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Supplementary Information for

Complementary interaction with peptide amphiphiles guides size-controlled assembly of small molecules for intracellular delivery

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Table of Contents

1. Materials and methods

Materials.

Cyanuric acid, ethyl 6-bromohexanoate, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 2,6-diamino4-chloro-[1,3,5]triazine, 4-Chloro-7-nitrobenzofurazan (NBD-Cl), Anti-Integrin β1 antibody produced in rabbit were purchased from Sigma Aldrich (St. Louis, MO, USA). Fmoc-Asp(OtBu)-Alko Resin, Fmoc-Gly-OH, Fmoc-Arg(pbf)-OH, Fmoc-Glu(OtBu)-OH・H_2O, Fmoc-Phe-OH, Fmoc-Acp(6)-OH, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxy-1H-benzotriazole monohydrate (HOBt), diisopropylethylamine (DIPEA), piperidine trifluoroacetic acid (TFA), ethanol (EtOH) were obtained from Watanabe Chemical Industries (Hiroshima, Japan). Reagents for Kaiser Test was purchased from Kokusan Chemical (Tokyo, Japan). N,N-dimethylformamide (DMF) and sodium hydroxide (NaOH), hydrochloric acid (HCl) were obtained from Kishida Chemical (Osaka, Japan). Methanol, dichloromethane, diethyl ether, acetonitrile, acetic acid, triethylamine, and sodium hydrogen carbonate ($NaHCO_3$), Acetonitrile (ACN) were obtained from FUJIFILM Wako Pure Chemical (Osaka, Japan). 1,2-Bis(2-aminoethoxy)ethane, di-tert-butyl dicarbonate (Boc_2O) were purchased from Tokyo Chemical Industry (Tokyo, Japan).

Methods.

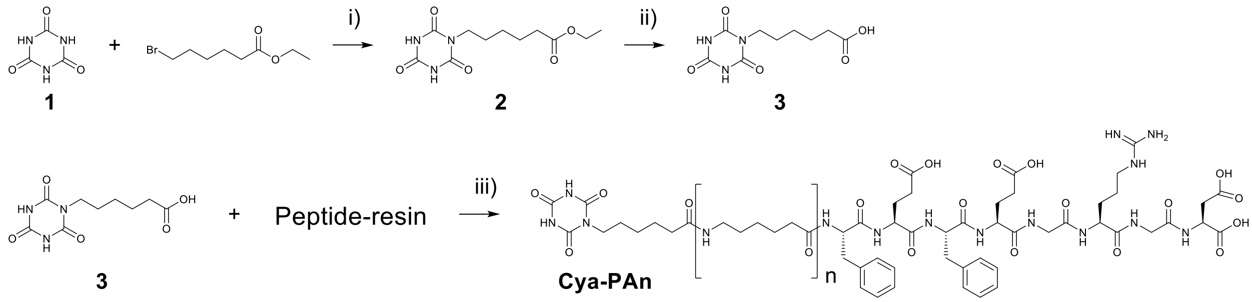
MALDI TOF MS was measured with Auto flex-Ⅲ (Bruker, Billerica, MA, USA) using dithranol or α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix. ^{1}H NMR spectra were recorded on a 300 MHz AV300M (Bruker) or on a 400 MHz ECZ400 (JEOL, Tokyo, Japan) for Mel-NBD or Cya-PA, respectively. Dimethylsulfoxide (DMSO)-d_6 (Wako) was used as the solvent. Chemical shifts were reported as δ/ppm relative to tetramethyl silane (δ/ppm = 0). Fourier-transform infrared (FT-IR) spectra were recorded on a Spectrum Two (PerkinElmer, Waltham, MA, USA).

Cya-PA/Mel-NBD assemblies were formed and lyophilized overnight to obtain powders that were used for the analysis. Resolution of 2 cm^{-1} was used. Dynamic light scattering (DLS) measurements were conducted using a Nano-ZS (Malvern Panalytical, Worcestershire, UK). Morphological observation was conducted by transmission electron microscopy (TEM) using a JEM-2010 (JEOL) or scanning probe microscopy (SPM) using a DimensionIcon (Bruker). For TEM, aggregates (Cya-PA1/Mel-NBD or Cya-PA2/Mel-NBD, 1.0 mM each) and control samples (Cya-PA or Mel-NBD alone) were drop-cast onto a STEM grid with an elastic carbon film (Okenshoji, Tokyo, Japan). The samples were stained with 2% uranyl acetate, dried in vacuo, and imaged at an accelerating voltage of 120 kV. For SPM, samples prepared at 1.5 mM were diluted to 0.5 mM using PBS and drop-cast onto freshly cleaved mica substrate, washed with water three times, and dried under ambient conditions. It was imaged using PeakForce Tapping mode with a SCANASYST-AIR probe (Bruker). Confocal fluorescence images were taken using a Zeiss LSM700 microscope (Carl Zeiss, Oberkochen, Germany) with diode lasers (405 nm for Hoechst 33342 and 488 nm for NBD). Flow cytometric analysis was conducted with an EC800 (Sony, Tokyo, Japan).

Cell culture.

HeLa cells were purchased from Riken Cell Bank (Ibaraki, Japan). MEM GlutaMAX™ Supplement (MEM, Thermo Fisher Scientific) supplemented with 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific) and Antibiotic-Antimycotic (Thermo Fisher Scientific) was used as a medium. Cells were cultured on 10 cm TC dish (Greiner bio-one, Frickenhausen, Germany) and incubated in a 5% CO_2 incubator (MCO-5 AC (UV), SANYO Electric) at 37 °C.

2. Synthesis of Cya-PA



Scheme S1. Synthesis of Cya-PA. Reagents and conditions: i) DBU, dry DMF, 70 °C, 4 h then r.t., 11 h; ii) EtOH, Water, NaOH, r.t., 19.5 h; iii) HBTU, HOBt, DIPEA, TFA, TIPS, H_2O.

Compound 2.[S1]

Cyanuric acid (**1**) (6.5 g, 50 mmol) was added to a 300 mL three-necked flask and purged with nitrogen by three degassing operations. Subsequently, dry DMF (50 mL) and ethyl 6-bromohexanoate (1.8 mL, 10 mmol) were added using a syringe. DBU (1.6 mL) was then slowly added dropwise over 13 min and the mixture was stirred at 70 °C for 4 h. After disappearance of the raw materials was confirmed by thin layer chromatography (TLC), the heating was stopped and the mixture was stirred at room temperature (r.t.) for 11 h. The solution was filtered under reduced pressure to remove insoluble materials. Subsequently, DMF in the filtrate was removed by distillation under reduced pressure. DCM:MeOH = 9:1 (150 ml) was added and the resulting white precipitate was removed by vacuum filtration. The crude product was purified by silica gel column chromatography (WAKO C-300, DCM: MeOH = 19: 1) to give 2.2 g of Compound **2** (yield 72%).

