Supporting Information

Poly(oxyethylene sugaramide)s: Unprecedented Multihydroxyl Building Blocks for Tumor-Homing Nanoassembly

Keunsoo Jeong,[a,b] Yong-Deok Lee,[a] Solji Park,[a,c] Eunjung Lee,[a] Chang-Keun Lim,[a] Kyung Eun Lee,[a] Hyesung Jeon,[a] Jungahn Kim,[c] Ick Chan Kwon,[a] Chong Rae Park,*[b] and Sehoon Kim*[a]

[a] Center for Theragnosis, Korea Institute of Science and Technology, 39-1 Hawolgok-dong, Seongbuk-gu, Seoul 136-791(Korea) and [2] Carbon Nanomaterials Design Laboratory

[b] Global Research Laboratory and Department of Materials Science and Engineering, Seoul National University, Seoul 151-744 (Korea)

[c] Department of Chemistry, Kyung Hee University, Dongdaemoon-gu, Seoul 130-701

Experimental Section

Materials and Characterization

All chemical reagents were purchased from Aldrich and TCI, and used without further purification. Chemical structures were identified by FT-IR (Thermo Mattsonmodel Infinity Gold FT-IR), ^{1}H- and ^{13}C-NMR (Varian unity plus 300). Elemental analysis was performed on a CHNS-O Analyzer (EA 1180, FISONS Instruments). The molecular weights of polymers were determined by ^{1}H-NMR spectral analysis and gel permeation chromatographic (GPC) analyses with Agilent Technologies 1200 series. UltrahydrogelTM 120, 500, and 1000 (Waters, USA) and pullulan were used for GPC columns and the standard, respectively. The flow rate was maintained at 1.0 mL/min at 30 oC. Transmission electron microscopic (TEM) images were recorded with a CM30 electron microscope (FEI/Philips) operated at 200 kV. For the TEM sample preparation, a drop of particle dispersion was dried on a 300 mesh copper grid coated with carbon and negatively stained with a 2 wt% uranyl acetate solution. Cryo-TEM images were recorded with a Tecnai G2 F20 electron microscope (FEI/Philips) operated at -170 ^{o}C and with 200 kV of acceleration voltage. For the TEM sample preparation, a drop of particle dispersion was loaded onto a holey-carbon film-supported grid, than frozen with liquid nitrogen. The size of aqueous dispersed nanoparticles was determined using a zeta-sizer (Nano-ZS, Malvern) equipped with a multi-purpose titrator (MPT-2, Malvern). Fluorescence spectra were acquired using a fluorescence spectrophotometer (Hitachi F-7000, wavelength calibrated for excitation and emission).

Synthesis of Dimethyl Galactarate (DMGA)

Galactaric acid (30 g, 0.143 mol) was refluxed in methanol (720 mL) and concentrated sulfuric acid (3 mL) overnight. The product was purified by recrystallization from mixture of methanol (800 mL) and triethylamine (5 mL) and dried under vacuum at room temperature. Yield: 28.65g (84 %). ^{1}H-NMR (DMSO-*d6*, 300 MHz, ppm): δ 3.33 (s, 6H), 3.79 (d, J= 9.2 MHz, 2H), 4.31 (d, J=8.7 MHz, 2H), 4.82 (m, 2H), 4.94 (d, J=9.2 MHz, 2H). ^{13}C-NMR (DMSO-*d6*, 75 MHz, ppm): δ 52.5, 71.4, 72.3, 175.9. FT-IR (KBr): 3305 cm^{-1} (O-H stretching), 2966 cm^{-1} (C-H stretching), 1729 cm^{-1} (ester C=O stretching), 1386 cm^{-1} (ester C-O stretching). Calcd for $C_8H_{14}O_8$: C, 40.3; H, 5.9. Found: C, 40.3; H, 5.9.

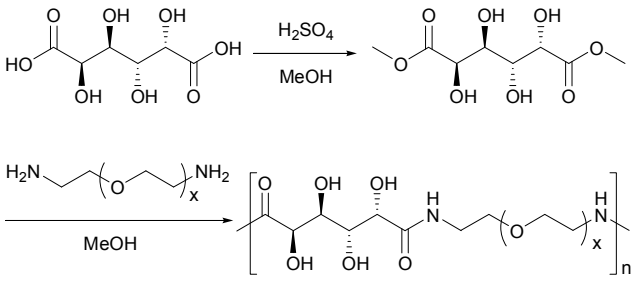
General Procedure for Synthesis of Poly(oxyethylene galactaramide)s (PEGAs)

Each polymer was synthesized by polycondensation between DMGA and oligo-EG, which are ethylenediamine (PEGA0), 2.2'-oxybis(ethylamine) (PEGA1), and 2,2′-(ethylenedioxy)bis(ethylamine) (PEGA2). The reaction mixture of DMGA (1 g, 4.2 mmol) and oligo-EG diamine (5.0 mmol) was refluxed in methanol (30 mL) for 6 - 24 hours, and the precipitate formed was filtrated, washed with methanol, and dried under vacuum at room temperature.

