SUPPORTING INFORMATION

Correlation between polymer architecture and Polyion Complex Micelle Stability with Proteins in Spheroid Cancer Models as seen by Light Sheet Microscopy

Fan Chen, Radhika Raveendran, Cheng Cao, Robert Chapman, Martina H. Stenzel

Materials. Chemicals were used as received unless otherwise specified. Poly (ethylene glycol) methyl ether acrylate (PEGMEA, M_n = 480 g mol^{-1}), 2-carboxyethyl acrylate (CEA, M_n = 144.13 g mol^{-1}), 1,4-dioxane (>99%), fluorescein isothiocyanate (FITC, ⩾90%), lysozyme from chicken egg white (HEWL, >90%), *N*-hydroxysuccinimide (NHS, 98%), *N*-(3-(dimethylamino)propyl)-*N'*-ethylcarbodiimide hydrochloride (EDC, BioXtra) were purchased from Sigma-Aldrich. 2,2′-Azobis(isobutyronitrile) (AIBN, Fluka, 98%). Diethyl ether (99%), toluene (>99.5%), dimethyl sulfoxide (DMSO, 98%) were purchased from Ajax Finechem. 3-benzylsulfanylthiocarbonylsufanylpropionic acid (BSPA) was synthesized according to a previous report.[1] Cyanine5 maleimide (Cy5) was purchased from Lumiprobe. Dialysis tubings (molecular weight cut-off 3.5 kDa) were purchased from Thermo Scientific. The cell line MCF-7 was purchased from CellBank Australia.

Synthesis of polymers

Synthesis of PPEGMEA

PEGMEA (5 g, 1.04 × 10^{-2} mol), BSPA (56.75 mg, 2.08 × 10^{-4} mol) and AIBN (3.42 mg, 2.08 × 10^{-5} mol) were dissolved in toluene (5.83 mL) in a clean 50 mL round-bottom flask. The mixed solution was purged with nitrogen for 40 min, followed by the polymerisation at 60 °C for 2 h. The polymerisation was terminated by placing the vial into ice bath and exposure to air. The conversion of the monomers was determined by ^{1}H NMR in $CDCl_3$ on a Bruker Avance III HD (400 MHz). The crude solution was purified by precipitating in a mixture of diethyl ether and hexane (1:1, v/v) once and another two times in diethyl ether alone. The molecular weight was determined by size exclusion chromatography (SEC) to be 11,000 g mol^{-1} with a $Đ$ of 1.13 using *N,N*-dimethylacetamide (DMAc) as the mobile phase and poly(methyl methacrylate) (PMMA) standards.

Synthesis of PPEGMEA-*b*-PCEA

The PPEGMEA-*b*-PCEA polymers with different lengths of the second charged blocks were prepared by chain extension of $PPEGMEA_{19}$ as a macro-RAFT agent. PPEGMEA (391 mg,

4.16×10^{-5} mol) was mixed with CEA (600 mg, 4.16×10^{-3} mol) and AIBN (1.37 mg, 8.33×10^{-6} mol) in 1,4-dioxane (3.69 mL) in a feed ratio of 1:100:0.2. The solution was equally split into three 5 mL round-bottom flask and purged with nitrogen for 40 min. Polymerizations were carried out at 60 °C in an oil bath for 1 h, 1.5 h and 2 h respectively before terminated by immersing the flasks into ice bath and exposure to air. The crude was extensively dialysed against Milli-Q water for two days followed by lyophilisation. The number of repeating units of the CEA block was determined by ^{1}H NMR in DMSO-d6. The dispersities ($Đ$) of the polymers were obtained from SEC using DMAc as the mobile phase and PMMA standards.

Synthesis of PPEGMEA-*b*-PAA

The same procedures were used as PPEGMEA-*b*-PCEA, where CEA was replaced by AA (300 mg, 4.16×10^{-3} mol).

Synthesis of PPEGMEA-*b*-(PCEA-*co*-PBA)

PPEGMEA (261 mg, 2.78×10^{-5} mol) was mixed with CEA (200 mg, 1.39×10^{-3} mol), BA (71 mg, 5.54×10^{-4} mol) and AIBN (0.91 mg, 5.54×10^{-6} mol) in 1,4-dioxane (2 mL) in a 5 mL round-bottom flask. The mixture was degassed by purging nitrogen for 40 min before the polymerised at 60 °C overnight. The reaction was terminated by immersing the flask into ice bath and exposure to air. The repeating units of CEA and BA were determined by ^{1}H NMR in DMSO-d6. The crude was transferred to 3.5 kDa MWCO dialysis tubings and purified by dialysing against methanol for one day and Milli-Q for another day followed by lyophilisation. $M_{n,SEC}$ = 34,000 g mol^{-1}, $Đ$ = 1.14.

