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Supporting Information

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Well-Defined, Reversible Boronate Crosslinked Nanocarriers for Targeted Drug Delivery in Response to Acidic pH Values and *cis*-Diols**

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Supporting Information

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1. Materials and Methods

1.1 Materials

Monomethylterminated poly(ethylene glycol) monoamine (MeO-PEG-NH_2, M_w: 5000 Da) was purchased from Rapp Polymere (Germany). 4-Carboxyphenylboronic acid, 4-Carboxyphenylboronic acid pinacol ester, 3-Carboxy-5-nitrophenylboronic acid and 3-Carboxy-5-nitrophenylboronic acid pinacol ester were obtained from Combi-Blocks (San Diego, CA). (Fmoc)lys(Boc)-OH, (Fmoc)Lys(Dde)-OH, (Fmoc)Lys(Fmoc)-OH and (Fmoc)Ebes-COOH were purchased from AnaSpec Inc. (San Jose, CA).$DiOC_{18}$ (DiO) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD) were purchased from Invitrogen (Carlsbad, CA). PTX was purchased from AK Scientific Inc. (Mountain View, CA). Taxol® (Mayne Pharma, Paramus, NJ) was obtained from the Cancer Center of University of California, Davis. 3,4-Dihydroxybenzoic acid, rhodamine B isothiocyanate, cholic acid, MTT [3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide] and all other chemicals were purchased from Sigma-Aldrich (St. Louis).

1.2 Synthesis of telodendrimers

The telodendrimers containing two and four 3,4-Dihydroxybenzoic acids (named as PEG^{5k}-$Catechol_2$-CA_8 and PEG^{5k}-$Catechol_4$-CA_8, respectively, Scheme S-1 and S-2) were synthesized via solution-phase condensation reactions from MeO-PEG-NH_2 via stepwise peptide chemistry [1]. The typical procedure for synthesis of PEG^{5k}-$Catechol_2$-CA_8 and PEG^{5k}-$Catechol_4$-CA_8 was as follows: (Fmoc)Lys(Boc)-OH (3 eq.) was coupled onto the N terminus of PEG using DIC and HOBt as coupling reagents until a negative Kaiser test result was obtained, thereby indicating completion of the coupling reaction. PEGylated molecules were precipitated by adding cold ether and then washed with cold ether twice. Fmoc groups were removed by the treatment with 20%(v/v) 4-methylpiperidine in dimethylformamide (DMF), and the PEGylated molecules were precipitated and washed three times by cold ether. White powder precipitate was dried under vacuum and one coupling of (Fmoc)Lys(Boc)-OH and three couplings of (Fmoc)lys(Fmoc)-OH were carried out respectively to generate a third generation of dendritic polylysine terminated with eight Fmoc groups on one end of PEG. Cholic acid NHS ester [1] were then coupled to the terminal end of dendritic polylysine. (Fmoc)Ebes-COOH was coupled to the amino groups of the proximal lysines between PEG and cholic acid upon the removal of Boc groups with 50% (v/v) trifluoroacetic acid (TFA) in dichloromethane (DCM).After the removal of Fmoc groups, one part of the polymer was coupled with 3,4-Dihydroxybenzoic acid resulting in PEG^{5k}-L_2-$Catechol_2$-CA_8 telodendrimer (Scheme S-1). The other part of the polymer was coupled with (Fmoc)lys(Fmoc)-OH and 3,4-Dihydroxybenzoic acid subsequently to generate PEG^{5k}-$Catechol_4$-CA_8 telodendrimer (Scheme S-1). The telodendrimers containing two or four 4-Carboxyphenylboronic acid and 3-Carboxy-5-nitrophenylboronic acids (named as PEG^{5k}-BA_2-CA_8, PEG^{5k}-BA_4-CA_8, PEG^{5k}-NBA_2-CA_8 and PEG^{5k}-NBA_4-CA_8, respectively, Scheme S-1 and S-2) were synthesized via the similar strategy as described above (Scheme S-1). 4-Carboxyphenylboronic acid pinacol ester and 3-Carboxy-5-nitrophenylboronic acid pinacol ester were coupled to the Ebes linkers or lysines between PEG and cholic acids at the last step. The four kinds of boronic acid containing telodendrimers were generated upon the removal of the pinacol esters with 50% (v/v) TFA in DCM. The telodendrimers was recovered from the mixture by three cycles of dissolution/reprecipitation with DMF and ether, respectively. Finally, the telodendrimers were dissolved in acetonitrile/water and lyophilized. The yields of the telodendrimers were in the range of 60-70% calculated based on the weight of the starting PEG. The PEG^{5k}-CA_8 parent telodendrimerwas synthesized to prepare the non-cross-linked micelles according to our previously reported method [1-2]. In order to prepare Rhodamine B labeled telodendrimers, (Fmoc)Lys(Dde)-OH was coupled to MeO-PEG-NH_2 initially to introduce an 1-(4,4-dimethyl-2,6-dioxocyclohex-1-yldine)ethyl (Dde) protected amino group. Rhodamine B isothiocyanate was conjugated to the amino group of the proximal lysine between PEG and cholic acids in the final telodendrimers after the removal of Dde protecting group by 2 % (v/v) hydrazine in DMF.

The mass spectra of the telodendrimers were collected on ABI 4700 MALDI TOF/TOF mass spectrometer (linear mode) using R-cyano-4-hydroxycinnamic acid as a matrix. It was difficult to obtain intense signals of boronic acid containing telodendrimers due to the difficulty of ionization and possible occurrence of dehydration and anhydride (boroxine) formation[3]. The mass spectrometry of these telodendrimers was recorded before the removal of the pinacol esters. The molecular weight of these telodendrimers was calculated by subtracting the molecular weight of pinacol esters from the detected values[3b]. ^{1}H NMR spectra of the polymers were recorded on an Avance 500 Nuclear Magnetic Resonance Spectrometer (Bruker) using DMSO-d6 as solvents. The ^{1}H NMR measurements were performed at 25^{o}C with the number of scans (nt) being 64, a 90^{o} pulse and a relaxation delay of 10 sec. The concentration of the polymers was kept at 5 ×10^{-4} M for NMR measurements. The solvent residual peak was used as reference (DMSO-d6: 2.49 ppm).

1.3 ARS based colorimetric and fluorescence assay

ARS is a catechol dye displaying dramatic changes in color and fluorescence intensity upon binding to boronic acid[4]. In this study, we utilized ARS indicator based colorimetric assay to estimate the concentration of boronic acid on telodendrimers. Briefly, as the concentration of boronic acid is increased, a visible color change from burgundy to yellow was observed. As shown in Figure S-3, the color and absorbance changes of ARS were observed with adding 3-Carboxy-5-nitrophenylboronic acid. The absorbance of the free ARS at 520 nm decreases as boronic acids added, and a new absorbance at 460 nm appears. A calibration curve was prepared by plotting the absorbance changes at 460 nm (ΔA) as function of concentrations of 4-Carboxyphenylboronic acid ([BA]) and 3-Carboxy-5-nitrophenylboronic acid ([NBA]) (Figure S-3). Based on the calibration curve, the number of boronic acids on the telodendrimers was calculated from the absorbance of samples in the colorimetric assay (Figure S-3, Table S-1).

ARS also displays a dramatic change in fluorescence intensity in response to the binding of boronic acids[4-5]. Boronic acid containing telodendrimers solutions (boronic acid concentrations: 0-5 mM) were mixed ARS solution in PBS at pH 7.4 and the fluorescence signal of the mixtures was measured by fluorescence spectrometry (Nanodrop3000, Microtrac). The final concentration of ARS was fixed at 0.1 mM. ARS fluorescence assay was further used to characterize the binding between the boronic acid containing telodendrimers and catechol containing telodendrimers. In this experiment, the final concentration of ARS and boronic acid of boronic acid containing telodendrimers were fixed at 0.1mM. Different molar ratio of catechol containing telodendrimers was premixed with boronic acid containing telodendrimers (0.1 mM) in anhydrous chloroform. The chloroform was evaporated and the thin film on the inner surface of flask was re-hydrated with PBS buffer to generate boronate crosslinked micelles. ARS solution was then mixed with the above micelle solutions and the fluorescence signal of the mixtures was measured by fluorescence spectrometry (Nanodrop3000, Microtrac).

1.4 Preparation of empty boronate cross-linked micelles (BCM)

Two distinct boronic acid-containing telodendrimer and catechol-containing telodendrimer (total 20 mg) were first dissolved in anhydrous chloroform in a 10 mL round bottom flask. The chloroform was evaporated under vacuum to form a thin film. PBS buffer (1 mL) was added to re-hydrate the thin film, followed by 30 min of sonication. Boronate ester bonds formed between boronic acids and catechols of adjacent telodendrimers, upon self-assembly in PBS, resulted in the formation of boronate cross-linked micelles (BCM). The micelle solution was filtered with 0.22 μm filter to sterilize the sample.

1.5 Preparation of drug or dye loaded micelles

Hydrophobic anti-cancer drug, such as paclitaxel (PTX), doxorubicin (DOX) and vincristine (VCR), were loaded into the micelles by the solvent evaporation method as described in our previous studies[1]. Briefly, drug (2.0 mg) and telodendrimers (total 20 mg) were first dissolved in anhydrous chloroform in a 10 mL round bottom flask. The chloroform was evaporated under vacuum to form a thin film. PBS buffer (1 mL) was added to re-hydrate

the thin film, followed by 30 min of sonication. The unloaded PTX was removed by running the micelle solutions through centrifugal filter devices (MWCO: 3.5 kDa, Microcon®). The PTX loaded micelles on the filters were recovered with PBS. The amount of drug loaded in the micelles was analyzed on a HPLC system (Waters) after releasing the drugs from the micelles by adding 9 times of acetonitrile and 10 min sonication. The drug loading was calculated according to the calibration curve between the HPLC area values and concentrations of drug standard. The loading efficiency is defined as the ratio of drug loaded into micelles to the initial drug content. Hydrophobic dye (DiO or DiD) was loaded into the micelles using the same strategy. The amount of dye loaded in the micelles was analyzed on a fluorescence spectrometry (SpectraMax M2, Molecular Devices, USA) after releasing the drugs from the micelles by adding 9 times of acetonitrile and 10 min sonication. The dye loading was calculated according to the calibration curve between the fluorescence intensity and concentrations of dye standard in acetonitrile.

1.6 Characterizations of micelles

The size and size distribution of the micelles were measured by dynamic light scattering (DLS) instruments (Microtrac). The micelle concentrations were kept at 1.0 mg/mL for DLS measurements. Each sample was measured for three times with an acquisition time of 30 seconds at room temperature. The data were analyzed by Microtrac FLEX Software 10.5.3 and values were reported as the means for each triplicate measurements. The morphology of micelles was observed on a Philips CM-120 transmission electron microscope (TEM). The aqueous micelle solution (1.0 mg/mL) was deposited onto copper grids, stained with phosphotungstic acid, and measured at room temperature. The apparent critical micelle concentration (CMC) of the NCM and BCMs was measured through fluorescence spectra by using pyrene as a hydrophobic fluorescent probe as described previously [1, 6]. Briefly, micelles were serially diluted in PBS to give the concentrations ranging from 5×10^{-7} to 5×10^{-4} M. The stock solution of pyrene in methanol was added into the micelle solution to make a final concentration of pyrene of 2×10^{-6} M. The solution was mildly shaken over night. Excitation spectra were recorded ranging from 300 to 360 nm with a fixed emission at 390 nm. The ratios of the intensity at 337 to 332 nm from the excitation spectra of pyrene were plotted against the concentration of the micelles. The CMC was determined from the threshold concentration, where the intensity ratio I337/I332 begins to increase markedly.

1.7 Stability of micelles in SDS and plasma

The stability study was performed to monitor the change in particle size of the NCM and BCMs in the presence of sodium dodecyl sulfate (SDS), which was reported to be able to efficiently break down polymeric micelles [7]. An SDS solution (7.5 mg/mL) was added to aqueous solutions of micelles (1.5 mg/mL). The final SDS concentration was 2.5 mg/mL and the micelle concentration was kept at 1.0 mg/mL. The size and size distribution of the micelle solutions was monitored continuously via dynamic light scattering (DLS) instruments (Microtrac) for 2 days. The stability of the micelles was also evaluated in PBS at different pH levels or in presence of mannitol and glucose (0, 10 mM, 50 mM, and 100 mM), together with SDS. Hydrogen chloride and sodium hydroxide solutions were used to prepare PBS at different pH levels. The pH values of the buffer were determined by a digital pH meter (Φ350 pH/Temp/mV meter, Beckman Coulter, USA) which gave pH values within 0.01 units. During the stability study, a small portion of the samples were taken out and further observed under TEM. The stability of NCM and BCMs was further studied in 50% (v/v) plasma from healthy human volunteers. The mixture was incubated at physiological body temperature (37 °C) followed by size measurements at predetermined time intervals up to 96 h.