PEGA0. Yield: 0.95 g. FT-IR (KBr): 3396 cm^{-1} (O-H and N-H stretching), 2947 and 2874 cm^{-1} (C-H stretching), 1640 cm^{-1} (amide C=O stretching), 1541 cm^{-1} (amide N-H bending). Calcd for $C_8H_{14}N_2O_6$: C, 41.0; H, 6.0; N, 13.0. Found: C, 40.0; H, 6.3; N, 11.8.

PEGA1. Yield: 1.10 g. ^{1}H-NMR (DMSO-d_6, 300 MHz, ppm): δ 3.44 (m, 4H), 3.80 (m, 2H), 4.14 (m, 2H), 4.42 (br), 5.29 (br), 7.60 (m, 2H). ^{13}C-NMR (DMSO-d_6, 75 MHz, ppm): δ 38.3, 69.1, 71.0, 174.4. FT-IR (KBr): 3355 cm^{-1} (O-H and N-H stretching), 2938 and 2875 cm^{-1} (C-H stretching), 1647 cm^{-1} (amide C=O stretching), 1537 cm^{-1} (amide N-H bending). Calcd for $C_{10}H_{18}N_2O_7$: C, 43.16; H, 6.52; N, 10.07. Found: C, 42.3; H, 6.8; N, 10.0.

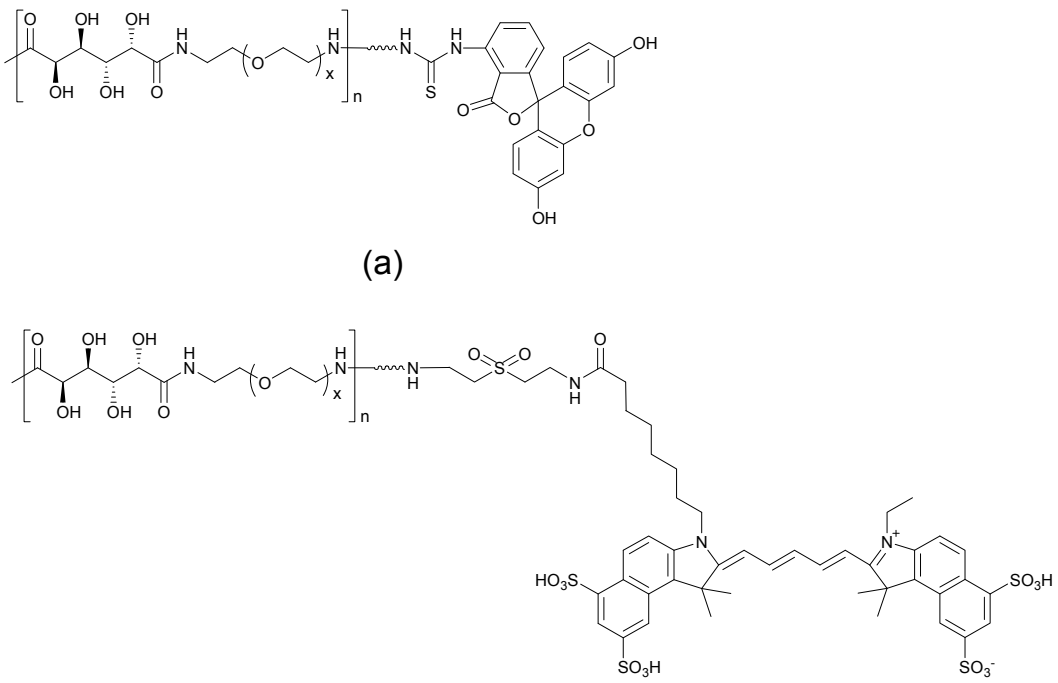
PEGA2. Yield: 1.15 g. ^{1}H-NMR (DMSO-d_6, 300 MHz, ppm): δ 3.23 (m, 4H), 3.43 (m, 4H), 3.52 (m, 4H), 3.81 (m, 2H), 4.15 (m, 2H), 4.41 (br), 5.29 (br), 7.58 (m, 2H). ^{13}C-NMR (DMSO-d_6, 75 MHz, ppm): δ 39.2, 70.2, 70.7, 72.8, 175.2. FT-IR (KBr): 3397 cm^{-1} (O-H and N-H stretching), 2936 and 2873 cm^{-1} (C-H stretching), 1644 cm^{-1} (amide C=O stretching), 1539 cm^{-1} (amide N-H bending). Calcd for $C_{12}H_{22}N_2O_8$: C, 44.7; H, 6.9; N, 8.7. Found: C, 44.4; H, 7.0; N, 8.7.



Scheme S1 Synthetic procedure of PEGAs.