^{1}H NMR (DMSO-*d6*, 400 MHz, δ/ppm) 1.17 (t, 3H, CH_3), 1.23-1.27 (m, 2H, CH_2), 1.46-1.54 (m, 4H, CH_2), 2.27 (t, 2H, CH_2), 3.61 (t, 2H, CH_2), 4.04 (q, 2H, CH_2), 11.22-11.58 (br, 1H, NH).

Compound 3.

Compound **2** (0.92 g, 3.0 mmol), EtOH (5.0 mL), distilled water (4.4 mL) and 10 M NaOH aq. (0.27 mL) were added to a 50 ml eggplant-shaped flask and stirred at r.t. for 19.5 h. After disappearance of the raw material peak was confirmed by TLC, the reaction was terminated. 1 M HCl aq. (1.4 mL) was added to the reaction mixture to neutralize to pH 6–7. The solvent was evaporated and concentrated under reduced pressure for 1 h to obtain compound **3** as a mixture with NaCl (Fig. S10).

^{1}H NMR (DMSO-d_6, 400 MHz, δ/ppm) 1.23 (t, 2H, CH_2), 1.40-1.55 (m, 4H, CH_2), 2.11 (t, 2H, CH_2), 3.61 (t, 2H, CH_2).

Cya-PA.

Cya-PAn (n = 1, 2) were synthesized by a standard *N*-α-9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide synthesis method in 0.2 mmol scale on the Fmoc-Asp(OtBu)-Alko resin. In brief, the coupling cycle of each amino acid or compound **2** were done by adding a mixture of coupling reagents (Fmoc-amino acid:HBTU:HOBt:DIEA = 3:3:3:6 mol equivalent to reactive sites on resin) in DMF and the reaction was conducted for 1 h at r.t. After coupling reactions, protective Fmoc group was removed using 20% piperidine in DMF. Cya-PA was cleaved from resin using the mixture of TFA:TIS:water = 95:2.5:2.5 (vol:vol:vol) for 2 h. After the removal of the solvents under reduced pressure, the peptides were precipitated and washed with cold diethyl ether. The crude peptide solids were collected and purified by HPLC on Inertsil ODS-3 column (GL science, Tokyo, Japan) using a gradient of water and ACN both containing 0.1% TFA. The fractions with products were collected and lyophilized, and stored at −20°C. The obtained Cya-

PAs were analyzed by HPLC (Inertsil ODS-3 column, GL science) and MALDI TOF MS using CHCA as the matrix.

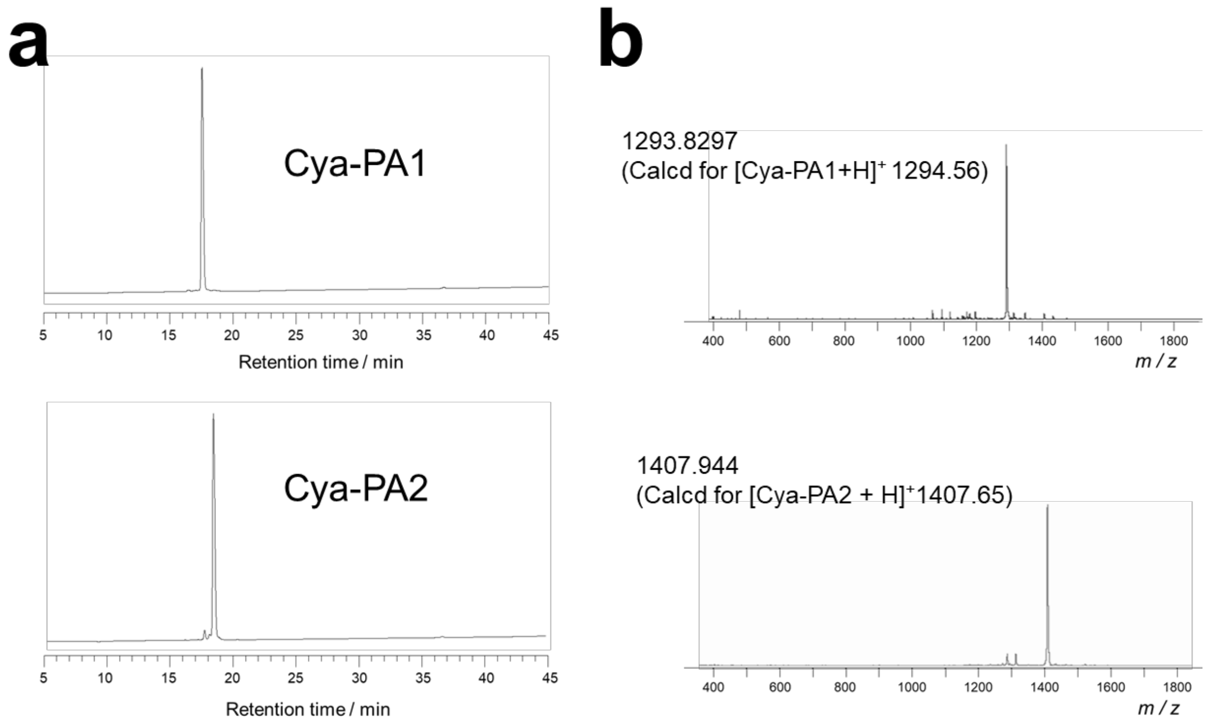
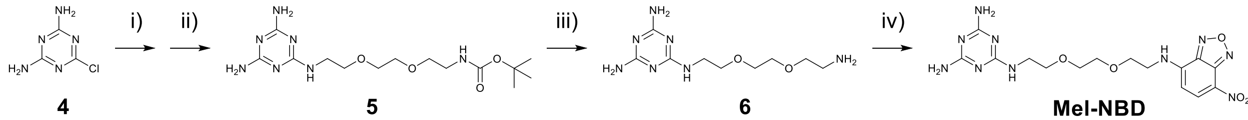


Fig. S1 Characterization of Cya-PA1 (top) and Cya-PA2 (bottom) by HPLC (a) and MALDI TOF MS (b). Conditions: (a) Inertsil ODS-3 (GL science, 4.6mm 250 mm), 20%B to 60%B in 40 min (A: water, B: acetonitrile, both containing 0.1% TFA), 1 mL/min, 220 nm detection. (b) Reflector positive mode using α-CHCA as the matrix.