1.8 PTX release study

PTX-loaded NCM and BCMs was prepared to determine the *in vitro* release profile. The PTX loading for NCM, BCM1, BCM2, BCM3 and BCM4 were 9.9%, 9.8%, 9.8%, 9.9%, 10.0% (w/w, PTX/micelle) (Table S-3) in the presence of total 20 mg telodendemers measured as described in Section 2.5. Aliquots of PTX-loaded micelle solution were injected into dialysis cartridges (Pierce Chemical Inc.) with a 3.5 kDa MWCO. In order to make an ideal sink condition, 10 g charcoal was added in the release medium. The cartridges were dialyzed against PBS at different pH levels (pH6.5, pH6.0, pH5.5 and pH5.0) or in the

presence of various concentrations of glucose or mannitol (0, 10mM, 50 mM, and 100 mM) at 37 ^{o}C. The release medium was stirred at a speed of 100 rpm. The concentration of PTX remaining in the dialysis cartridge at various time points was measured by HPLC. In some experiments, the release medium (pH7.4) was replaced with fresh medium at different pH levels (pH6.5, pH6.0, pH5.5 and pH5.0) and/or in the presence of mannitol or glucose (10 and 100 mM) at a specific release time (5 h). Values were reported as the means for each duplicate samples.

1.9 Cell uptake and MTT assay

SKOV-3 ovarian cancer cells were seeded at a density of 50000 cells per well in eight-well tissue culture chamber slides (*BD* Biosciences, Bedford, MA, USA), followed by 24 h of incubation in McCoy's 5a Medium containing 10% FBS. The medium was replaced, and DiD labeled micelles (100 μg/mL) were added to each well. After 30 min, 1h, 2h and 3h, the cells were washed three times with PBS, fixed with 4% paraformaldehyde and the cell nuclei were stained with DAPI. The slides were mounted with cover slips and observed under confocal laser scanning microscope (Olympus, FV1000). For the DiD channel, the excitation was set to 625 nm while the emission was set to 700 nm.

SKOV-3 ovarian cancer cells were seeded in 96-well plates at a density of 5000 cells/well 24 h prior to the treatment. The culture medium was replaced with fresh medium containing various formulations of PTX with different dilutions at pH 7.4 or 5.0, in the absence or in the presence of 100 mM mannitol. The cells were washed with PBS and incubated for another23 h in a humidified 37 ^{o}C, 5% CO_2 incubator. MTT was added to each well and further incubated for 4 h. The absorbance at 570 nm and 660 nm was detected using a micro-plate ELISA reader (SpectraMax M2, Molecular Devices, USA). Untreated cells served as a control. Results were shown as the average cell viability $[(OD_{treat} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100\%]$ of triplicate wells. The cells were also treated with telodendrimers and empty crosslinked micelles with different dilutions and incubated for total 72 h in order to evaluate telodendrimer related toxicity.

1.10 Animal and tumor xenograft model

Female athymic nude mice (Nu/Nu strain), 6-8 weeks age, were purchased from Harlan (Livermore, CA). All animals were kept under pathogen-free conditions according to AAALAC guidelines and were allowed to acclimatize for at least 4 days prior to any experiments. All animal experiments were performed in compliance with institutional guidelines and according to protocol No. 07-13119 and No. 09-15584 approved by the Animal Use and Care Administrative Advisory Committee at the University of California, Davis. The subcutaneous xenograft model of ovarian cancer was established by injecting 7×10^6 SKOV-3 ovarian cells in a 100 μL of mixture of PBS and Matrigel (1:1 v/v) subcutaneously into the right flank of female nude mice.

1.11 In vivo FRET studies

PEG^{5k}-CA_8 (15 mg), rhodamine B conjugated PEG^{5k}-CA_8 (5 mg) and DiO (0.5 mg) were used to prepare non-crosslinked FRET micelles (FRET-NCM) as described in Section 1.5 while PEG^{5k}-NBA_4-CA_8(5 mg), rhdamine B conjugated PEG^{5k}-NBA_4-CA_8 (5 mg), PEG^{5k}-$Catechol_4$-CA_8 (10 mg) and DiO (0.5 mg) were used to prepare crosslinked FRET micelles (FRET-BCM4). Micelles with DiO alone and micelles with rhodamine B alone at the same dye contents were also prepared for comparison. The micelle solution was filtered with 0.22 μm filter to sterilize the sample. The particle size of these micelles was measured by DLS. The absorbance and fluorescence spectra of these micelles diluted by PBS were characterized by fluorescence spectrometry (SpectraMax M2, Molecular Devices, USA). 100 μL of FRET micelles (2.0 mg/mL) were injected into nude mice (n=3) via tail vein to investigate their *in vivo* stability by monitoring FRET efficiency. 50 μL blood was collected at different time points post-injection to measure the fluorescence spectra[8]. The excitation was set to 480 nm while the emission was recorded from 500 nm to 660 nm. The FRET ratio was calculated by the formula of $(I_{\text{rhodamine B}}/(I_{\text{rhodamine B}} + I_{\text{DiO}}))$, where $I_{\text{rhodamine B}}$ and I_{DiO} were fluorescence intensity of rhodamine B at 580 nm and DiO at 530 nm, respectively. According to the body weight, we assume the blood volume of a nude mouse is around 2 mL, and thus the micelle concentration in blood was estimated to be 100 μg/mL.

1.12 In vivo blood elimination kinetics

Rhodamine B labeled NCM and BCMs were prepared for the blood elimination study as described in Section 1.5. The concentration of rhodamine B conjugated micelles was 2.0 mg/mL. The absorbance and fluorescence spectra of these micelles diluted 20 times by PBS were characterized by fluorescence spectrometry (SpectraMax M2, Molecular Devices, USA). 100 μL of Rhodamine B conjugated NCM and BCMs were injected into tumor free nude mice via tail vein. 50 μL blood was collected at different time points post-injection to measure the fluorescence signal of Rhodamine B.

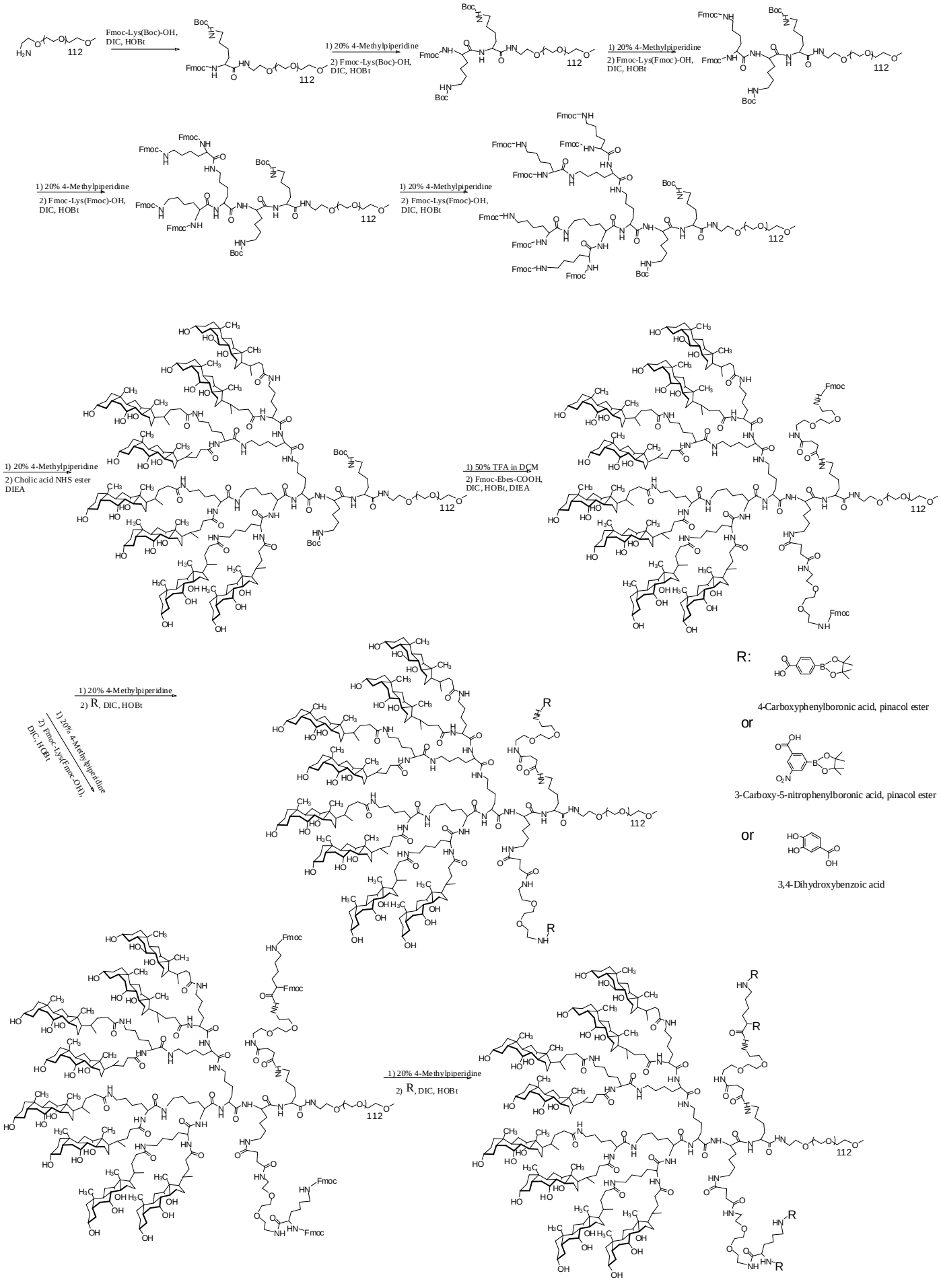
1.13 In vivo optical imaging

Nude mice with subcutaneous SKOV-3 tumors of an approximate 8~10 mm diameter were subjected to *in vivo* optical imaging. At different time points post injection of DiD (a hydrophobic near infrared dye) and PTX co-loaded BCM4 (the concentrations of DiD and PTX were both 0.5 mg/mL), mice were scanned using a Kodak multimodal imaging system IS2000MM with an excitation band pass filter at 625 nm and an emission at 700 nm. The mice were anaesthetized by intraperitoneal injection of pentobarbital (60 mg/kg) before each imaging. After *in vivo* imaging, animals were euthanized by CO_2 overdose at 32 h after injection. Tumors and major organs were excised and imaged with the Kodak imaging station.

1.15 Statistical analysis

The level of significance in all statistical analyses was set at a probability of $P< 0.05$. Data are presented as means±standarderror (SEM). Statistical analysis was performed by Student's t-test for comparison of two groups, and one-way analysis of variance (ANOVA) for multiple groups, followed by Newman-Keuls test if overall $P < 0.05$.

2. Results


Fmoc-Lys(Boc)-OH, DIC, HOBt
1) 20% 4-Methylpiperidine
2) Fmoc-Lys(Boc)-OH, DIC, HOBt
1) 20% 4-Methylpiperidine
2) Fmoc-Lys(Fmoc)-OH, DIC, HOBt
1) 20% 4-Methylpiperidine
2) Fmoc-Lys(Fmoc)-OH, DIC, HOBt
1) 20% 4-Methylpiperidine
2) Fmoc-Lys(Fmoc)-OH, DIC, HOBt
1) 20% 4-Methylpiperidine
2) Cholic acid NHS ester DIEA
1) 50% TFA in DCM
2) Fmoc-Ebes-COOH, DIC, HOBt, DIEA
1) 20% 4-Methylpiperidine
2) R, DIC, HOBt
1) 20% 4-Methylpiperidine
2) Fmoc-Lys(Fmoc-OH), DIC, HOBt
1) 20% 4-Methylpiperidine
2) R, DIC, HOBt
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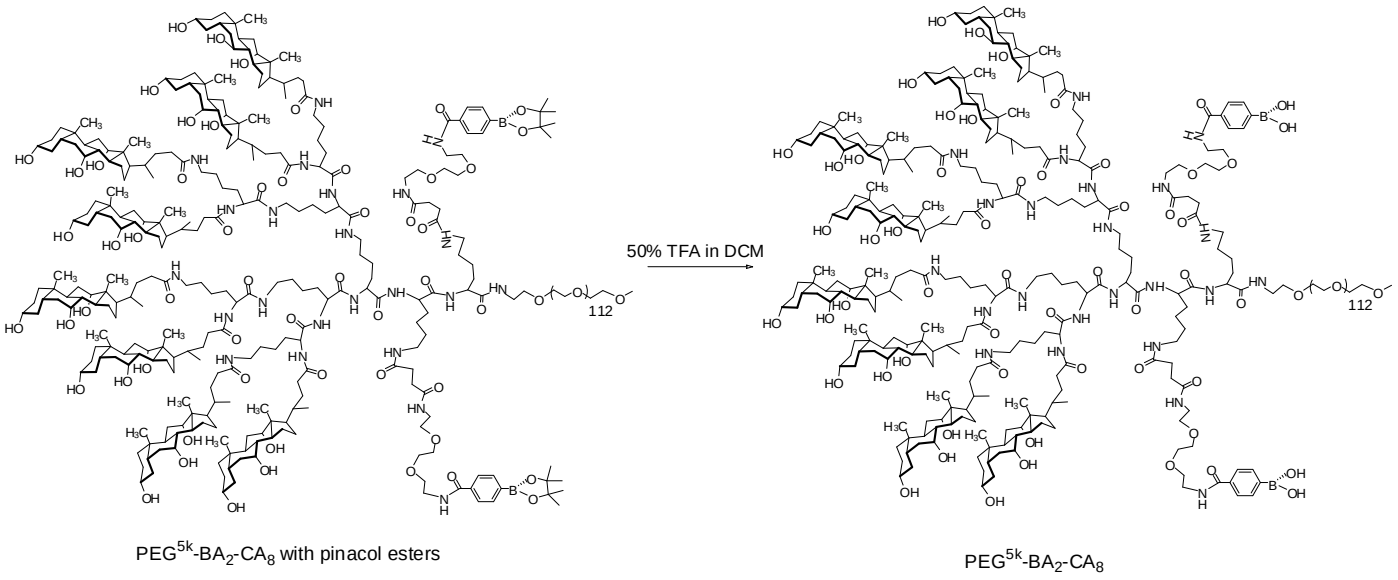
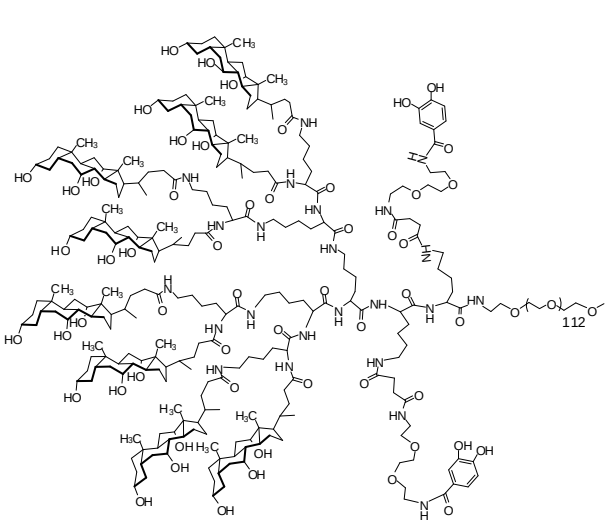
or
3,4-Dihydroxybenzoic acid