Conjugation of Dyes to PEGA2 and mPEG-amine

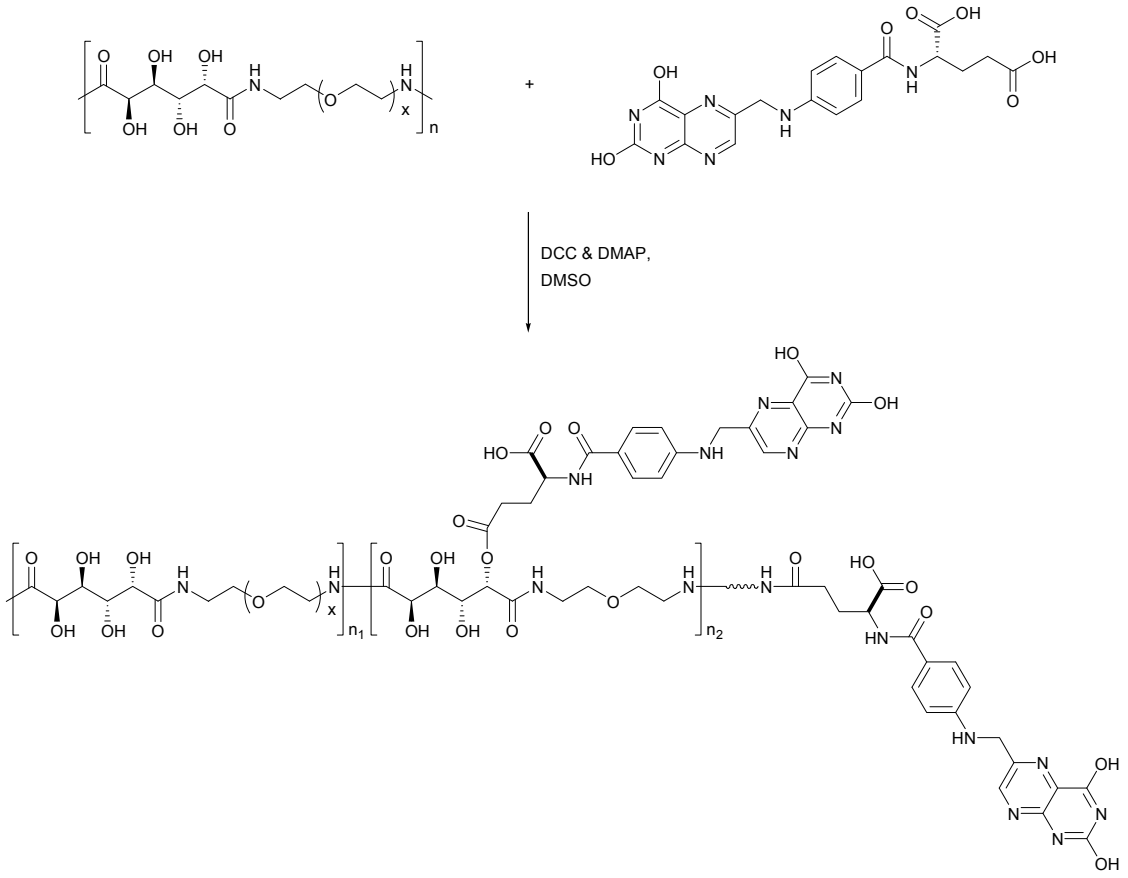
PEGA2 and mPEG-amine were labeled by direct conjugation with fluorescein isothiocyanate (FITC) or vinylsulfone-functionalized Cy5.5 (Cy5.5-VS, Bioacts). To label with Cy5.5, each polymer (200 mg) and Cy5.5-VS (1 mg) were stirred in DMSO (10 mL) at room temperature for 2 days, dialyzed with Spectra/Por membrane® (MWCO: 3,500), and lyophilized. In case of FITC labeling, 7.7 μmol of FITC and same equivalent of polymers were stirred with DMSO (10 mL) for 2 days, followed by the same purification procedure for Cy5.5-labeled polymers.



Scheme **S2** Chemical structure of dye-labeled PEGA2: a) PEGA2-FITC and b) PEGA2-Cy5.5.

Conjugation of Folic Acid (FA) to PEGA2

FA was conjugated to the hydroxyl groups and terminal amine groups of PEGA2 via esterification and amidation, respectively. PEGA2 (32.2 mg, 0.1 mmol of repeat unit) and FA (0.1 mmol or 0.01 mmol) were dissolved in DMSO (10 mL). N,N'-dicyclohexylcarbodiimide (DCC, 30.9 mg, 0.15 mmol) and 4-dimethylaminopyridine (DMAP, 18.3 mg, 0.15 mmol) were added into the reaction mixture, then stirred for 2 days at room temperature. The precipitates (dicyclohexylurea, DCU) were carefully removed by 2 times of filtration, and the filtrate was dialyzed with Spectra/Por membrane® (MWCO: 3,500), and lyophilized.



Scheme **S3** Synthetic procedure of folic acid (FA)-conjugated PEGA2.

Cellular Uptake Behavior of PEGA2 Nanoparticles

A human cervical epitheloid carcinoma (HeLa) cell line was provided by Korea Basic Science Institute, Korea. The cell line was maintained in Dulvecco's modified eagle medium with 10 % FBS,

L-glutamine (5×10^{-3} M) and gentamicin (5 μg mL^{-1}), in a humidified 5 % CO_2 incubator at 37 °C. The tested cells were seeded onto 35 mm coverglass bottom dishes and allowed to grow until a confluence of 70 %. Prior to the experiment, cells were washed twice with PBS (pH 7.4) to remove the remnant growth medium, and then incubated in medium (1.8 mL) containing PEGA2-Cy5.5 or PEGA2-Cy5.5-FA nanoparticles (200 μL, 2 mg mL^{-1}) for 1 hour. The cells were then washed twice with ice-cold PBS (pH 7.4) and directly imaged using a microscope (Ziess Axioskop2 FS Plus) equipped with an Axiocam black and white CCD camera and a fluorescence filter set for Cy5.5 (Omega Optical).