3. Synthesis of Mel-NBD



Scheme S2. Synthesis of Mel-NBD. Reagents and conditions: i) 1,2-Bis(2-aminoethoxy)ethane 100 °C, 2 h; ii) DMF, Boc_2O, r.t., 3h; (iii) TFA/DCM, r.t., 1h; (iv) NBD-Cl, $NaHCO_3$, 50% ACN aq., r.t., 24h.

Compound 5.[S2]

Compound **4** (2.0 g, 14 mmol) and 1,2-Bis(2-aminoethoxy)ethane (12 mL, 79 mmol) were added to a 50 mL eggplant-shaped flask and stirred at 100 °C for 2 h. After disappearance of the raw material peak was confirmed by TLC, the reaction was terminated. DCM was added to the obtained reaction solution and washing with DCM by decantation. Subsequently, DMF (15 mL) and Boc_2O (11 g, 46 mmol) were added to the residual oil and the mixture was stirred at r.t. for 3 h. After disappearance of the raw material peak was confirmed by TLC, the reaction was terminated. Ethyl acetate was added to the reaction mixture and insoluble materials were filtered off. The obtained filtrate was concentrated under reduced pressure for 5 h to obtain a crude product as a transparent oil. The crude product was purified by silica gel column chromatography (WAKO C-300, DCM: MeOH = 15: 1 and 9: 1) to yield compound **4** as a transparent oil (2.1 g, 41%) (Fig. S11).

^{1}H NMR ($CDCl_3$, 300 MHz, δ/ppm) 1.45 (s, 9H, C*H*$_3$), 3.32 (s, 2H, C*H*$_2$), 3.50-3.71 (m, 10H, C*H*$_2$), 5.30-5.80 (br, 2H, N*H*$_2$), 6.10-6.60 (br, 3H, N*H*&N*H*$_2$), 7.15-7.22 (br, 1H, amide); MALDI TOF MS (positive, dithranol) *m/z* found 358.2 $[M+H]^+$ (Calcd for $C_{14}H_{27}N_7O_4$: 357.2).

Compound 6.[S3]

Compound **5** (0.27 g, 0.75 mmol) and TFA/DCM (6.0 mL, 31.0 mmol, 41 eq./**5**) were added to a 200 mL eggplant-shaped flask and the mixture was stirred at r.t. for 1 h. After disappearance of the raw material peak was confirmed by TLC, the reaction was terminated. The obtained reaction solution was concentrated and evaporated under reduced pressure for 2 h to obtain a crude product containing TFA and DCM, which was used in the next step without further purifications (Fig. S12).

^{1}H NMR (DMSO-d_6, 300 MHz, δ/ppm) 2.97 (q, 2H, CH_2), 3.45 (t, 2H, CH_2), 3.50-3.62 (m, 8H, CH_2), 7.70-7.90 (br, 6H, NH_2), 8.07 (t, 1H, NH); MALDI TOF MS (positive, dithranol) m/z found 258.0 $[M+H]^+$ (Calcd for $C_9H_{19}N_7O_2$: 257.2).

Mel-NBD.[S4]

A crude product **6** obtained above (0.19 g, 0.75 mmol) was added with NBD-Cl (0.15 g, 0.75 mmol), $NaHCO_3$ (0.61 g, 7.3 mmol), and 50% ACN aq. (20 mL), and the mixture was stirred at r.t. for 24 h. After removing insoluble materials by filtration under reduced pressure, the filtrate was concentrated under reduced pressure. Methanol was added, the suspension was filtrated, and the filtrate was concentrated. The resulting crude product was purified by silica gel column chromatography (WAKO C-300, DCM:Methanol = 15:1 and 9:1) to yield Mel-NBD (Fig. S13).

^{1}H NMR (DMSO-d_6, 300 MHz, δ/ppm) 3.55-3.89 (m, 12H, CH_2), 5.90-6.20 (br, 4H, NH_2), 6.31 (t, 1H, NH), 6.47 (d, 1H, Ar-H), 8.51 (d, 1H, Ar-H), 9.15-9.90 (br, 1H, NH); MALDI TOF MS (positive, dithranol) m/z found 421.3 $[M+H]^+$ (Calcd for $C_9H_{19}N_7O_2$: 420.2).