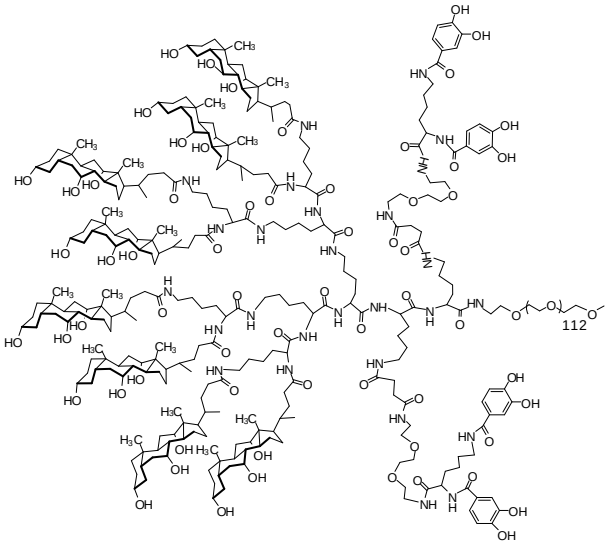
PEG^{5k}-BA$_2$-CA$_8$ with pinacol esters

PEG^{5k}-BA$_2$-CA$_8$

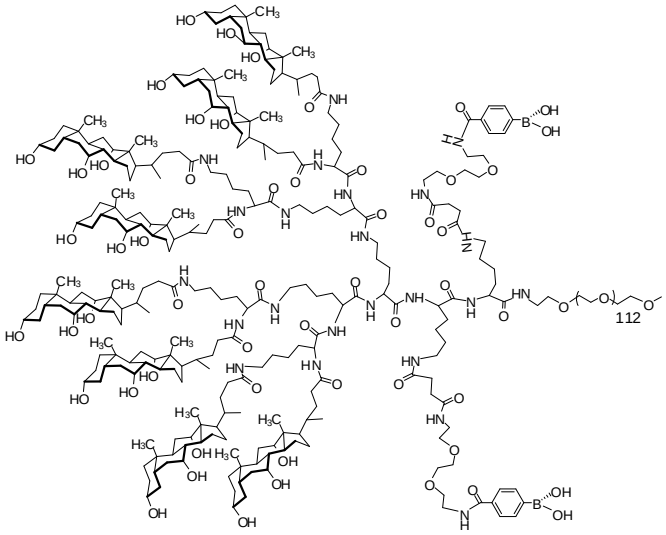
Scheme S-1. Synthetic scheme for the catechol containing telodendrimers and boronic acid containing telodendrimers. The pinacol esters of boronic acid containing telodendrimers were removed via DCM/TFA (1/1, v/v) at the last step. The synthetic scheme of PEG^{5k}-BA$_2$-CA$_8$ was shown as an example.



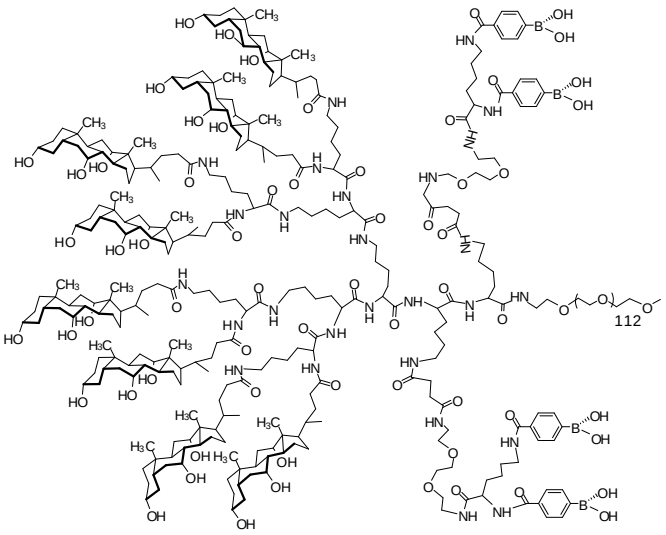
PEG^{5k}-Catechol$_2$-CA$_8$



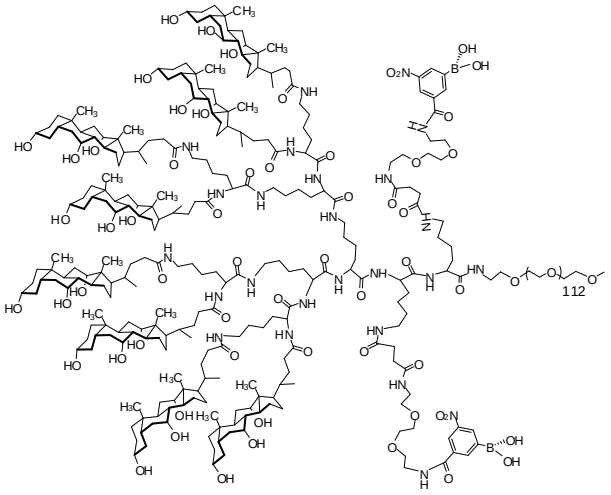
PEG^{5k}-Catechol$_4$-CA$_8$



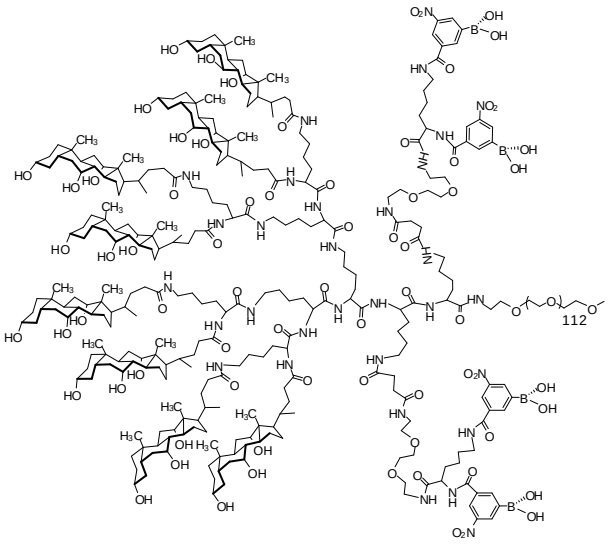
PEG^{5k}-BA$_2$-CA$_8$



PEG^{5k}-BA$_4$-CA$_8$



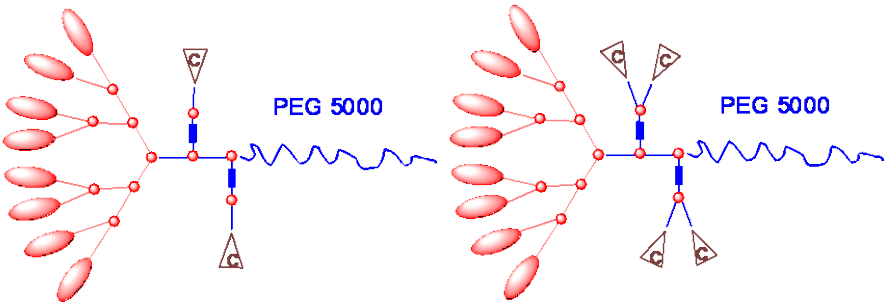
PEG^{5k}-NBA$_2$-CA$_8$



PEG^{5k}-NBA$_4$-CA$_8$

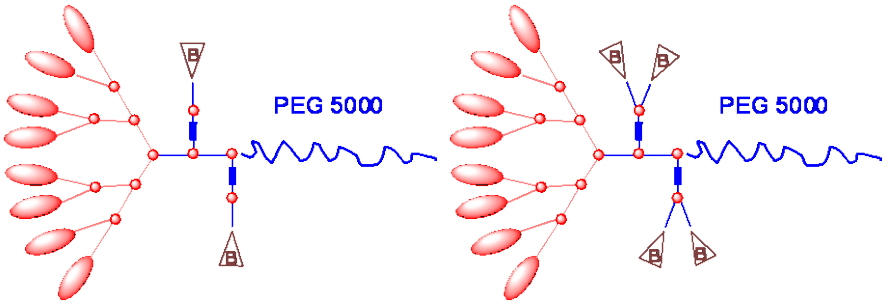
Scheme S-2. The chemical structure of the catechol containing telodendrimers and boronic acid containing telodendrimers.

Catechol containing telodendrimers

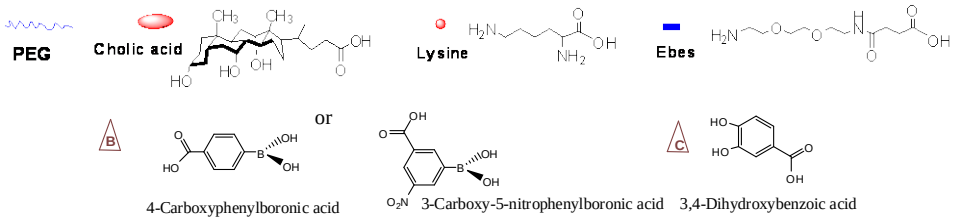


PEG^{5k}-$Catechol_2$-CA_8 PEG^{5k}-$Catechol_4$-CA_8

Boronic acid containing telodendrimers



PEG^{5k}-(boronic acid)$_2$-CA_8 PEG^{5k}-(boronic acid)$_4$-CA_8



Scheme S-3. Schematic representation of the catechol containing telodendrimers and boronic acid containing telodendrimers.

Table S-1. Characterization of the telodendrimers. The mono-dispersed mass traces were detected for the starting PEG and the telodendrimers, and the molecular weights of the telodendrimers from MALDI-TOF MS (Figure S-1) were almost identical to the theoretical value. The number of boronic acids, catechols and cholic acids determined by ^{1}H-NMR for the telodendrimers was consistent with the molecular formula of the target telodendrimers (Figure S-2). As determined by the colorimetric assay based on the indicator of alizarin red S (ARS)[4-5] (Figure S-3), the number of covalently attached nitro-phenylboronic acids and phenylboronic acids in the telodendrimers was also in line with the molecular formula of the telodendrimers. These results demonstrated the well-defined structure of telodendrimers.