Cell Attachment onto the Surface of 96-well Plate

PEGA2 in PBS (50 μM) was directly immobilized on the well of maleic anhydride-activated 96-well plate (Pierce). After incubating overnight at room temperature, unimmobilized PEGA2 was washed out 3 times with PBS. A human cervical epitheloid carcinoma (HeLa) cells was seeded onto PEGA2-immobilized, maleic anhydride-activated or non-treated bare 96-well plates, and allowed to attach onto the plates for 12 h. The number of attached cells on the plates was determined by the colorimetric MTT assay, and the microscopic images were taken with CK40-SL optical microscope (Olympus, Japan).

***In Vivo/Ex Vivo* NIRF Imaging**

The animal study was approved by the animal care and use committee of Korea Institute of Science and Technology and all handling of mice was performed in accordance with institutional regulations. For animal experiments, Balb/c mice (male, 5 weeks of age; Orient Bio Inc., Korea) were shaved and anaesthetized with intraperitoneal injection of 0.5 % pentobarbital sodium (0.10 mL/10 g). All *in vivo* NIRF images were taken frequently for 1 week after intravenous injection of PEGA2-Cy5.5 nanoparticles and mPEG-amine-Cy5.5 solution (200 μL) with the eXplore Optix system (ART Advanced Research Technologies, Inc., Canada) and IVIS Spectrum imaging system (Caliper, USA). The concentration of PEGA2-Cy5.5 nanoparticles was 2 mg mL^{-1}, and that of mPEG-amine-Cy5.5 was adjusted so as to have the same Cy5.5 absorbance with PEGA2-Cy5.5. At 1 h, 1 d, and 7 d after intravenous injection, the organs and tumor of mice were resected and directly imaged with a 12-bit CCD camera (Kodak Image Station 4000 MM, New Haven, CT, USA) equipped with a special C-mount lens and Cy5.5 bandpass emission filter (680-720 nm; Omega Optical, USA).

Supplementary Figures

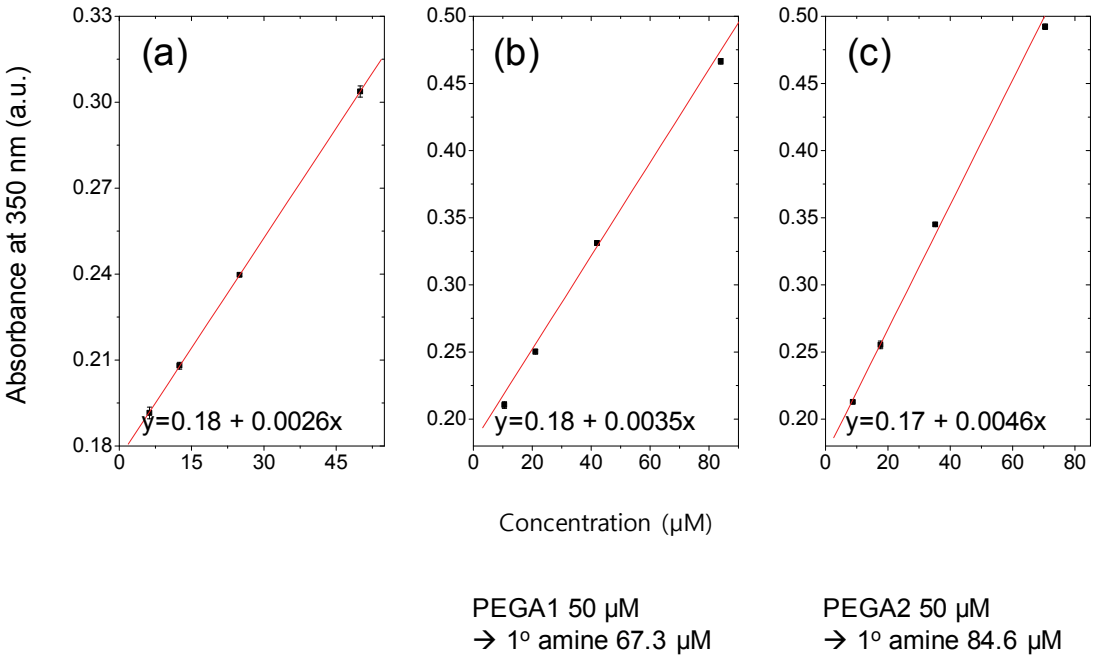


Fig. S1. Determination of the primary amine groups at the chain ends of PEGAs via the TNBSA (2,4,6-trinitrobenzene sulfonic acid) chromogenic assay. TNBSA reacts readily with the primary amine groups in aqueous solution at pH 8 to form yellow adducts having absorption at 335-345 nm.