Synthesis of PPEGMEA-*b*-PBA

PPEGMEA (293 mg, 3.12×10^{-5} mol) was mixed with BA (200 mg, 1.56×10^{-3} mol) and AIBN (1.02 mg, 6.24×10^{-6} mol) in toluene (2 mL) in a 5 mL round-bottom flask. The mixture was degassed by purging nitrogen for 40 min before the polymerised at 60 °C for 1 h 45 min. The reaction was terminated by immersing the flask into ice bath and exposure to air. The repeating units of BA were determined by ^{1}H NMR in $CDCl_3$. The crude was transferred to 3.5 kDa MWCO dialysis tubings and purified by dialysing against methanol for one day and Milli-Q for another day followed by lyophilisation. $M_{n,SEC}$ = 13,000 g mol^{-1}, $Đ$ = 1.12.

Synthesis of PPEGMEA-*b*-PBA-*b*-PCEA

PPEGMEA-b-PBA (204 mg, 1.66×10^{-5} mol) was mixed with CEA (239.5 mg, 1.66×10^{-3} mol) and AIBN (0.55 mg, 3.32×10^{-6} mol) in 1,4-dioxane (1.5 mL) in a 5 mL round-bottom flask. The mixture was degassed by purging nitrogen for 40 min before the polymerised at 60 °C for 2 h 10 min. The reaction was terminated by immersing the flask into ice bath and exposure to air. The repeating units of BA were determined by ^{1}H NMR in $CDCl_3$. The crude

was transferred to 3.5 kDa MWCO dialysis tubings and purified by dialysing against Milli-Q for 2 days followed by lyophilisation. $M_{n,SEC}$ = 26,000 g mol^{-1}, $Đ$ = 1.20.

Synthesis of PPEGMEA-*b*-PCEA-*b*-PBA

PPEGMEA-*b*-PCEA (200 mg, 1.25× 10^{-5} mol) was mixed with BA (80.1 mg, 6.25 × 10^{-4} mol) and AIBN (0.41 mg, 2.50 × 10^{-6} mol) in 1,4-dioxane (1.7 mL) in a 5 mL round-bottom flask. The mixture was degassed by purging nitrogen for 40 min before the polymerised at 60 °C for 4 h 40 min. The reaction was terminated by immersing the flask into ice bath and exposure to air. The repeating units of BA were determined by ^{1}H NMR in $CDCl_3$. The crude was transferred to 3.5 kDa MWCO dialysis tubings and purified by dialysing against methanol for one day and Milli-Q for another day followed by lyophilisation. $M_{n,SEC}$ = 31,000 g mol^{-1}, $Đ$ = 1.12.

Micellization. The polymers were dissolved in Milli-Q water (500 μL) at various concentrations. HEWL (0.7 mg) was also dissolved in Milli-Q water (500 μL). PIC micelles were formed by dropwise adding HEWL solution into polymer solution under stirring.

Size Exclusion Chromatography (SEC). SEC was conducted using a Shimadzu modular system containing a DGU-12A degasser, a LC-10AT pump, a SIL-10AD automatic injector, a CTO-10A column oven, and a RID-10A refractive index detector. A 50 × 7.8 mm guard column and four 300 × 7.8 mm linear columns (10^5, 10^4, 10^3, and 10^2 Å pore size, 5 μm particle size) were used for the analyses. *N*, *N*-Dimethylacetamide (DMAc, HPLC grade, 0.05% w/v of 2,6- dibutyl-4-methylphenol (BHT), 0.03% w/v of LiBr) with a flow rate of 1 mL min^{-1} was used for analyses. The calibration of the instrument was conducted by commercial polymethyl methacrylate standards (0.5−1000 kDa, Polymer Laboratories). Chromatograms were processed using Cirrus 2.0 software (Polymer Laboratories).

Dynamic light scattering (DLS)

The measurements were performed at 25°C using Malvern Nano-ZS equipped with a He-Ne laser (4 mW, λ = 632 nm) and scattering light was detected at an angle of 173°. All measurements were taken in triplicate.

Isothermal titration calorimetry

Stock solutions of PPEGMEA-b-PCEA (0.02 mM), PPEGMEA-b-PAA (0.02 mM) and Lys (0.5 mM) were separately prepared in Milli-Q water. For each measurement, polymer solution (200 µL) was added in the sample cell of a GE ITC200 isothermal calorimeter and equal volume of Milli-Q water was place in the reference cell. Lys solution (40 µL) was loaded into the syringe. Measurement was carried out at 25 °C and titrant (1 µL) was injected into the sample cell 20 times in a row with 180 s spacing between 2 injections. The mixture was constantly stirred at 750 rpm throughout the experiment. Control experiments of Lys into Milli-Q water, Milli-Q water into polymer and Milli-Q to Milli-Q were also conducted using the same procedure. The raw data was analysed using a MicroCal LLC ITC program in Origin® and the data from the control experiments were subtracted from sample data before they were fitted.