Telodendrimers	*M*w (theo.)[a]	*M*w(MS)[b]	N_{CA}[c] (NMR)	N_{BA}[e] (NMR)	N_{NBA}[f] (NMR)	$N_{catechol}$[d] (NMR)	N_{BA}[g] (ARS assay)	N_{NBA}[h] (ARS assay)
PEG^{5k}-CA_8	9059	8918	7.5	--	--	--	--	--
PEG^{5k}-$Catechol_2$-CA_8	9893	9928	8.1	--	--	2.1	--	--
PEG^{5k}-$Catechol_4$-CA_8	10419	10376	7.6	--	--	4.1	--	--
PEG^{5k}-BA_2-CA_8	9917	9801	7.6	1.7	--	--	2.5	--
PEG^{5k}-BA_4-CA_8	10467	10393	7.6	3.8	--	--	4.2	--
PEG^{5k}-NBA_2-CA_8	9971	9837	7.7	--	1.8	--	--	2.3
PEG^{5k}-NBA_4-CA_8	10650	10278	7.5	--	4.0	--	--	4.1

[a] Theoretical molecular weight.
[b] Obtained via MALDI-TOF MS analysis (linear mode). The boronic acid containing telodendrimers were measured in the pinacol ester form and the molecular weight was calculated as described in Section 1.2.
[c]Number of cholic acids, calculated based on the average integration ratio of the peaks of methyl proton 18, 19, and 21 in cholic acid at 0.66, 0.87 and 1.01 ppmand methylene proton of PEG at 3.5-3.7 ppm in^{1}H-NMR spectra in DMSO-d6 (90^o pulse). The molecular weight of the starting PEG was 4912 (Figure S-1A).
[d]Number of phenylboronic acids, calculated based on the average integration ratio of the peaks of the phenyl protons of phenylboronic acids (6.7-7.2 ppm) and methylene proton of PEG to in^{1}H-NMR spectra in DMSO-d6 (90^o pulse).
[e]Number of nitro-phenylboronic acids, calculated based on the average integration ratio of the peaks of the phenyl protons of nitro-phenylboronic acids (8.6-8.9 ppm) and methylene proton of PEG to in^{1}H-NMR spectra in DMSO-d6 (90^o pulse) (Figure S-2).
[f]Number of 3,4-Dihydroxybenzoic acids (catechols), calculated based on the average integration ratio of the peaks of the phenyl protons of catechols (6.7-7.5 ppm) and methylene proton of PEG to in^{1}H-NMR spectra in DMSO-d6 (90^o pulse) (Figure S-2).
[g]Number of phenylboronic acids, determined by ARS colorimetric assay, as described in Section 1.3.
[h]Number of nitro-phenylboronic acids, determined by ARS colorimetric assay, as described in Section 1.3.

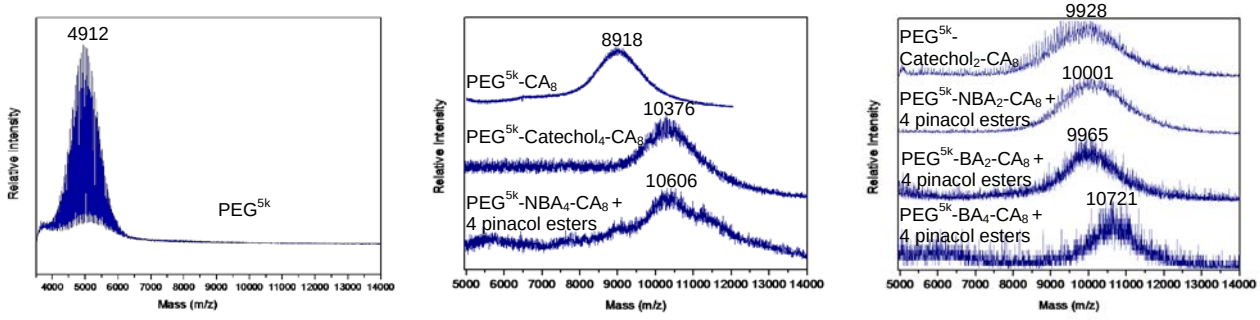
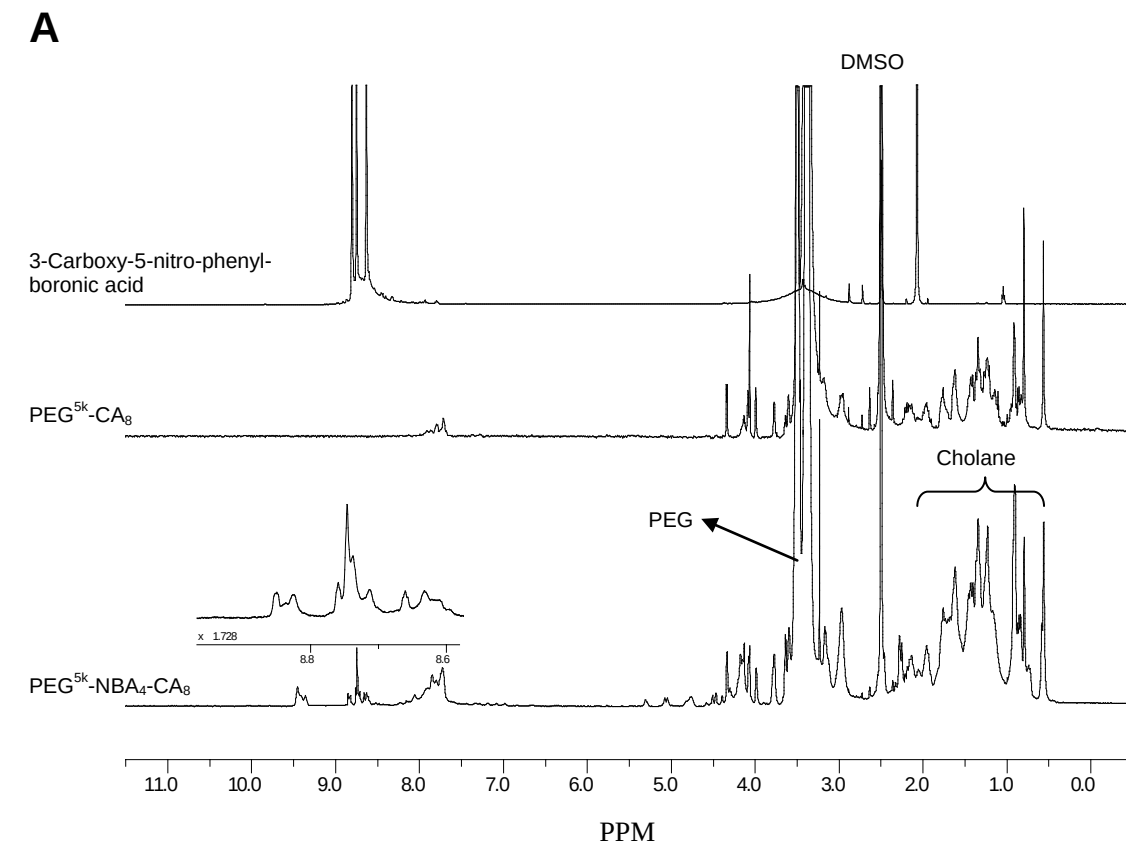
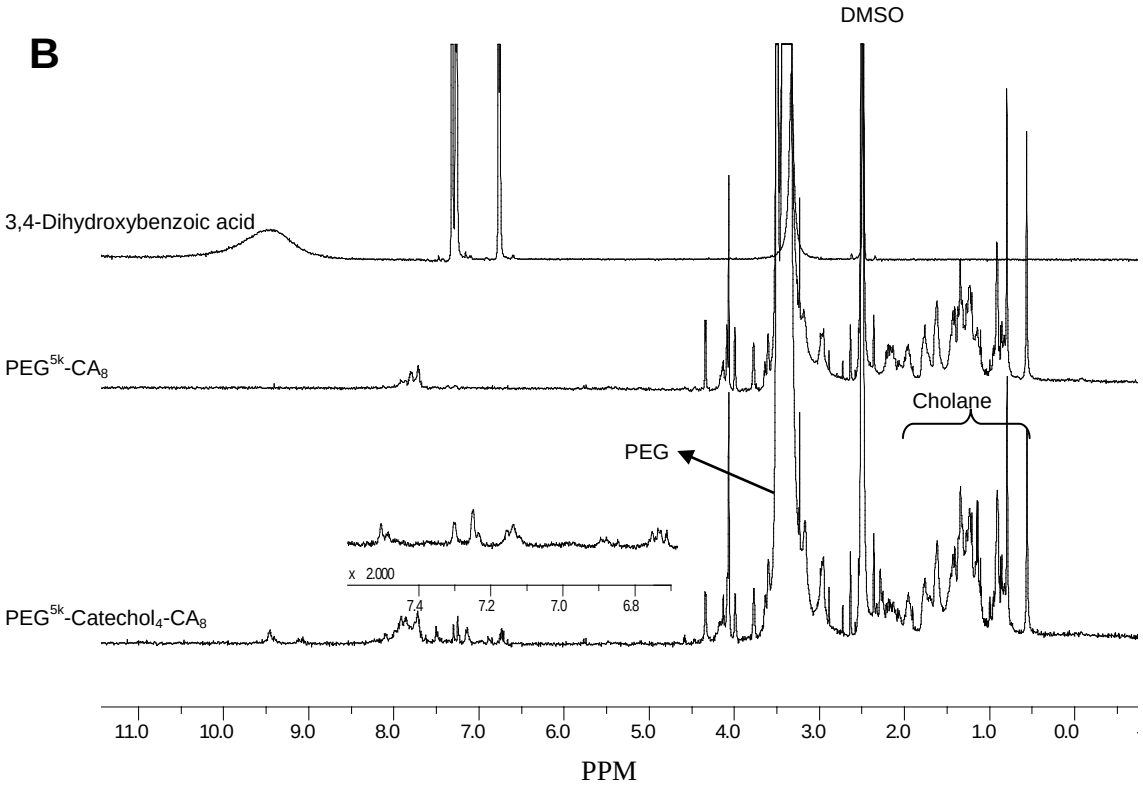
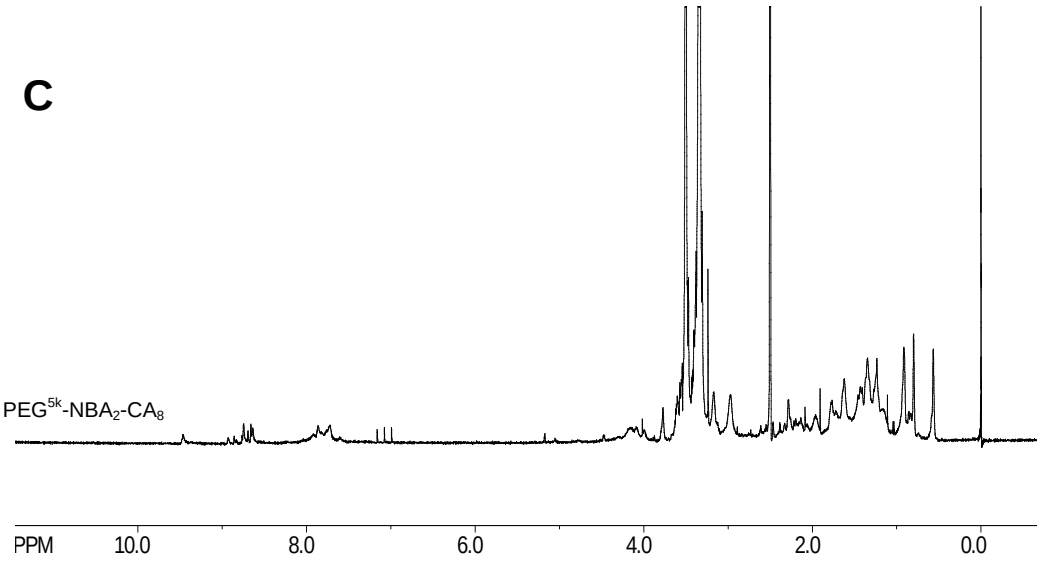
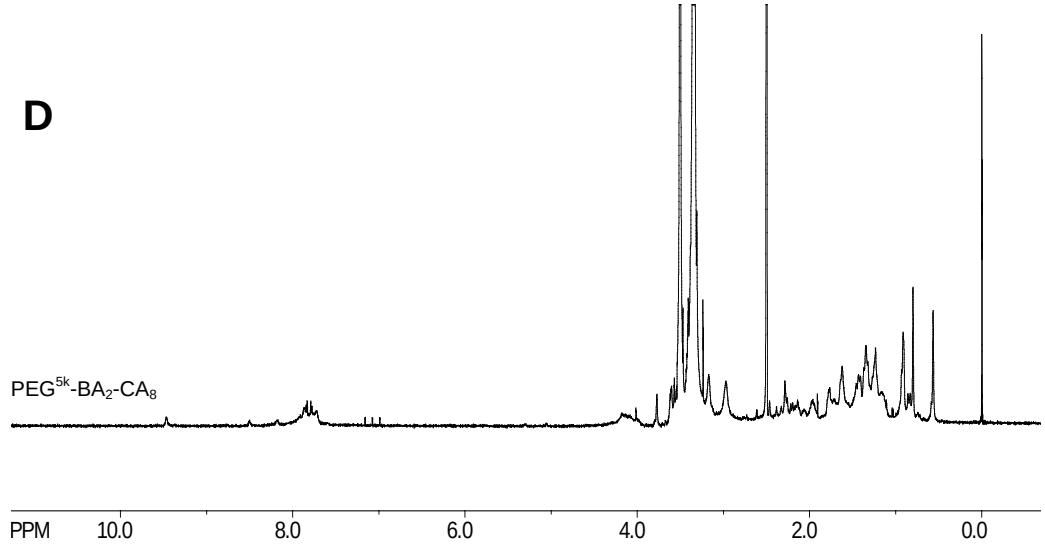