The measurement was performed with a solution of TNBSA (Pierce, Rockford, IL) in $NaHCO_3$ reaction buffer, following the standard procedure [1]: the absorbance of TNBSA adducts at 335 nm was measured at a series of PEGA1 (b) and PEGA2(c) concentrations, and the results were compared to a standard curve generated with glycine (a). From this comparison, the primary amine groups introduced to the chain end of the polyamides were estimated to be 1.3 - 1.7 on each chain.

[1] G. Hermanson, Bioconjugate Techniques, Academic Press, San Diego, California, **1996**, pp. 112-113.

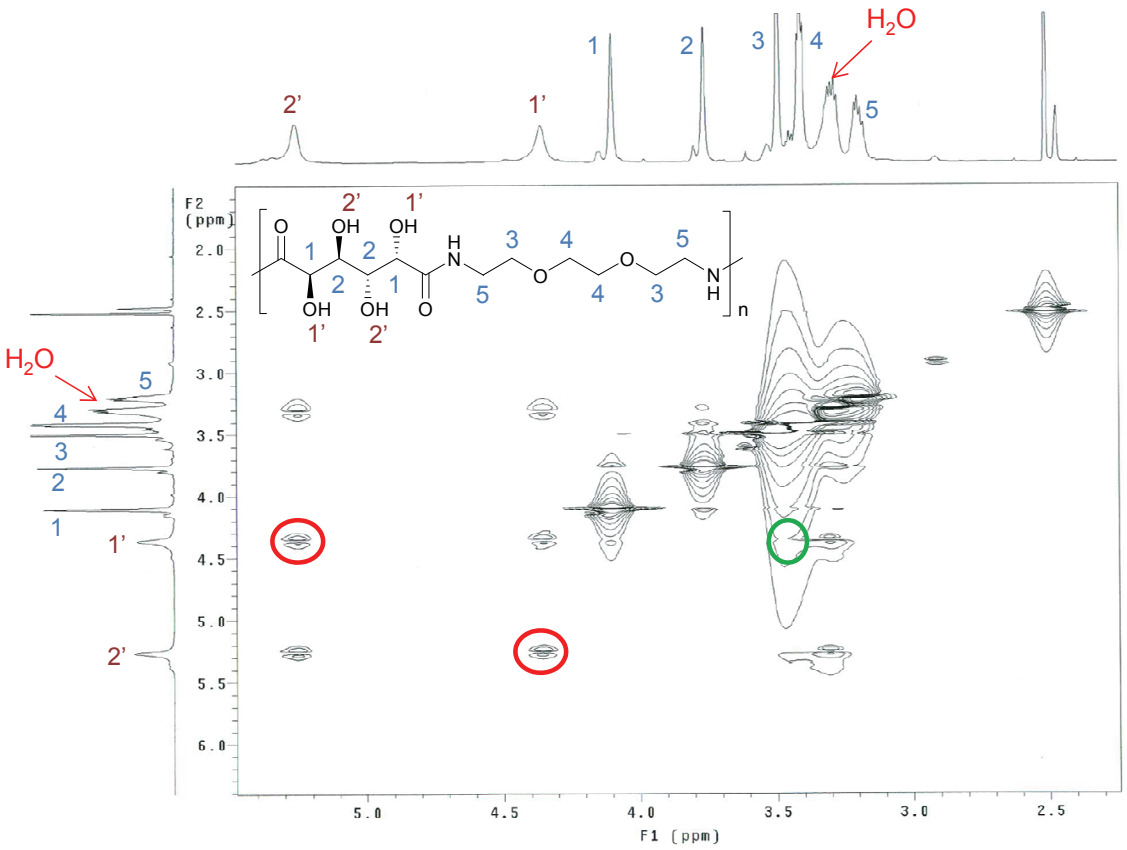


Fig. S2 Hydrogen bonding behavior of PEGA2 in hydrogen bonding disturbing solvent. 2D ^{1}H-NOESY NMR spectrum was taken from PEGA2 solution in DMSO-*d6* at 30 mg mL^{-1}. The cross peaks (H1'↔H2' and H1'↔H3(H4)) are indicated by red and green circles.

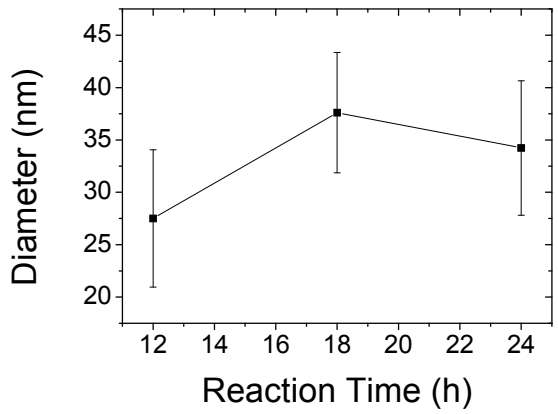


Fig. S3 Number averaged hydrodynamic diameter (measured by DLS) of PEGA2 with varying polymerization reaction time. PEGA2 were dispersed into fetal bovine serum (FBS), a model physiological fluid, with 5 -10 mg mL^{-1} of concentration.