4. FT-IR spectra of Cya-PA/Mel-NBD assemblies

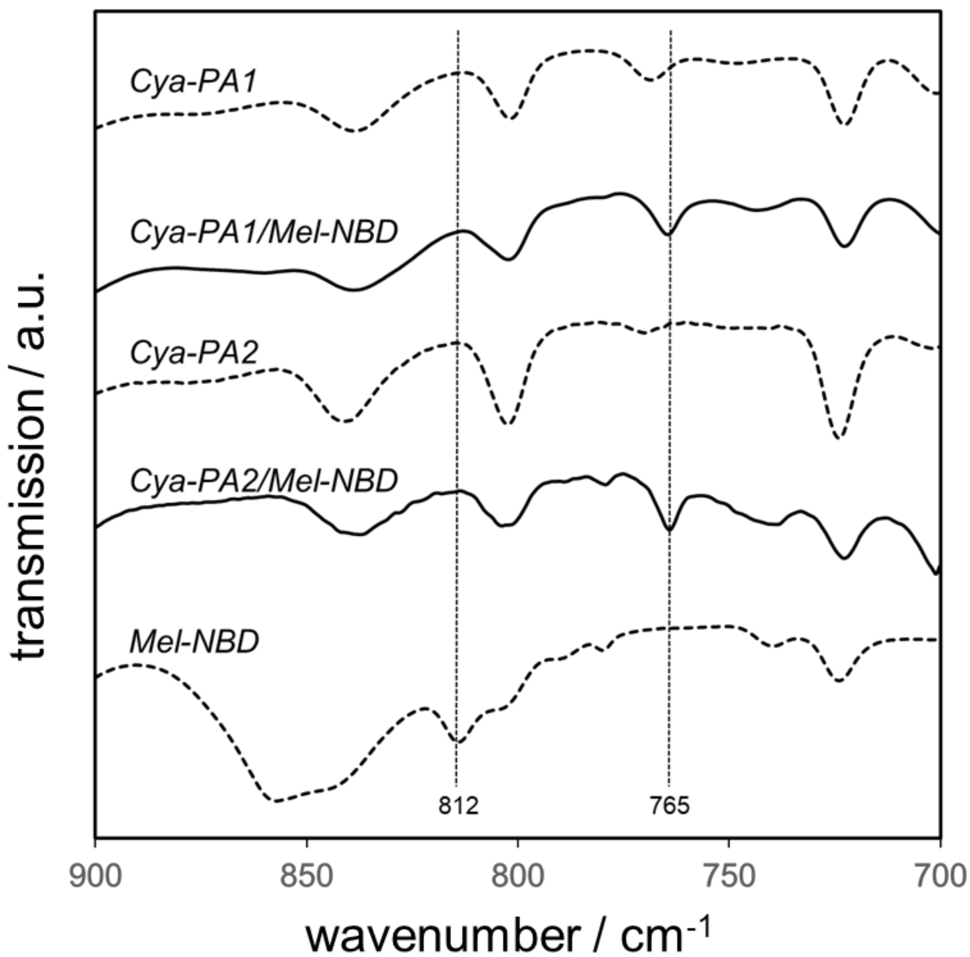


Fig. S2 FT-IR spectral changes upon mixing of Cya-PA and Mel-NBD.

5. Dynamic light scattering measurement

Table S1. Count rates of each sample measured by DLS.

	Cya-PA1	Cya-PA2	Mel-NBD	Cya-PA1/ Mel-NBD	Cya-PA2/ Mel-NBD
Count rates (kcps)	123	353	1 408	5 123	12 594

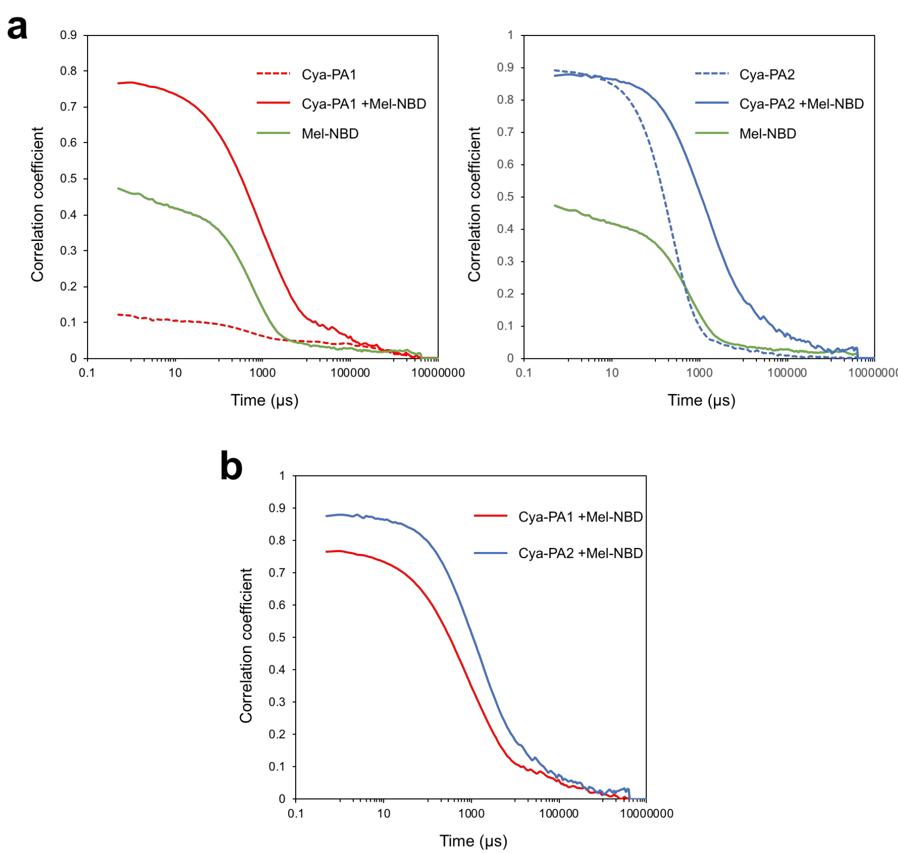


Fig. S3 Correlation coefficient determined by DLS measurement. (a) Cya-PA/Mel-NBD mixtures compared with Cya-PA or Mel-NBD alone. (b) Comparison of Cya-PA1/Mel-NBD with Cya-PA2/Mel-NBD.

6. **Morphological analysis of Cya-PA/Mel-NBD assemblies using transmission electron microscopy**