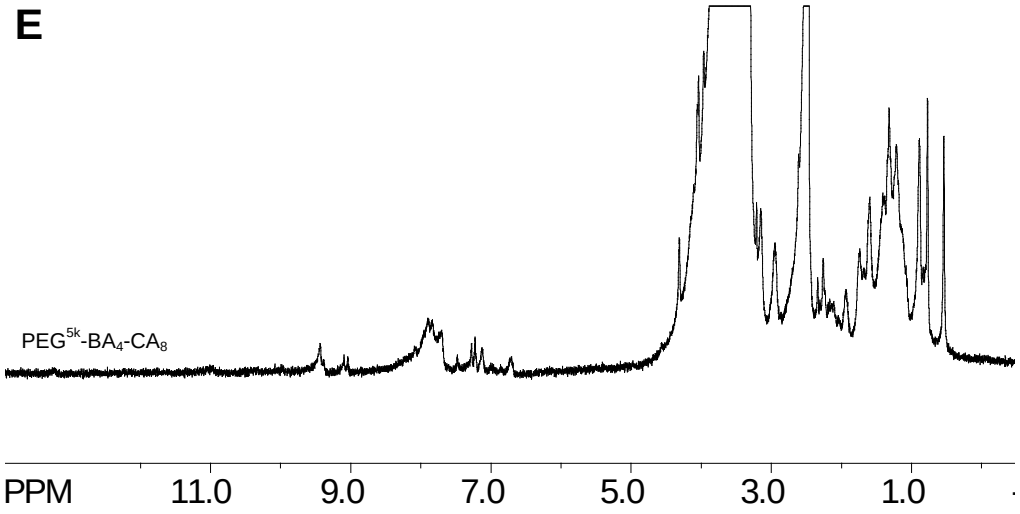
Figure S-1. The MALDI-TOF MS of the starting PEG, PEG^{5k}-CA_8 telodendrimer and the boronic acid and catechol containing telodendrimers. The mass spectra of the pinacol ester form of boronic acid containing telodendrimers was shown.


A
DMSO
3-Carboxy-5-nitro-phenyl-
boronic acid
PEG5k-CA8
Cholane
PEG
x 1.728
8.8
8.6
PEG5k-NBA4-CA8
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
PPM


B
DMSO
3,4-Dihydroxybenzoic acid
PEG5k-CA8
Cholane
PEG
x 2.000
7.4
7.2
7.0
6.8
PEG5k-Catechol4-CA8
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
PPM


C
PEG5k-NBA2-CA8
PPM
10.0
8.0
6.0
4.0
2.0
0.0


D
PEG5k-BA2-CA8
PPM
10.0
8.0
6.0
4.0
2.0
0.0


E
PEG5k-BA4-CA8
PPM
11.0
9.0
7.0
5.0
3.0
1.0

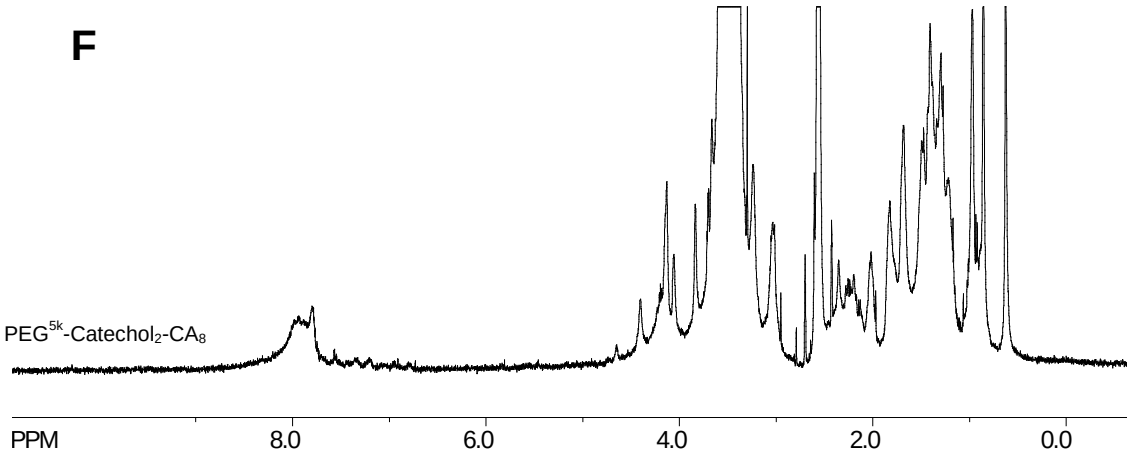
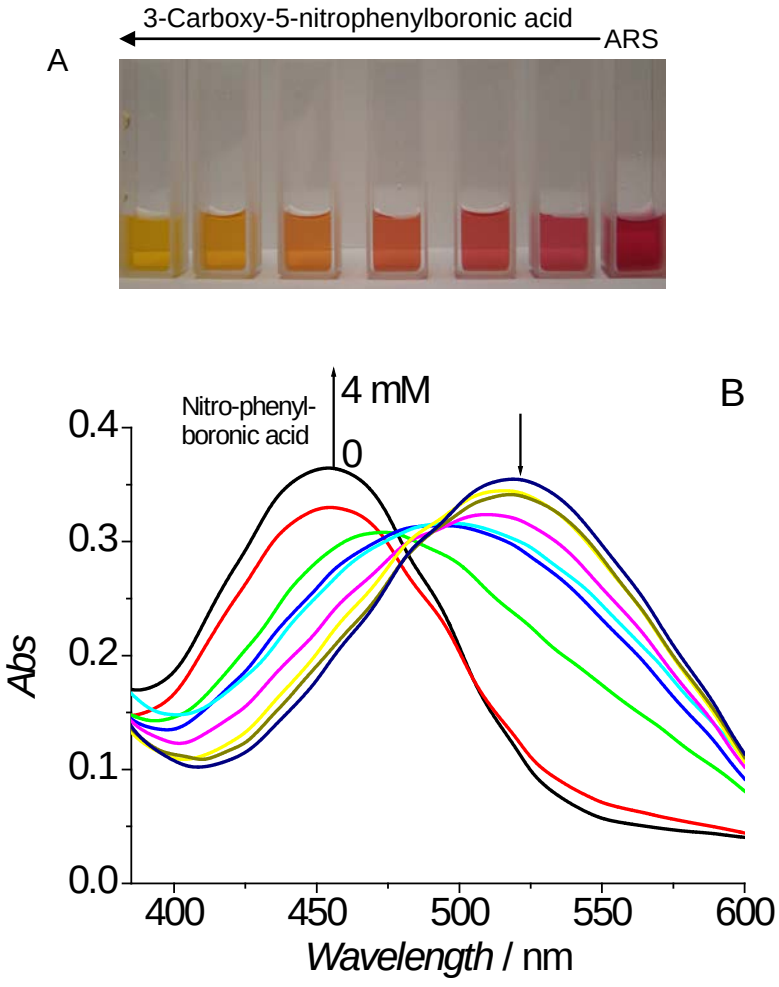


Figure S-2. ^{1}H NMR spectra of the representative telodendrimer pair (PEG^{5k}-NBA$_4$-CA$_8$ **(A)** / PEG^{5k}-Catechol$_4$-CA$_8$ **(B)**) comparing with the corresponding small molecular 3-Carboxy-5-nitrophenylboronic acid and 3,4-Dihydroxybenzoic acid as well as PEG^{5k}-CA$_8$ telodendrimer recorded in DMSO-d6. ^{1}H NMR spectra of PEG^{5k}-NBA$_2$-CA$_8$ **(C)**, PEG^{5k}-BA$_2$-CA$_8$ **(D)**, PEG^{5k}-BA$_4$-CA$_8$ **(E)** and PEG^{5k}-Catechol$_2$-CA$_8$ **(F)**.



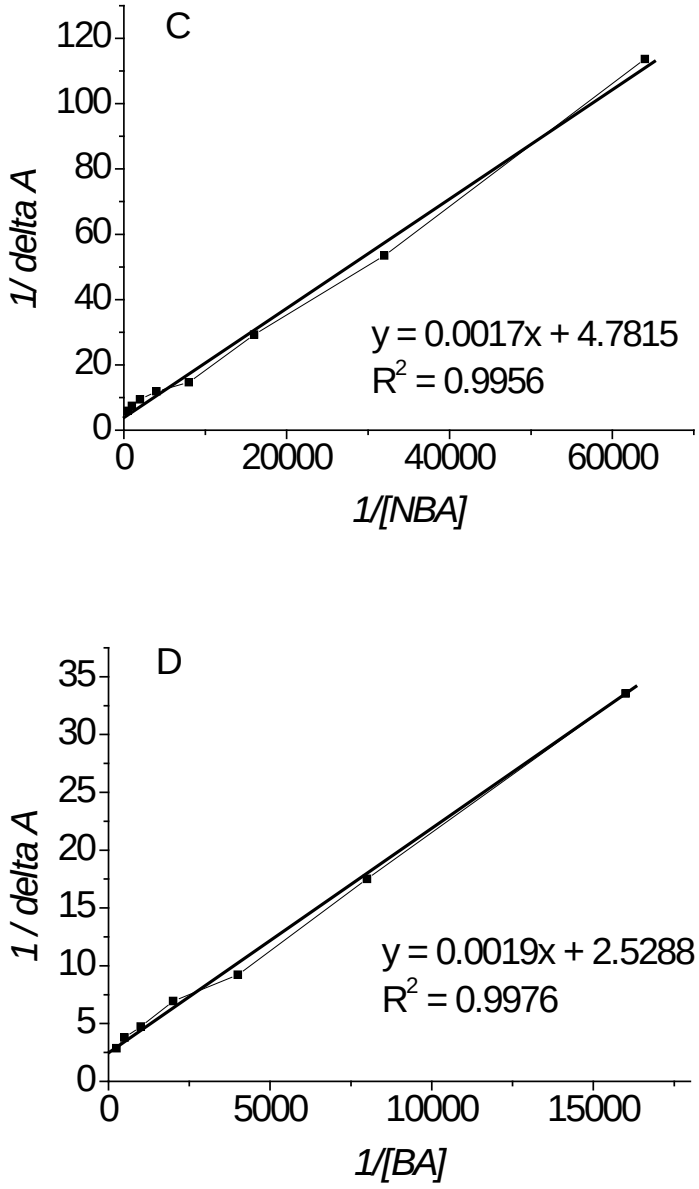
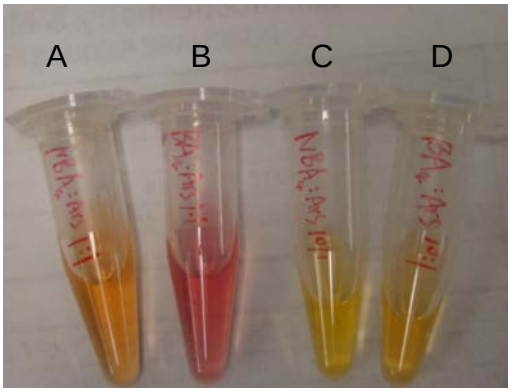
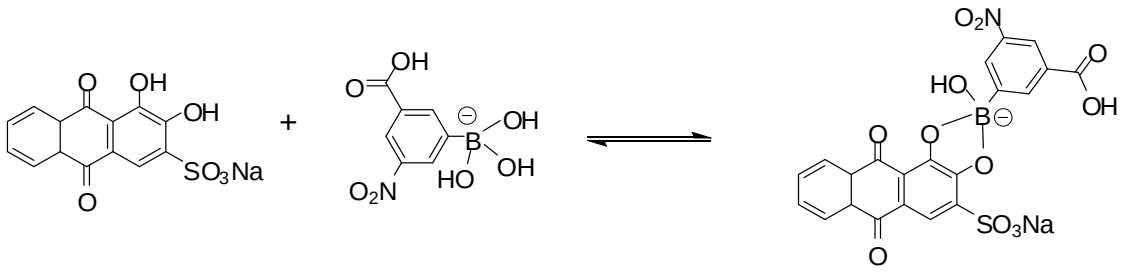


Figure S-3. (**A**) Color changes and (**B**) absorption spectral changes of ARS (0.1 mM) with increasing concentration of 3-Carboxy-5-nitrophenylboronic acid (0-4mM). (**C**) Standard curve between 1/ΔA and 1/[3-Carboxy-5-nitrophenylboronic acid] in the presence ARS (0.1 mM) and 3-Carboxy-5-nitrophenylboronic acid (0-4 mM) in PBS at pH7.4 with data being taken at 460 nm. (**D**) Standard curve between 1/ΔA and 1/[4-Carboxy-phenylboronic acid] in the presence ARS (0.1 mM) and 4-Carboxy-phenylboronic acid (0-4 mM) in PBS at pH7.4 with data being taken at 460 nm.