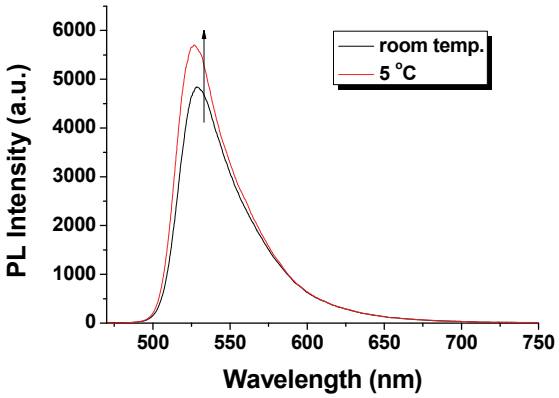


Fig. S4 Fluorescence evolution of aqueous FITC solution upon cooling from room temperature to 5 °C. A change upon cooling is indicated by an arrow.

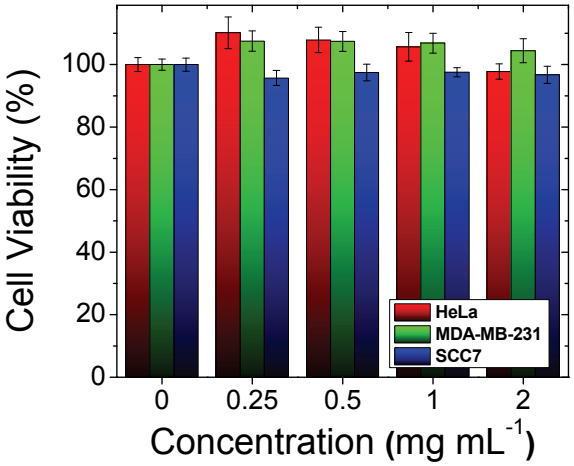


Fig. S5 *In vitro* cytotoxicity of PEGA2 against tumor cells (HeLa, MDA-MB-231, and SCC7 cell), evaluated by the colorimetric MTT assay. Cells were treated at various concentrations for 1 h and assayed according to the literature procedure. [1]

[1] T. Mosmann, *J. Immunol. Methods*, **1983**, 65, 55.

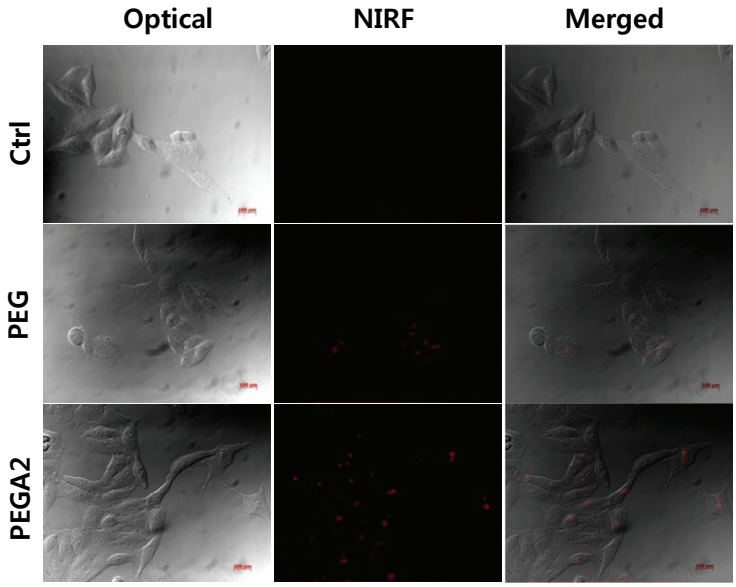
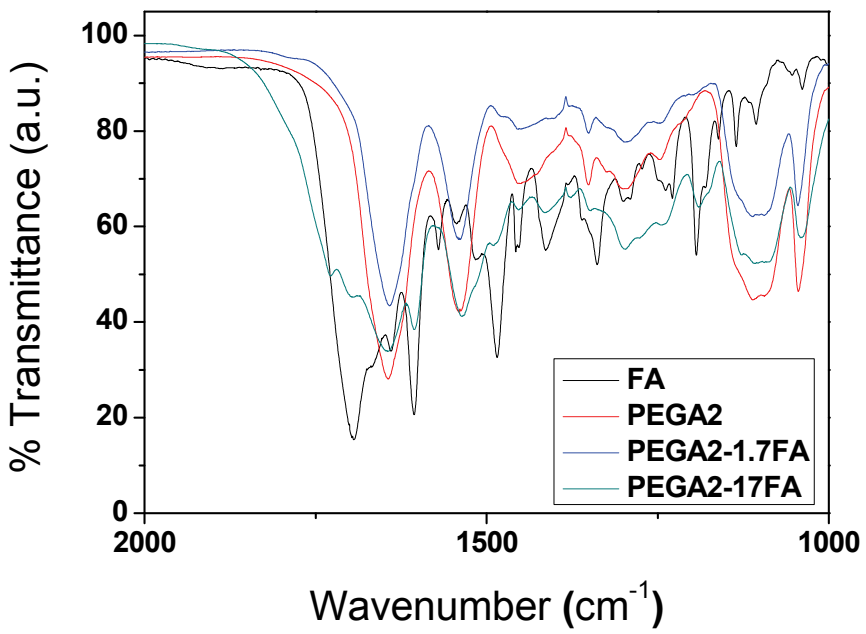


Fig. S6 Optical and near-infrared fluorescence (NIRF) images of HeLa cells untreated (ctrl) and treated with mPEG-amine-Cy5.5 and the PEGA2-Cy5.5 assembled nanoparticles.