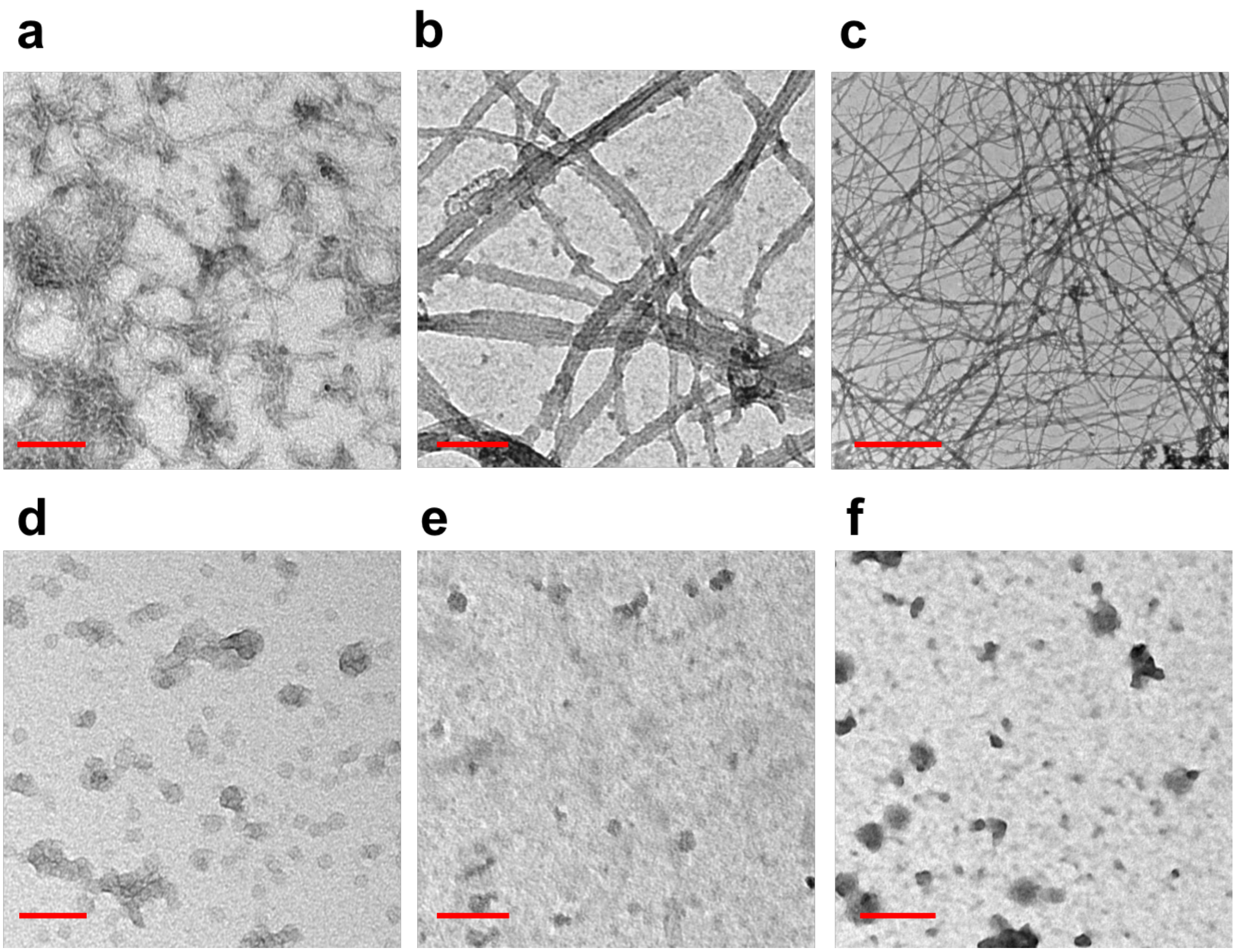


Fig. S4 TEM images of (a) Cya-PA1/Mel-NBD; (b, c) Cya-PA2/Mel-NBD (b (10000x), c (1000x)); (d) Cya-PA1; (e) Cya-PA2; (f) Mel-NBD. Bars: (a, b, d–f) 100 nm, (c) 1 μm.

7. Study on the influence of the mixing ratio in Cya-PA2/Mel-NBD assemblies

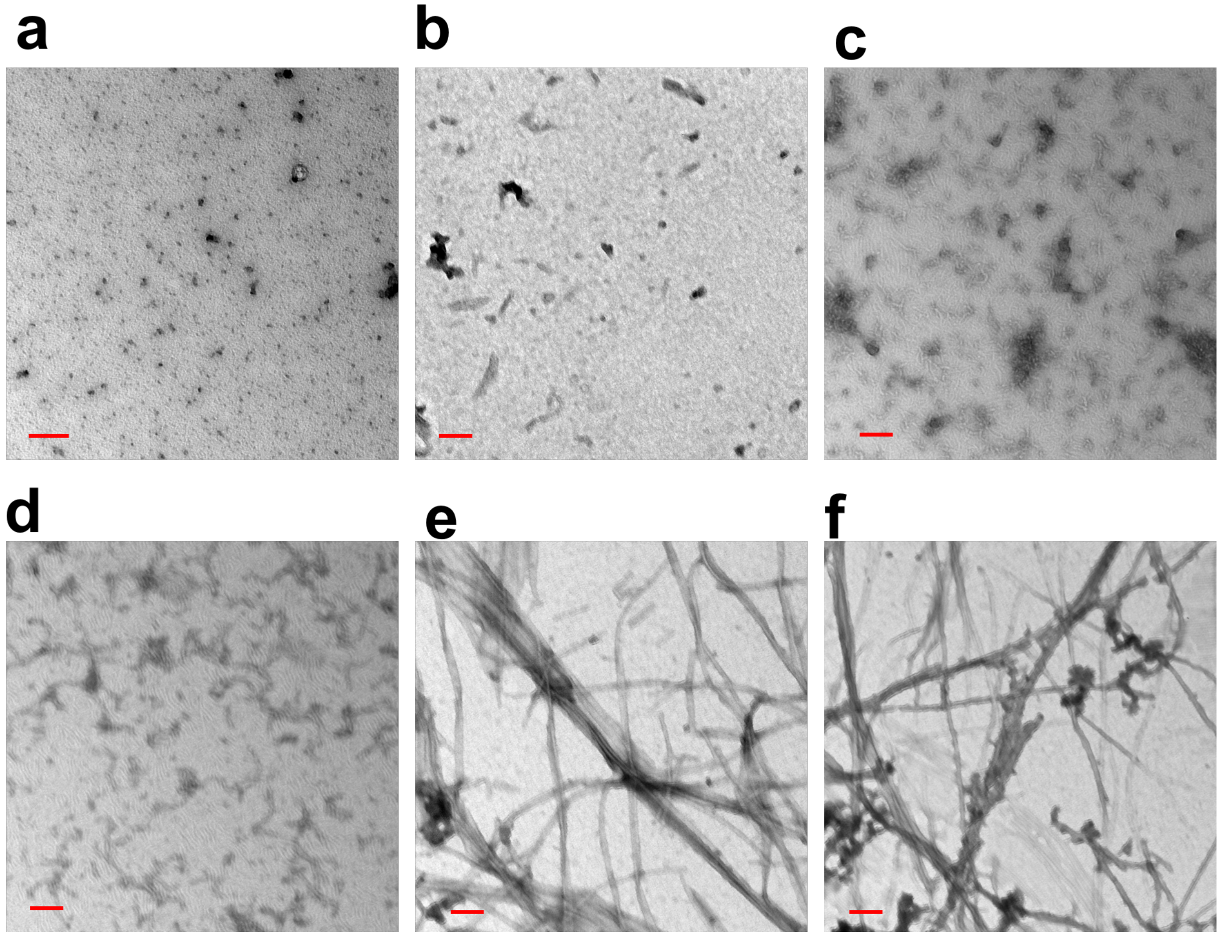


Fig. S5 TEM images of Cya-PA2/Mel-NBD assemblies. [Mel-NBD] = 1 mM and [Cya-PA2] = (a) 0 mM, (b) 0.25 mM, (c) 0.5 mM, (d) 0.75 mM, (e) 1mM and (f) 1.5 mM. Bars: 100 nm.