A: PEG^{5k}-NBA$_4$-CA$_8$ 0.025 mM + ARS
B: PEG^{5k}-BA$_4$-CA$_8$ 0.025 mM + ARS
C: PEG^{5k}-NBA$_4$-CA$_8$ 0.25 mM + ARS
D: PEG^{5k}-BA$_4$-CA$_8$ 0.25 mM + ARS

Figure S-4. Color changes of ARS (0.1 mM) with different concentrations of PEG^{5k}-NBA$_4$-CA$_8$ and PEG^{5k}-BA$_4$-CA$_8$.



Non-Fluorescent ARS

Fluorescent ARS Complex

Scheme S-4. Alizarin Red S (ARS), which fluoresces only when present as a boronate complex[4]. The complex between ARS and 3-Carboxy-5-nitrophenylboronic acid was shown as an example.

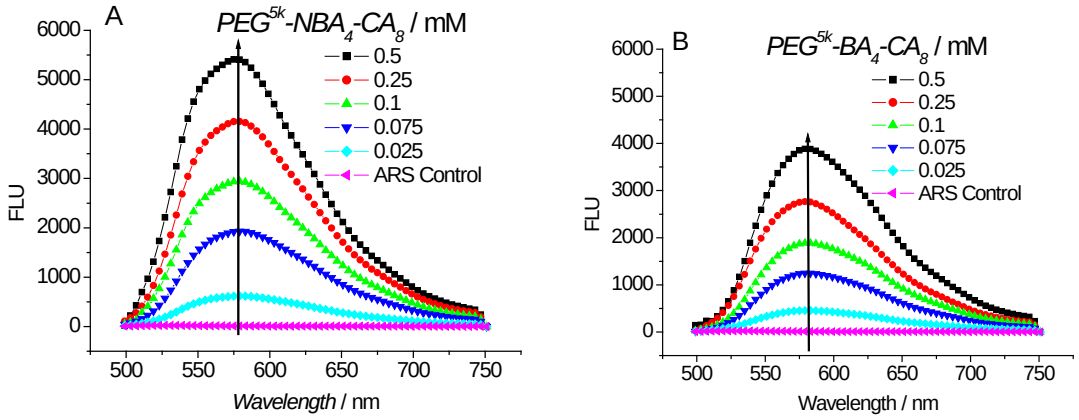


Figure S-5. Fluorescence spectra for the complexes formed between ARS (0.1 mM) and (**A**) PEG^{5k}-NBA$_4$-CA$_8$ (0-0.5 M) or (**B**) PEG^{5k}-BA$_4$-CA$_8$ (0-0.5M) in PBS at pH7.4. Excitation: 468 nm.

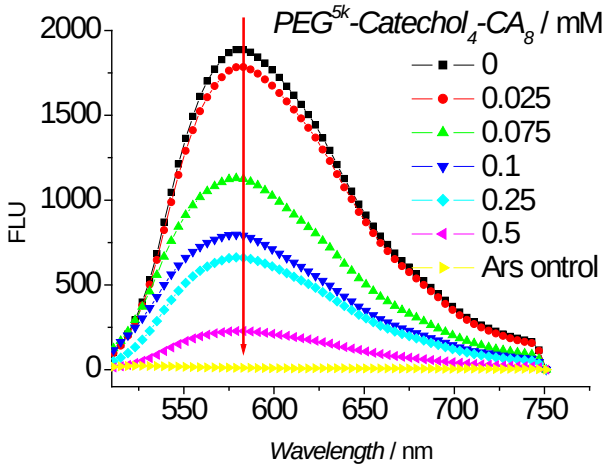
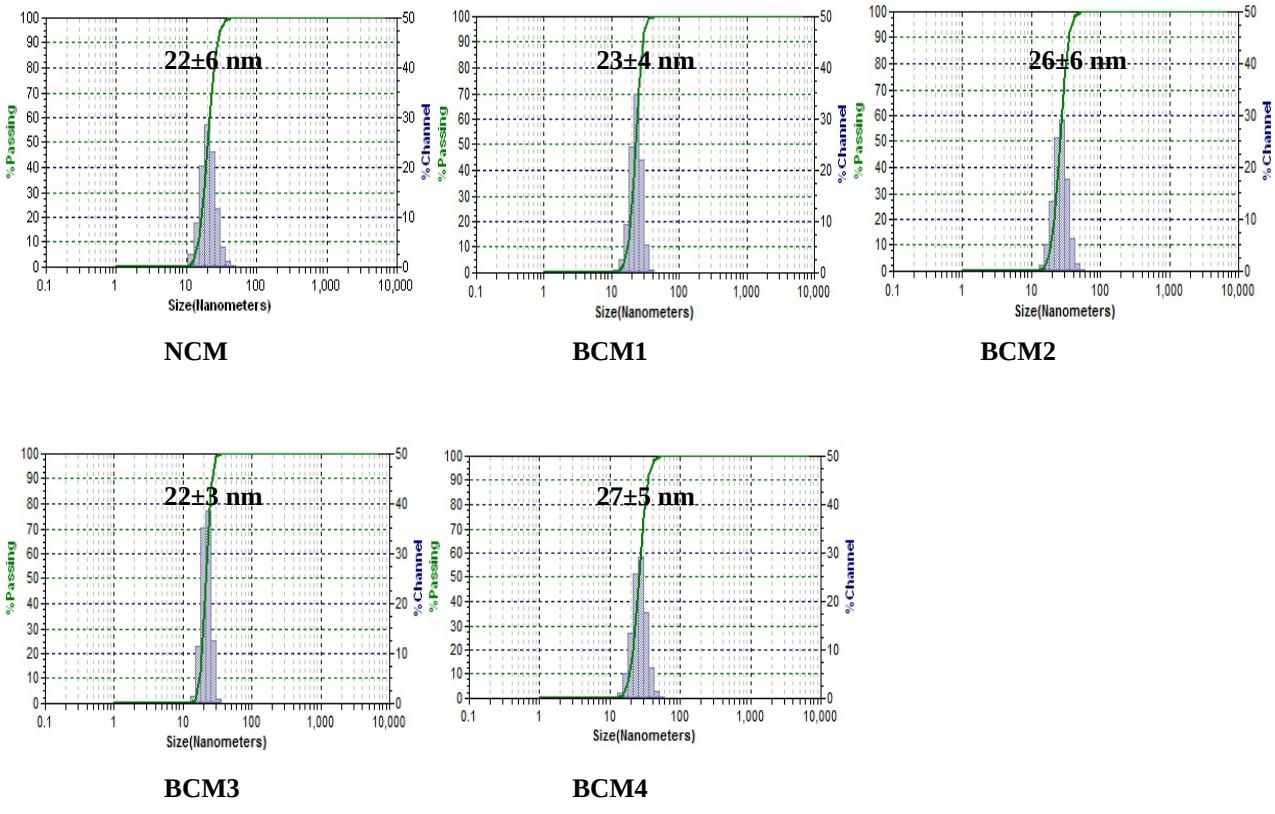


Figure S-6. The fluorescent intensity of ARS (0.1 mM) upon mixing with micelles formed by PEG^{5k}-BA_4-CA_8 (0.1 mM) with different ratios of PEG^{5k}-$Catechol_4$-CA_8 (0-0.5 mM) in PBS at pH7.4.Excitation: 468 nm.



NCM **BCM1** **BCM2**

BCM3 **BCM4**

Figure S-7. The particle size of NCM, BCM1, BCM2, BCM3 and BCM4, measured by dynamic light scattering (Microtrac®).

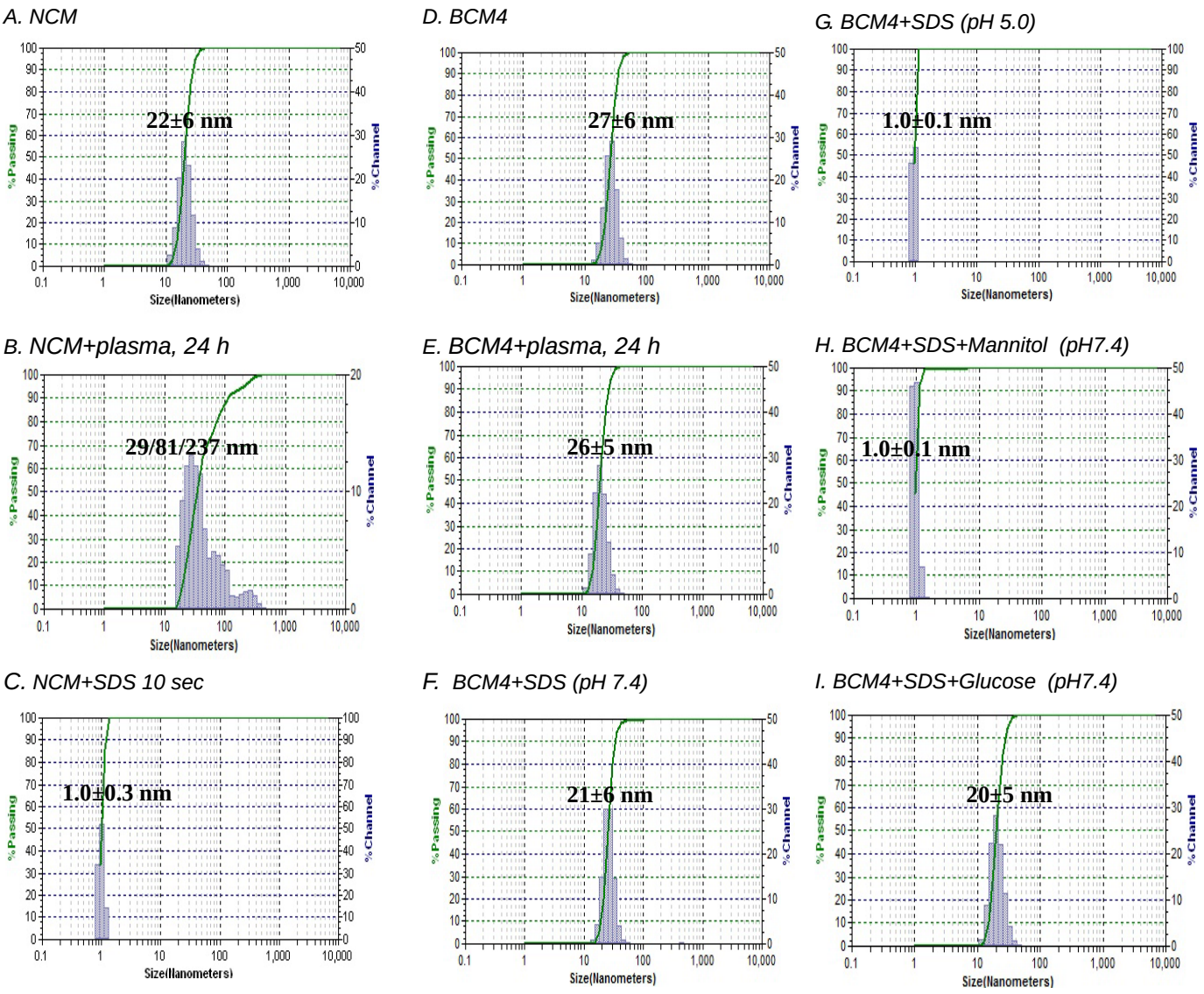


Figure S-8. The particle size of NCM in the absence (**A**) and in the presence (**B**) of plasma 50% (v/v) for 24 h; The particle size of NCM in the presence of 2.5 mg/mL SDS for 10 sec (**C**);The particle size of BCM4 in the absence (**D**) and in the presence of plasma 50% (v/v) for 24 h (**E**);The particle size of BCM4 in the presence of 2.5 mg/mL SDS for 120 min (**F**);The particle size of BCM4 in SDS for 120 min and then adjusted the pH of the solution to 5.0 for 20 min (**G**), The particle size of BCM4 in SDS for 120 min and then treated with mannitol (100 mM) for 20 min (**H**) The particle size of BCM4 in SDS for 120 min and then treated with glucose (100 mM) for 20 min. The particle size was measured by dynamic light scattering (Microtrac).The concentration of micelles was kept at 1.0 mg/mL in PBS.

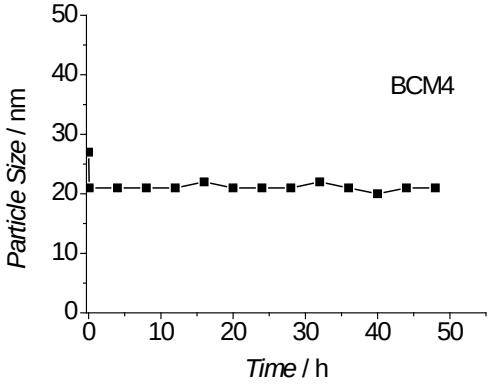


Figure S-9. Continuous particle size measurements of BCM4 in the presence of 2.5 mg/mL SDS at pH 7.4 via DLS. The concentration of micelles was kept at 1.0 mg/mL.