(a)



(b)

	Feeding Ratio[a]	Calculated			Found		
		C	H	N	C	H	N
PEGA2-1.7FA	1.7	44.9	6.8	9.1	44.1	6.8	9.0
		Number of PEGA2 repeat unit : FA = 32.3 : 1					
		→ 0.5 FA per PEGA2 chain					
PEGA2-17FA	17	46.6	6.0	13.1	46.4	6.2	13.1
		Number of PEGA2 repeat unit : FA = 2 : 1					
		→ 8.6 FA per PEGA2 chain					

[a] The number of Feeding FA per PEGA2 chain

Fig. S7 Chemical structure identification of FA-conjugated PEGA2. a) FT-IR spectra of FA, PEGA2, and PEGA2-FA. b) Elemental analysis of PEGA2-FA. The peak corresponding to the ester C=O stretching vibration at 1728 cm^{-1} is only observed in the spectrum of PEGA2-FA (17 FA fed per chain), indicating that FA is conjugated onto the hydroxyl groups of PEGA2. From elemental analysis, 0.5 and 8.6 FA were attached per chain for PEGA2-1.7FA and PEGA2-17FA, respectively.

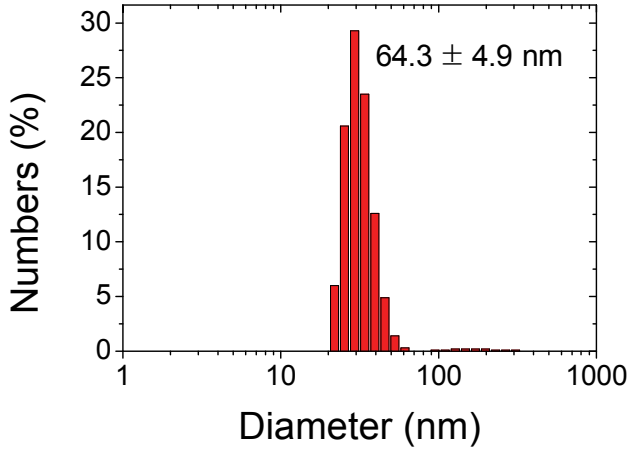


Fig. S8 Number averaged hydrodynamic size distribution of FA-conjugated PEGA2 nanoparticles. The size was measured from aqeous PEGA2-17FA dispersion with 2 mg mL^{-1} of concentration.