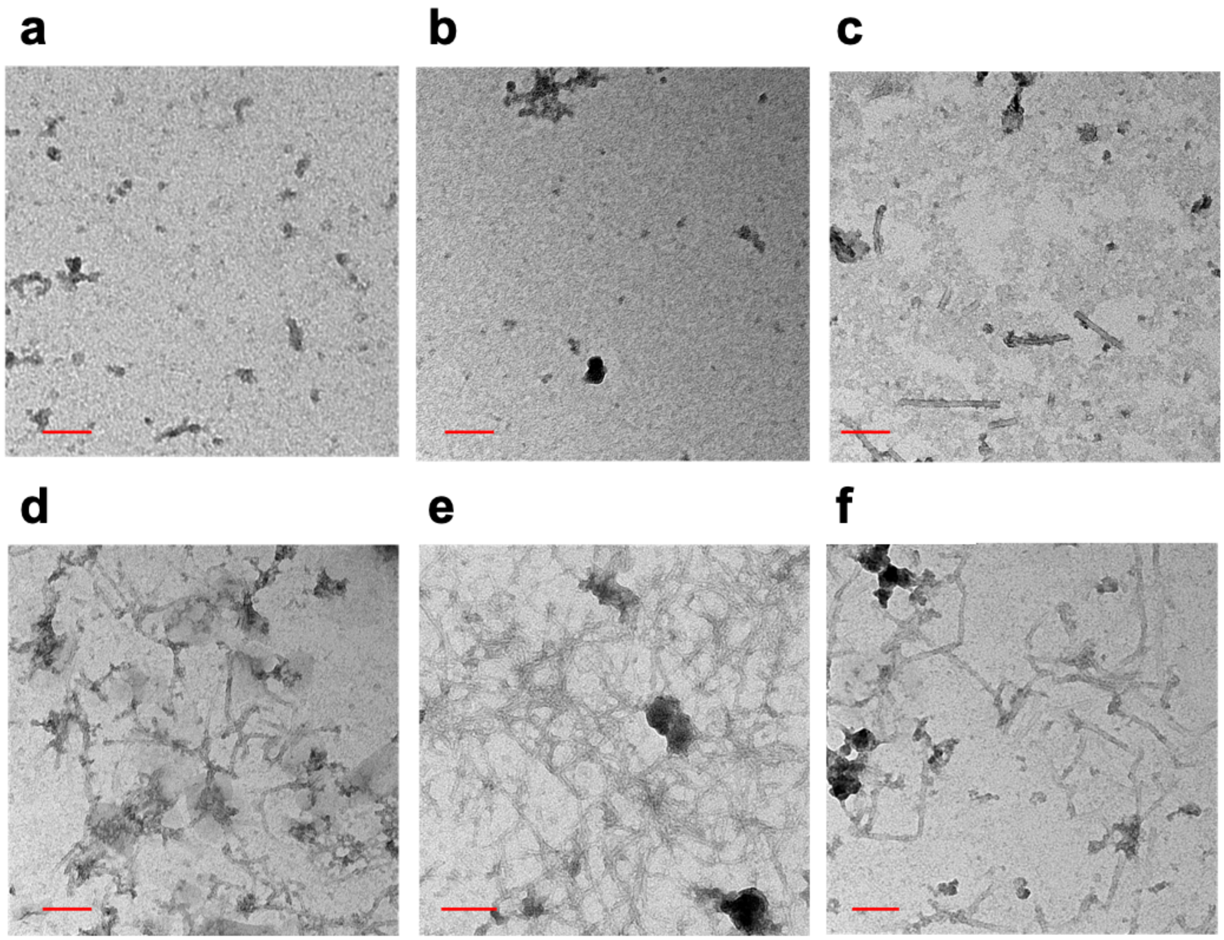


Fig. S6 TEM images of Cya-PA2/Mel-NBD assemblies. [Cya-PA2] = 1 mM and [Mel-NBD] = (a) 0 mM, (b) 0.25 mM, (c) 0.5 mM, (d) 0.75 mM, (e) 1mM and (f) 1.5 mM. Bars: 100 nm.

8. Confocal fluorescence microscope images of Cya-PA2/Mel-NBD assembly

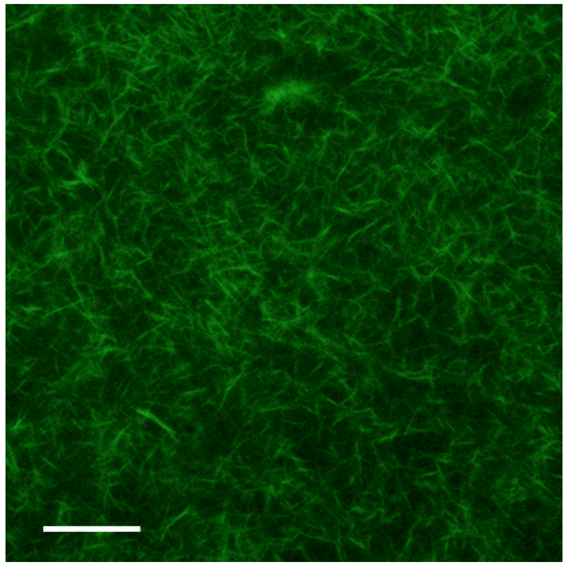


Fig. S7 Confocal microscopic image of Cya-PA2/Mel-NBD assembly ([Cya-PA2] = [Mel-NBD] = 1 mM). Bar: 10 μm.

9. Cytotoxicity evaluation of Cya-PA/Mel-NBD assemblies and each component

HeLa cells (4000 cells/well) were seeded in 96-well microplate (greiner) and incubated for 24 h at 37°C under 5% CO_2 atmosphere. Samples (Cya-PA1/Mel-NBD, Cya-PA2/Mel-NBD, Cya-PA1, Cya-PA2, Mel-NBD) were added at the final concentration of 0.4 mM for each component, incubated for 24 h, and the cell viability was examined by WST assay using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

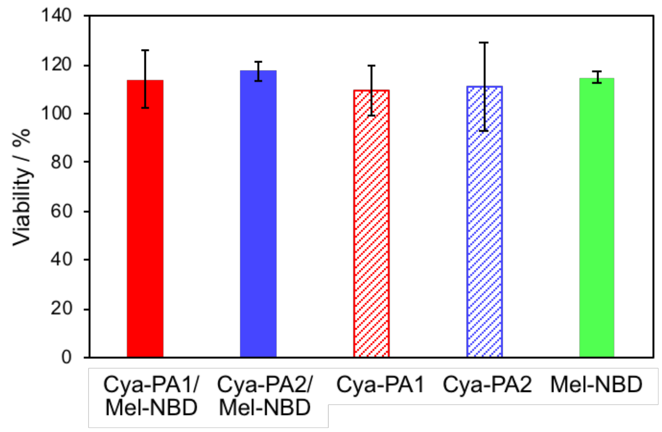


Fig. S8 Cytotoxicity of the materials used in this study. HeLa cells were incubated with Cya-PA1/Mel-NBD, Cya-PA2/Mel-NBD, Cya-PA1, Cya-PA2, and Mel-NBD for 24 h and the cell viability was examined using WST assay.