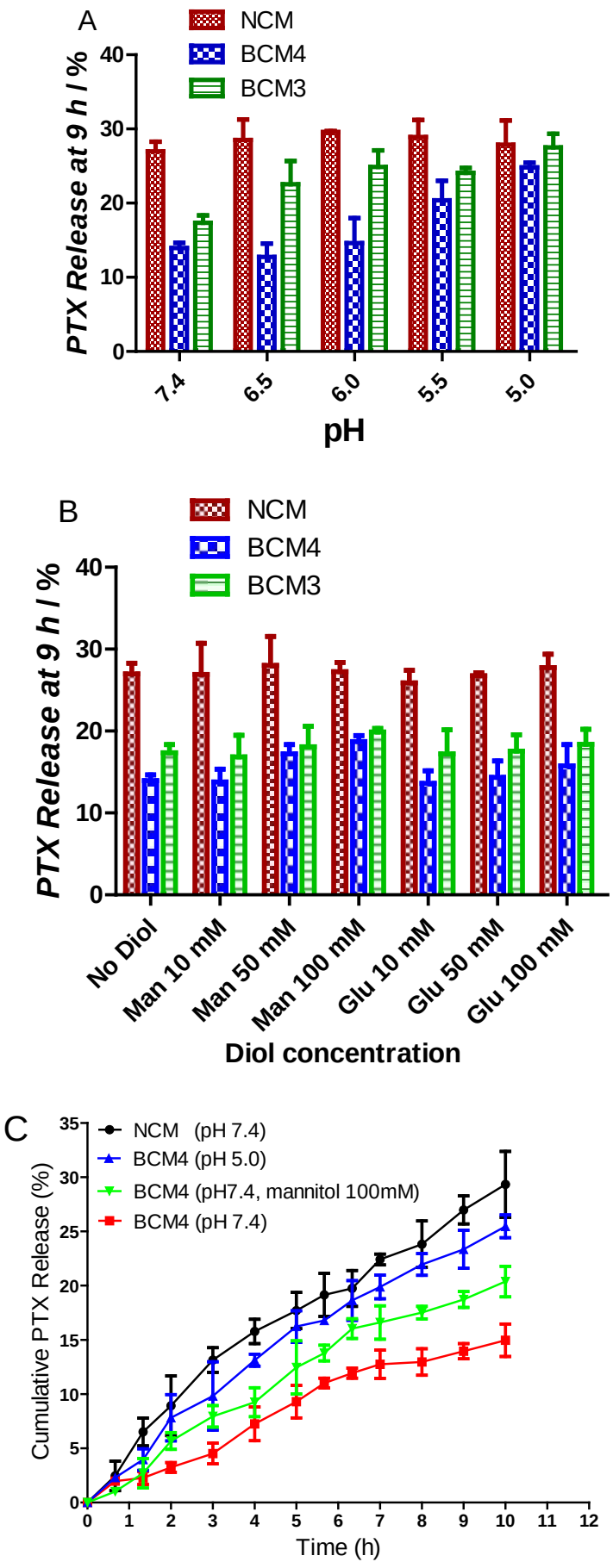


Figure S-10. PTXrelease from NCM, BCM3 and BCM4 at 9 h (**A**) at different pH levels and (**B**) in the presence of different concentrations of mannitol (Man) or glucose (Glu). (**C**) Cumulative PTX release profiles of BCM4 at different pH levels (5.0 and 7.4) and in the presence of mannitol (100 mM) compared with that of NCM at pH7.4.

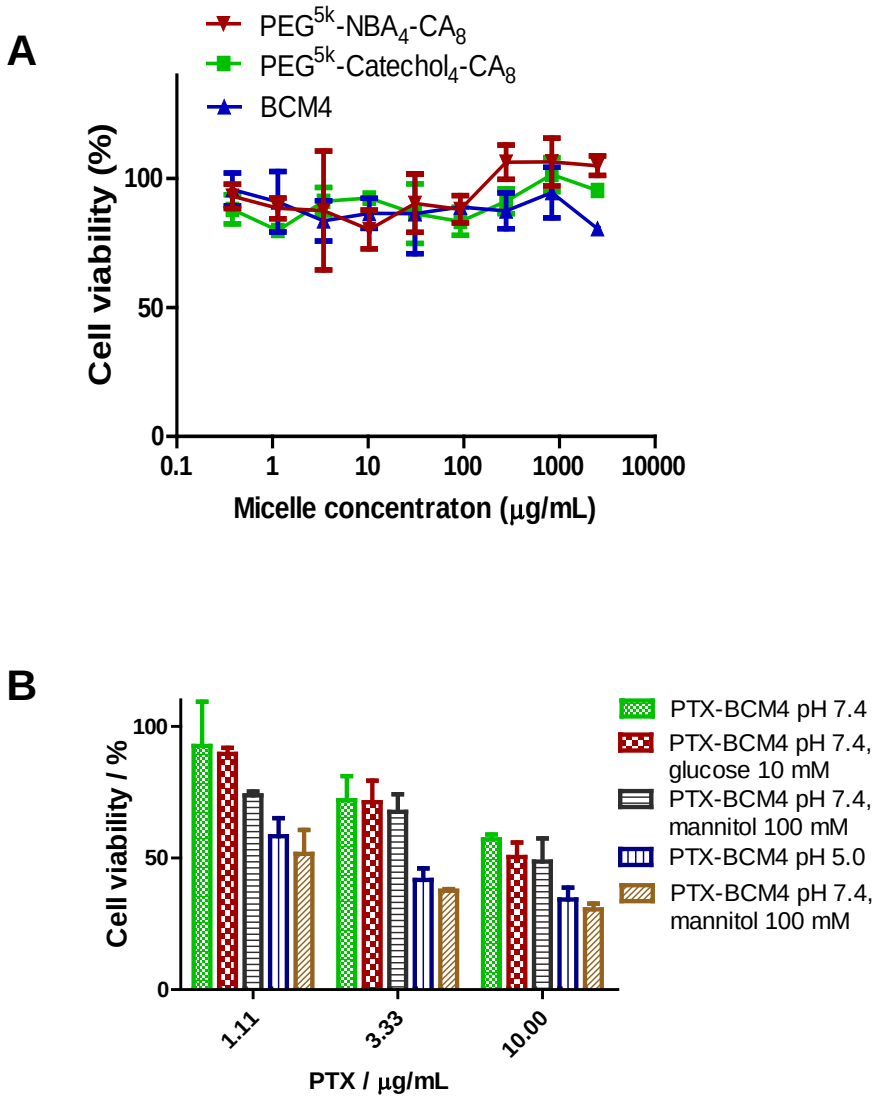


Figure S-11. (A) MTT assays showing the viability of SKOV-3 cells after 72 h incubation with different concentrations of PEG^{5k}-NBA_4-CA_8, PEG^{5k}-$Catechol_4$-CA_8 and empty BCM4. (**B**) MTT assays showing the viability of SKOV-3 cells after 1hr incubation with PTX-BCM4 at pH 7.4, PTX-BCM4 with 10 mM glucose at pH 7.4, PTX-BCM4 with 100 mM mannitol at pH 7.4, PTX-BCM4 at pH 5.0 and PTX-BCM4 with 100 mM mannitol at pH 5.0, followed by 3 times wash with PBS and additional 23hr incubation.

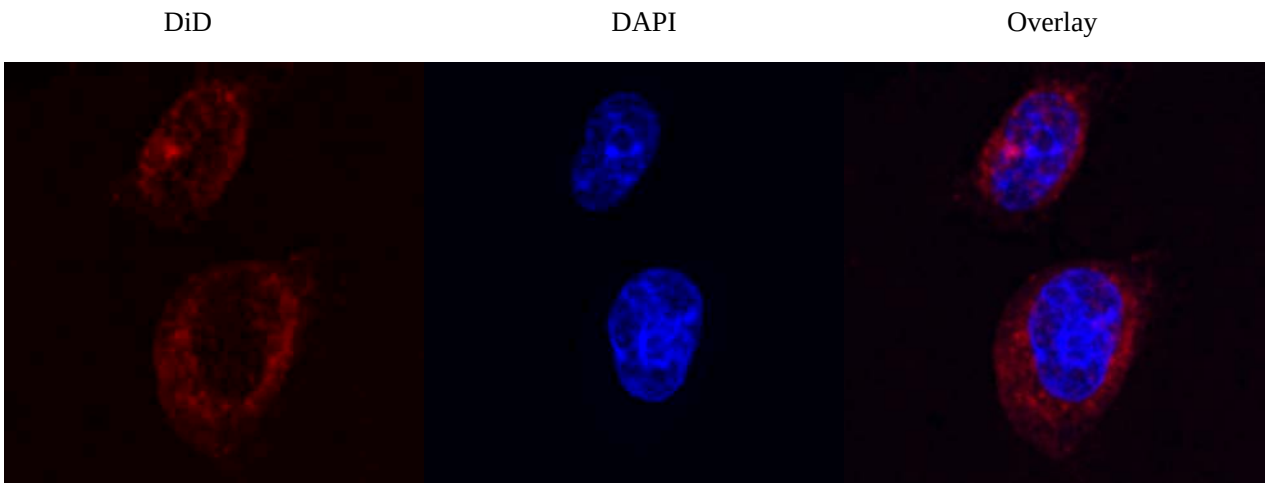


Figure S-12. Cellular uptake of DiD-labeled BCM4 in SKOV-3 ovarian cancer cells after 1hr incubation time, observed by confocal laser scanning microscope. The cell nuclei were stained with DAPI.

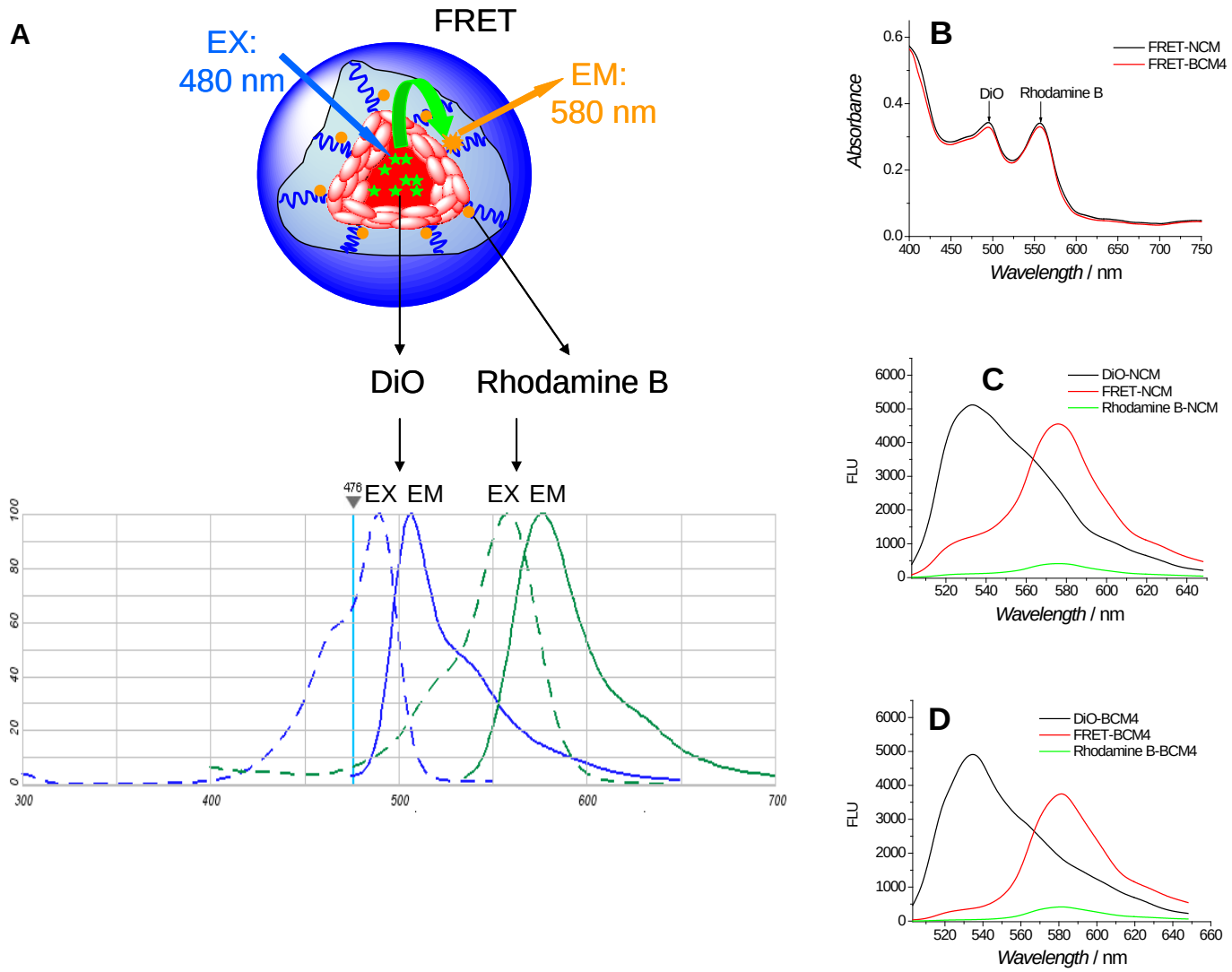


Figure S-13. (**A**) Schematic representation of a FRET micelle conjugated with rhodamine B and loaded with DiO. (**B**) Absorbance spectra of 2.0 mg/mL non-crosslinked FRET micelles (FRET-NCM) (black line) and 2.0 mg/mL FRET BCM4 micelles (FRET-BCM4) (red line) in PBS, indicating the identical dye contents of the two kinds of micelles. (**C**) Fluorescence spectra of NCM with DiO alone (DiO loading: 2.5%) (black line), NCM with rhodamine B alone (rhodamine B conjugated PEG^{5k}-CA_8: 5.0 mg) (green line), and FRET-NCM (DiO loading: 2.5%, rhodamine B conjugated PEG^{5k}-CA_8: 5.0 mg) (red line) in PBS with 480 nm excitation. (**D**) Fluorescence spectra of BCM4 with DiO alone (DiO loading: 2.5%) (black line), BCM4 with rhodamine B alone (rhdamine B conjugated PEG^{5k}-NBA_4-CA_8: 5.0 mg) (green line), and FRET-BCM4 (DiO loading: 2.5%, rhodamine B conjugated PEG^{5k}-NBA_4-CA_8: 5.0 mg) (red line) in PBS with 480 nm excitation. All the micelles were prepared with 20 mg total telodendrimers as described in Section 1.10. Prior to the fluorescence measurements, all the micelles solutions were diluted to 0.1 mg/mL in PBS.