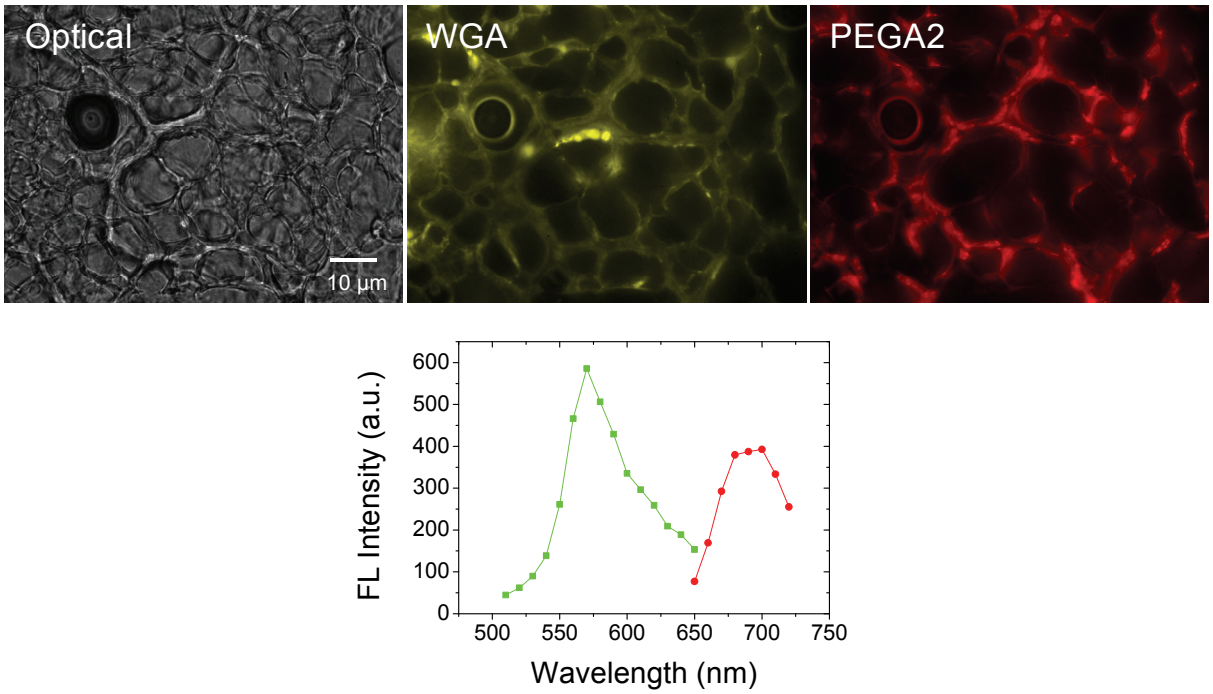


Fig. S9 Fluorescence images (upper) and spectra (lower) of the histological section of tumor at 1 hours after intravenous injection of PEGA2-Cy5.5 nanoparticles, taken with a LEICA DMI3000B equipped with a Nuance FX multispectral imaging system (CRI, USA). The section of tumor was labeled with wheat germ agglutinin-Alexa Fluor® 555 conjugate to stain the cell membrane. The green and red curves indicate the fluorescence spectrum of WGA and PEGA, respectively. In the microscopic images of tumor, PEGA2-Cy5.5 nanoparticles were observed in outside of tumor cells (the cell membrane or the extra-cellular matrix).

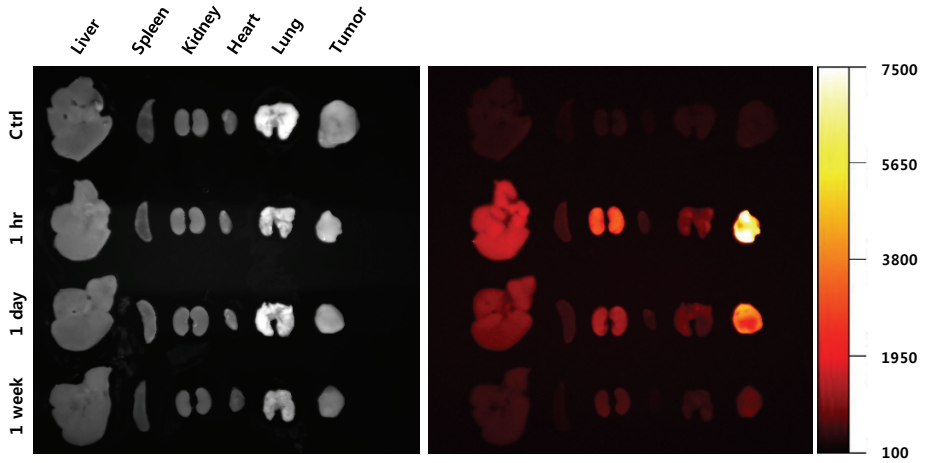


Fig. S10 Optical (left) and NIRF (right) images showing the *ex vivo* biodistribution of PEGA2-Cy5.5 nanoparticles in a mouse. Organs were collected from a mouse body at 1 hour, 1 day, and 1 week after intravenous injection.

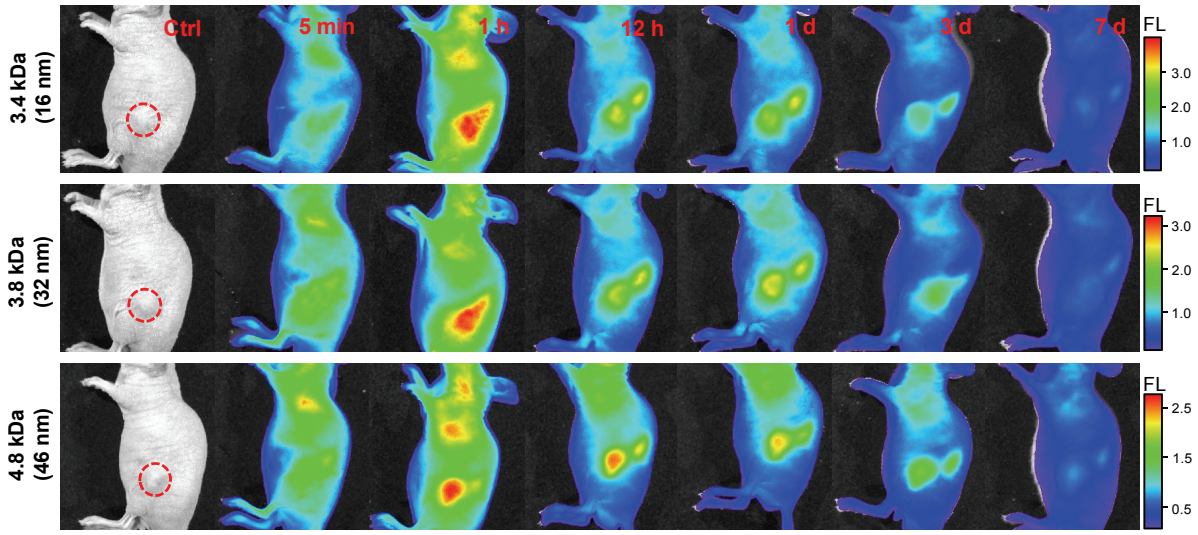


Fig. S11 *In vivo* NIRF images of SCC7 tumor-bearing mice taken at the predetermined time points before (Ctrl) and after tail vein injection of PEGA2-Cy5.5 nanoparticles with varying hydrodynamic size. The hydrodynamic sizes of PEGA2-Cy5.5 nanoparticles were varied by using different molecular weights of PEGA2. Red circles indicate the locations of tumor.