10. Intracellular localization of Mel-NBD in HeLa cells by 3D confocal fluorescence images

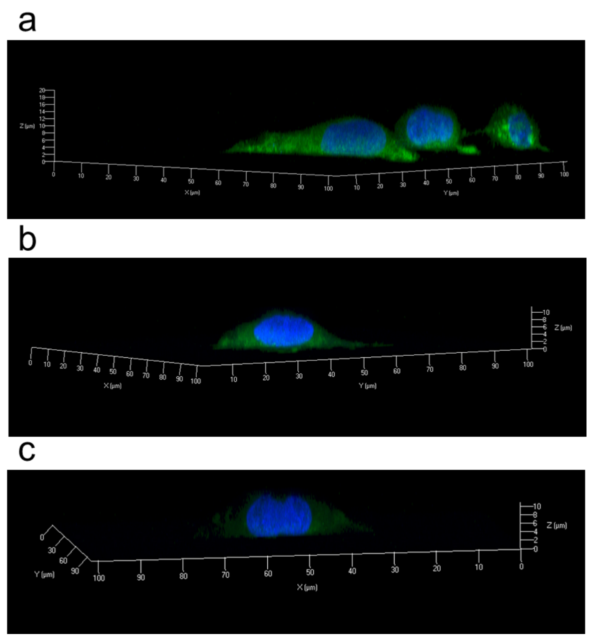


Fig. S9 3D confocal fluorescence images of HeLa cells after treatment with (a) Cya-PA1/Mel-NBD; (b) Cya-PA2/Mel-NBD; (c) Mel-NBD alone. Intracellular localization of Mel-NBD is visualized in green and nuclei are in blue (Hoechst33342).

11. Inhibition of intracellular delivery of Mel-NBD by anti-integrin antibody

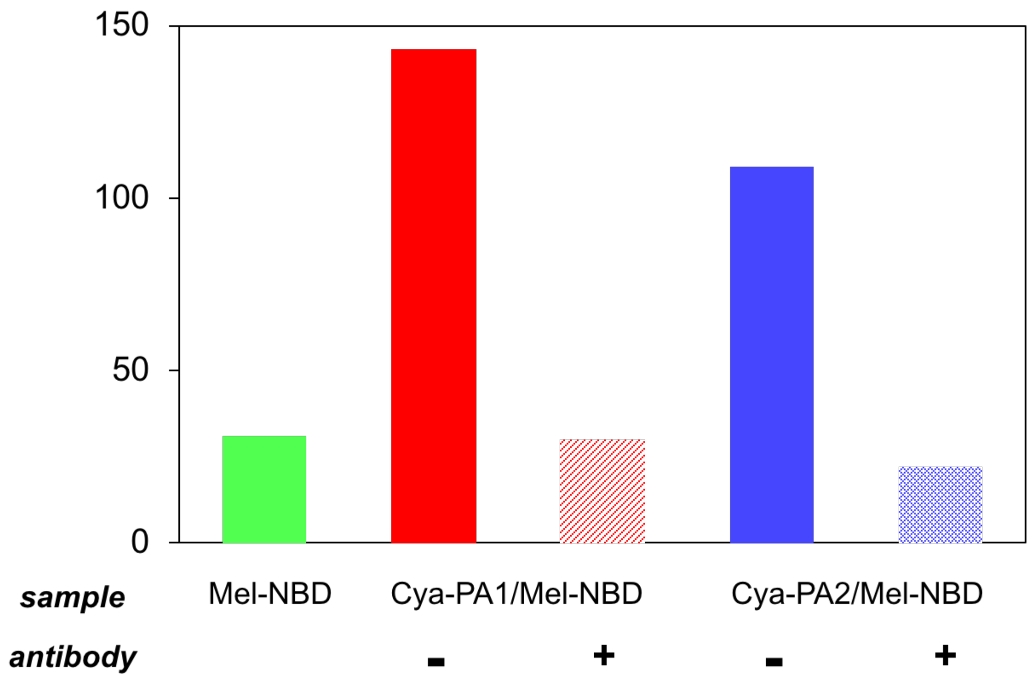


Fig. S10 Flowcytometric analysis of inhibition of intracellular delivery of Mel-NBD by pre-treatment with anti-integrin antibody. HeLa cells (2×10^5 cells) were incubated with rabbit anti-integrin β_1 antibody (Sigma SAB4300655, 50-fold dilution) at r.t. for 1 h, then each Cya-PA/Mel-NBD assembly was added at r.t. for 1 h. Samples were removed and cells were washed with PBS, fixed with 4% PFA, and suspended in 1% FBS/PBS for flow cytometric analysis. As controls, cells without pre-treatment with antibody were analysed.

12. Influence of RGD on the intracellular delivery of Mel-NBD by co-assembly formation

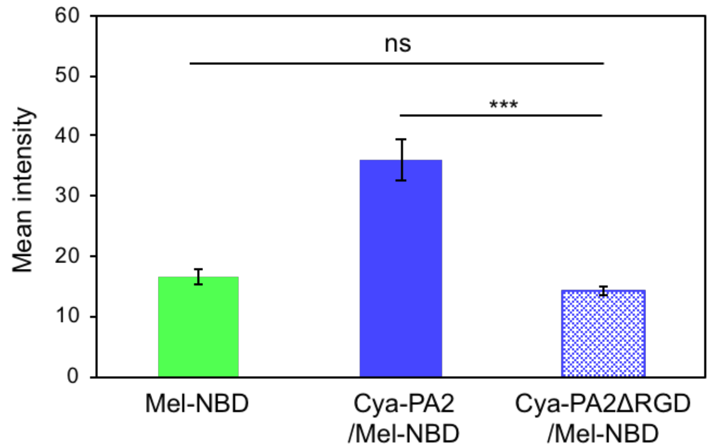


Fig. S11 Intracellular delivery of Mel-NBD by co-assembly formation with Cya-PA2 or Cya-PA2ΔRGD. HeLa cells (2×10^5 cells) were incubated with Cya-PA/Mel-NBD assemblies at r.t. for 1 h. Samples were removed and cells were washed with PBS, fixed with 4% PFA, and suspended in 1% FBS/PBS for flow cytometric analysis. N = 3, mean ± SD, ***$p < 0.001$.

