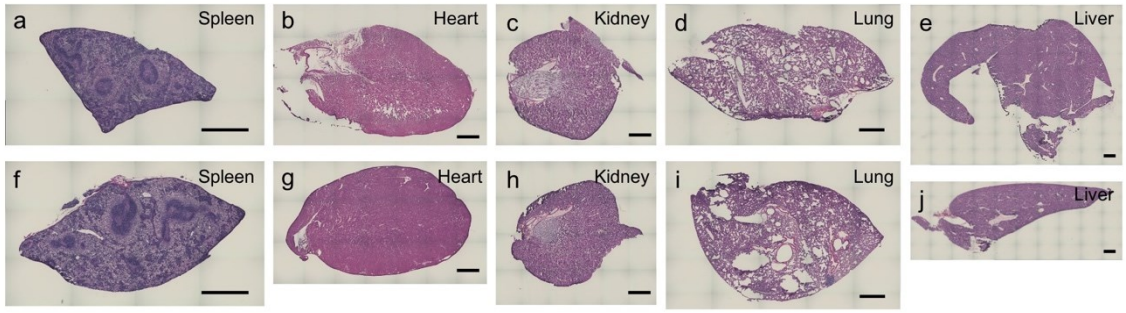


Fig. S19 Low magnification section images of Fig. S18. Histological analyses of the extracted (a, f) spleen, (b, g) heart, (c, h) kidney, (d, i) lung and (e, J) liver from the 4-MB20k-C (top, a-e) and PBS (bottom, f-j) treated mice stained with HE (scale bar: 1 mm). The mice are same as used in figure 4.

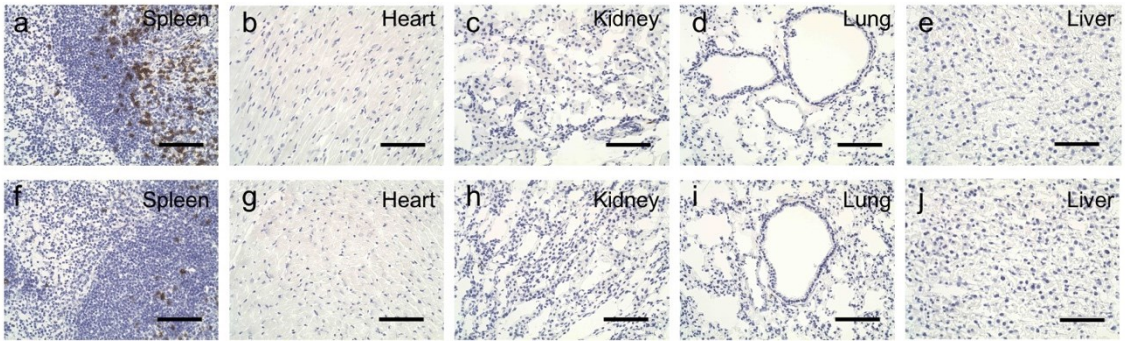


Fig. S20 Immunohistochemical observation of (a, f) spleen, (b, g) heart, (c, h) kidney, (d, i) lung, and (e, J) liver from the 4-MB20k-C (top, a-e) and the PBS (bottom, f-j) injected mice for CD3 (scale bar: 400 μm). The mice are same as used in figure 4.