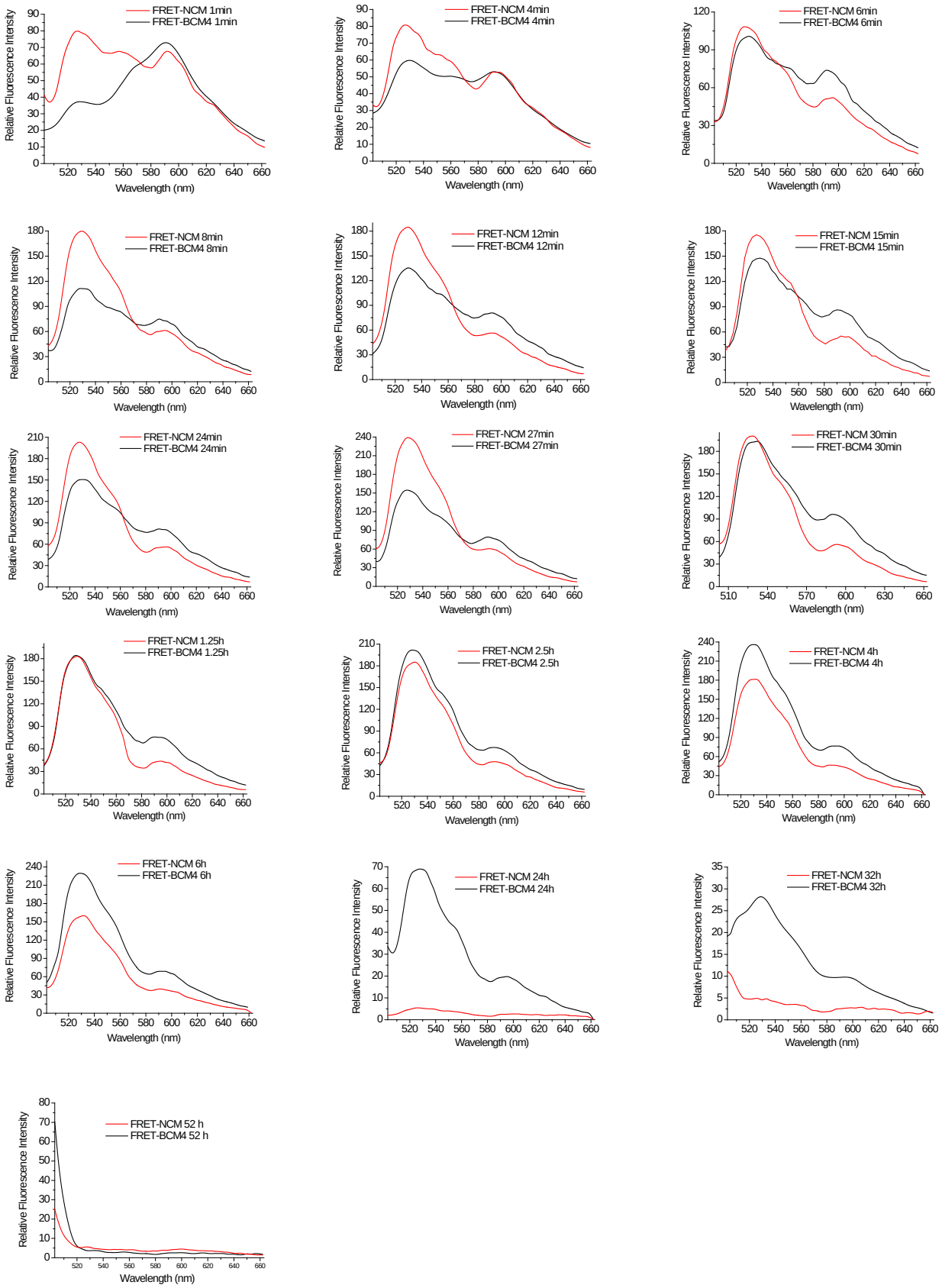


Figure S-14. Representative fluorescence spectra of FRET-NCM (red line) and FRET-BCM4 (black line) in blood at different time points after intravenous injection of 100 μL FRET micelles (2.0 mg/mL) in nude mice. Excitation: 480 nm.

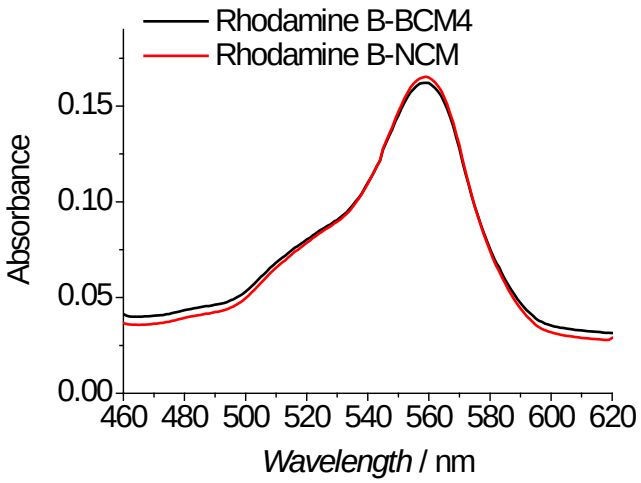


Figure S-15. Absorbance spectra of rhodamine B labeled NCM (red line) and BCM4 (black line) in PBS, indicating the identical dye contents.

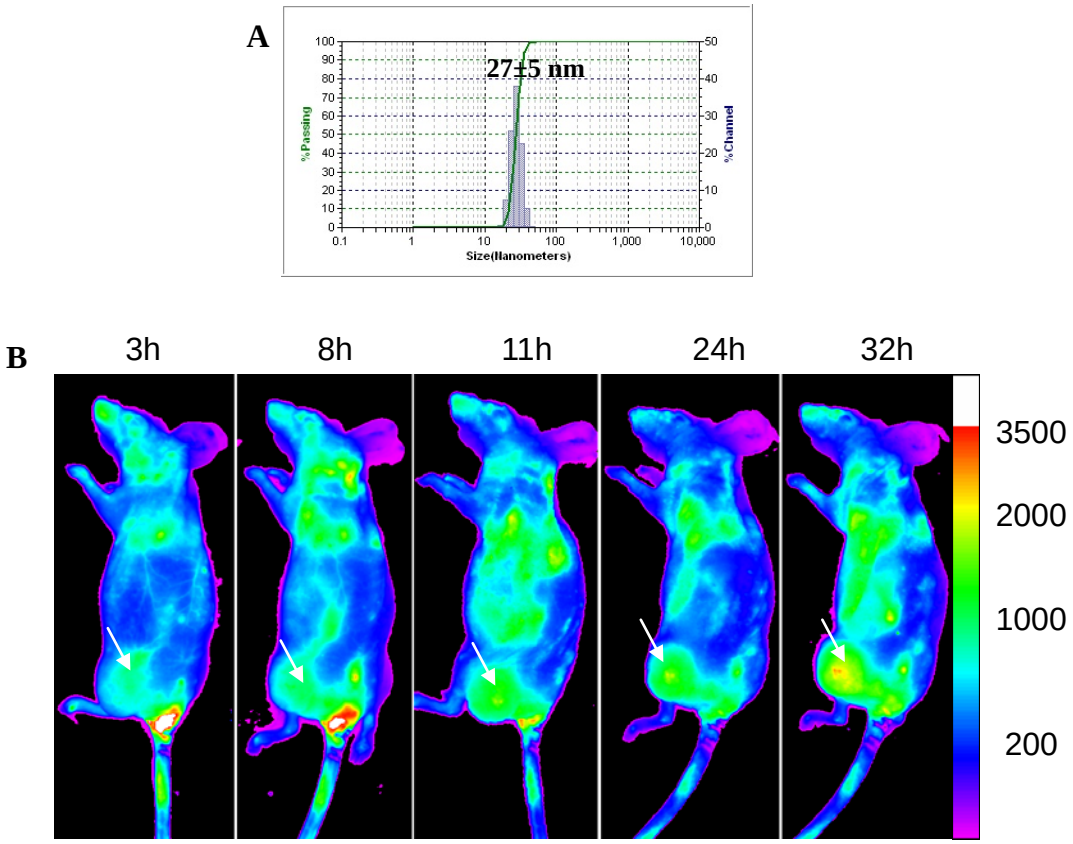


Figure S-16. (**A**)The DLS size distribution of DiD and PTX co-loaded BCM4 (the concentrations of DiD and PTX were both 0.5 mg/mL). (**B**) *In vivo* near infra-red fluorescence (NIRF) optical imaging of SKOV-3 xenograft bearing mouse obtained with Kodak imaging system at different time points after i.v. injection of BCM4 co-loaded with PTX and DiD. A significant contrast of fluorescence signal was observed between tumor and background at 3 hr after administration and sustained up to 32 hr. A relatively high uptake in the liver was observed compared to other organs, which is likely attributed to the nonspecific clearance of nanoparticles by the reticuloendothelial system (RES). Such liver uptake can probably be significantly decreased by introducing a carboxyl group (negative charge) to the distal terminus of the PEG of each telodendrimer as demonstrated in our previous study[9]. We have previously demonstrated the non-crosslinked PEG^{5k}-CA_8 micelles could preferentially accumulate in tumor due to EPR effect. However, NIRF optical imaging is not quantitative enough to distinguish the difference in the tumor uptake levels of BCM and NCM (comparison data was not shown). In contrast, fluorescence uptake of injected free DiD dye into the tumor was not seen[2].

3. References

[1] J. Luo, K. Xiao, Y. Li, J. S. Lee, L. Shi, Y. H. Tan, L. Xing, R. Holland Cheng, G. Y. Liu, K. S. Lam, *Bioconjug Chem* **2010**, *21*, 1216-1224.

[2] K. Xiao, J. Luo, W. L. Fowler, Y. Li, J. S. Lee, L. Xing, R. H. Cheng, L. Wang, K. S. Lam, *Biomaterials* **2009**, *30*, 6006-6016.

[3] aC. Longstaff, M. E. Rose, *Org Mass Spectrom* **1982**, *17*, 508-518; bB. C. J, W. Zhang, L. S. W, *Anal Chem* **2011**, *83*, 3548-3554.

[4] G. Springsteen, B. Wang, *Chem Commun* **2001**, 1608-1609.

[5] G. Springsteen, B. H. Wang, *Tetrahedron* **2002**, *58*, 5291-5300.

[6] aT. F. Yang, C. N. Chen, M. C. Chen, C. H. Lai, H. F. Liang, H. W. Sung, *Biomaterials* **2007**, *28*, 725-734; bM. Jones, J. Leroux, *Eur J Pharm Biopharm* **1999**, *48*, 101-111; cY. Li, S. Pan, W. Zhang, Z. Du, *Nanotechnology* **2009**, *20*, 065104.

[7] A. N. Koo, H. J. Lee, S. E. Kim, J. H. Chang, C. Park, C. Kim, J. H. Park, S. C. Lee, *Chemical Communications* **2008**, 6570-6572.

[8] Y. Li, K. Xiao, J. Luo, W. Xiao, J. S. Lee, A. M. Gonik, J. Kato, T. A. Dong, K. S. Lam, *Biomaterials* **2011**, *32*, 6633-6645.

[9] K. Xiao, Y. Li, J. Luo, J. S. Lee, W. Xiao, A. M. Gonik, R. G. Agarwal, K. S. Lam, *Biomaterials* **2011**, *32*, 3435-3446.