13. NMR spectra of synthesized compound

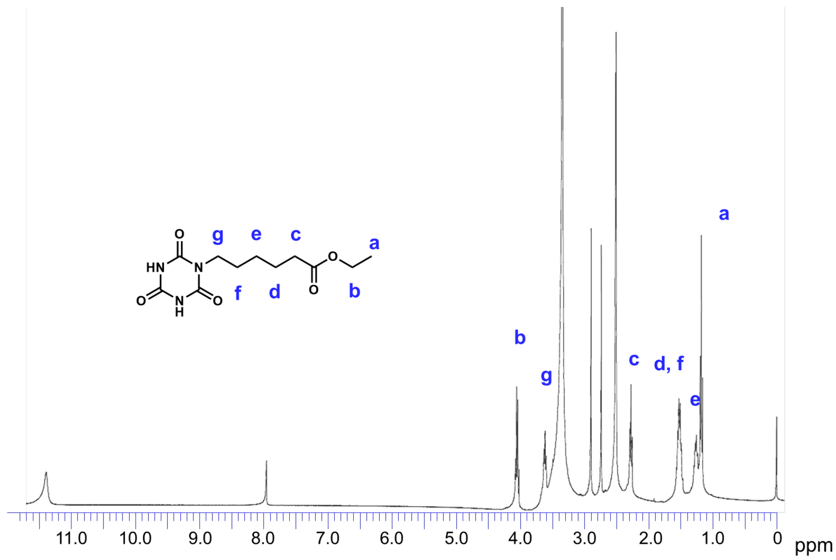


Fig. S12 ^{1}H NMR spectrum of Compound **2** (400 MHz, DMSO-d_6)

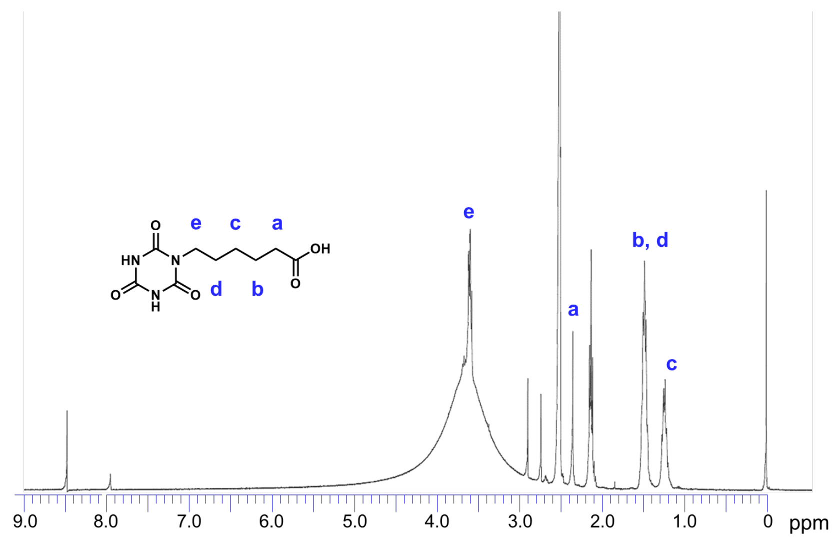


Fig. S13 ^{1}H NMR spectrum of Compound **3** (400 MHz, DMSO-d_6)

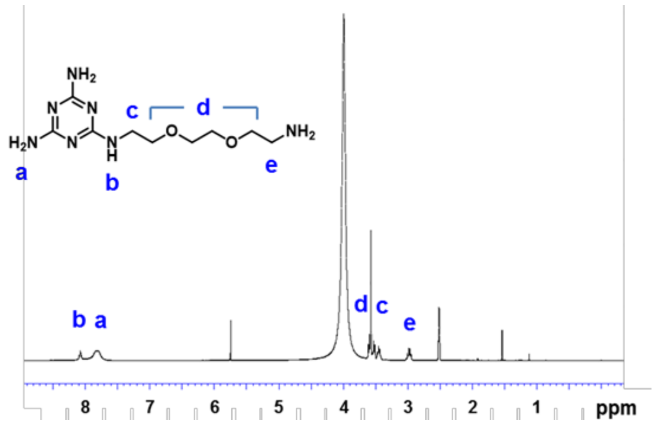


Fig. S14 [1]H NMR spectrum of Compound **5** (300 MHz, DMSO-d_6)

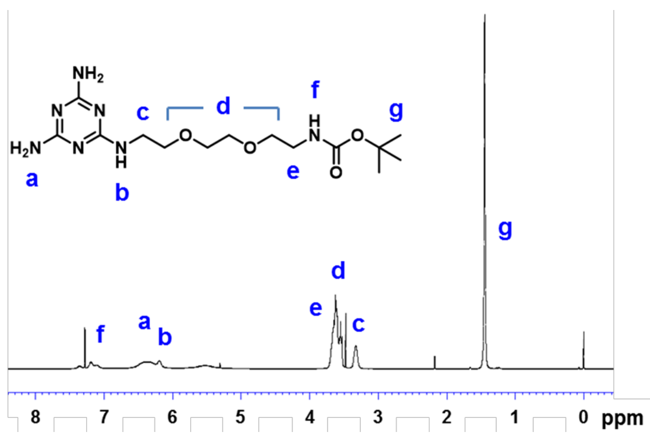


Fig. S15 [1]H NMR spectrum of Compound **6** (300 MHz, DMSO-d_6)

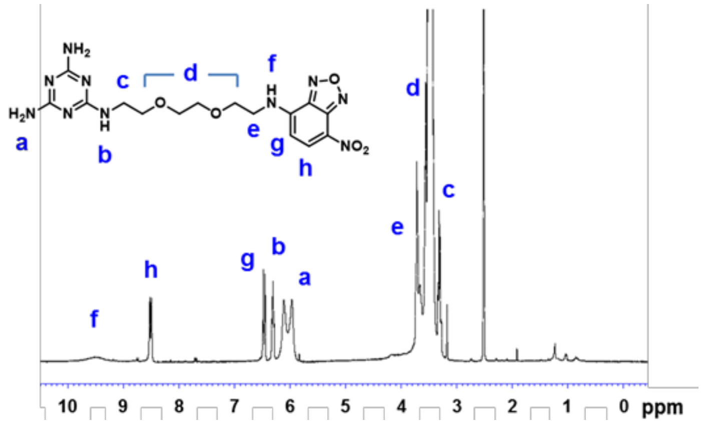


Fig. S16 ^{1}H NMR spectrum of Mel-NBD (300 MHz, DMSO-d_6)

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