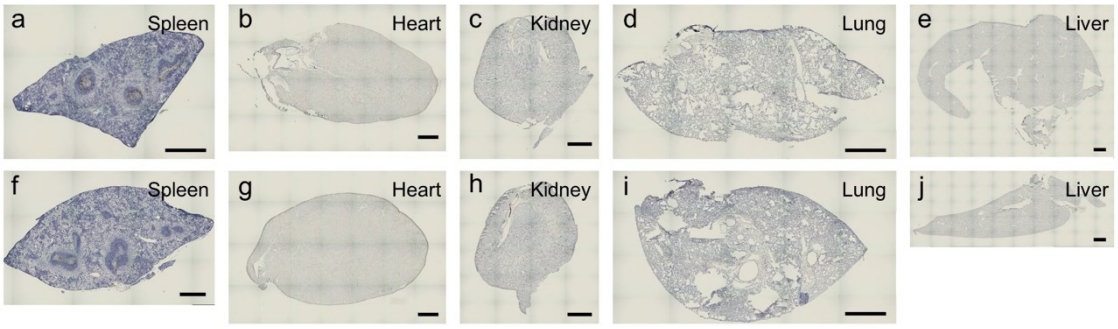


Fig. S21 Low magnification section images of Fig. S20. The immunohistochemical observation of (a, f) spleen, (b, g) heart, (c, h) kidney, (d, i) lung, and (e, J) liver from the 4-MB20k-C (top, a-e) and the PBS (bottom, f-j) injected mice for CD3 (scale bar: 1 mm). The mice are same as used in figure 4.

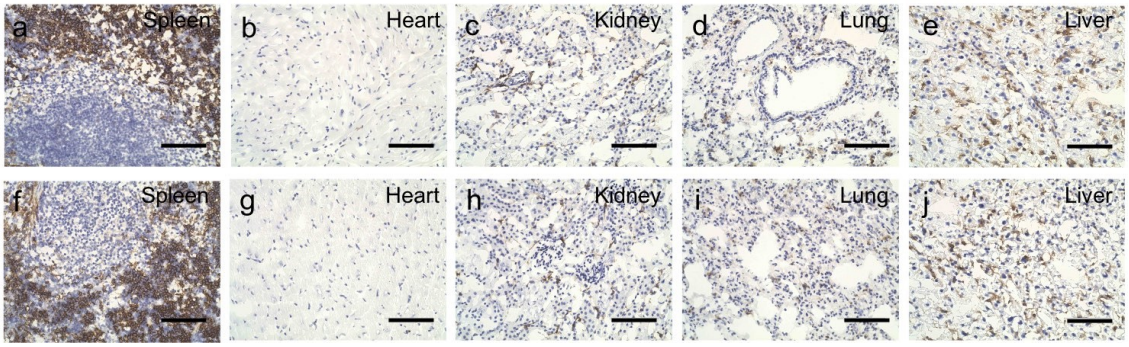


Fig. S22 Immunohistochemical observation of (a, f) spleen, (b, g) heart, (c, h) kidney, (d, i) lung, and (e, J) liver from the 4-MB20k-C (top, a-e) and the PBS (bottom, f-j) injected mice for F4/80 (scale bar: 400 μm). The mice are same as used in figure 4.

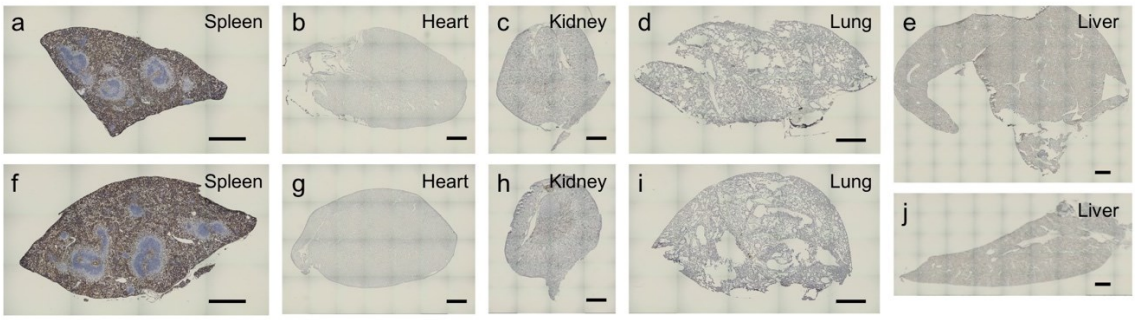


Fig. S23 Low magnification section images of Fig. S22. The immunohistochemical observation of (a, f) spleen, (b, g) heart, (c, h) kidney, (d, i) lung, and (e, J) liver from the 4-MB20k-C (top, a-e) and the PBS (bottom, f-j) injected mice for F4/80 (scale bar: 1 mm). The mice are same as used in figure 4.

Movie S1. Time-course of HT29 treated with 1mg/ml of 4-MB-F at pH6.2 from 0 to 24 h after treatment.