Disassembly at high ionic strength

Stock solutions of the polymers (1mg/ml) and lysozyme (1.4 mg/ml) were prepared in Milli-Q water. For the PIC micelle preparation, 500 µl of lysozyme solution was added dropwise to varying concentrations of polymer solution such that ratio of the negative to positive charges was 1:1. After stirring for 3 hours, the PIC micelles were left to equilibrate for 2 hours before addition of NaCl solution. 20 µl of 1 M NaCl was added, each time, followed by stirring for 60 secs and equilibration for 120 secs at 25°C before measuring the derived count rate using Malvern Nano-ZS equipped with a He-Ne laser (4 mW, $\lambda = 632$ nm) with scattering light detection at an angle of 173°.

Cell viability assay

The nanoparticles were prepared in Milli-Q water at a concentration of 1 mg/mL. The biocompatibility and cytotoxicity were evaluated by sulforhodamine B (SRB) assay using MCF-7 human breast cancer cell line. The cells were seeded into a 96-well plate (4000 cells/well) in 100 µL Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 0.002% plasmocin at 37 °C in a 5% CO_2 and 100% humidity environment for 24 h. The medium was then replaced by 2× concentrated fresh DMEM media. The micelles were sterilized by UV irradiation for 15 min and then serially diluted (2× dilution) by sterile Milli-Q water. 100 µL of each solution were separately added into 4 wells while 4 control samples were fed with 100 µL Milli-Q water. The final concentration of micelles ranged from 2 µg/mL to 500 µg/mL. After 72 h of incubation, the culture medium

was discarded and 100 μL of cold trichloroacetic acid (TCA) was added into each well, followed by an additional incubation at 4 °C for 30 min. After that, the cells were washed 5 times with Milli-Q water and dyed with 100 μL of SRB solution (0.4% w/v in 1% acetic acid). After incubated at room temperature for 20 min, unbound dye was washed out by 1% acetic acid (100 μL) for 5 times. The plate was then allowed to air dry in the dark. Tris buffer (200 μL, 10mM) was added into each well to solubilize bound dyes. The absorbance of the solution was measured on a Bio-Rad BenchMark microplate reader at 490 nm. The relative viability of cell (%) was determined as a percentage of the treated cells to the untreated control cells.

Polymer labelling with Cy5

For each polymer, it was firstly dissolved in DMSO (1.5 mL) at a concentration of 1.53 μM 50 eq, containing EDC (11.7 μg, 61.2 nM, 2 eq) and NHS (3.1 μg, 30.6 nM, 1 eq) and stirred for 20 min at room temperature. Cy5 stock solution (20 μg in 2 mL DMSO, 1 eq) was then added into the polymer solution and continuously stirred overnight in the dark. Unreacted cy5 was removed by dialysing against methanol for one day and Milli-Q water for another day, followed by lyophilization. Lys was labelled with FITC as follows: Lys (14 mg) was dissolved in 7 mL 0.05 M carbonate buffer (7 mL, pH 9.5) while FITC (2.34 mg) was dissolved in DMSO (1 mL). The reaction was carried out by mixing both the solution at 4 °C overnight in the dark. The crude product was purified by dialyzing against Milli-Q water for 2 days at 4 °C, followed by lyophilization.

Flow cytometry

MCF-7 breast cancer cells were seeded into 6-well plate at a concentration of 5×10^5 cells/well and cultured for 2 days. The cells were then incubated with 3 mL fresh DMEM culture media containing FITC labelled micelles (Lys 15 μM, polymer 30 μM) for (Lys 7.1 μM, polymer 14.2 μM) 2 h before the cells were washed with cold PBS for 4 times and detached from the plate well using trypsin/EDTA solution. Cell suspensions were then centrifuged and resuspended in cold Hank's buffer. The cellular uptake was determined by measuring fluorescence intensity from 20000 cell events per well (λ_{ex} = 488 nm, λ_{ex} = 530/30 nm) using flow cytometry (BD FACSCanto ™ Ⅱ Analyser). Median fluorescence intensity (MFI) was averaged from 3 individual wells for one sample using a FlowJo® software.

Protein labelling with FITC

FITC was used to fluorescently label Lys using a previously reported method. Briefly, Lys was dissolved in 0.05 M carbonate buffer (7 mL, pH 9) at a concentration of 2 mg/mL while FITC stock solution was prepared in DMSO (1 mL) at a concentration of 2.34 mg/mL. The reaction was carried out by dropwise adding the FITC solution into Lys solution. The mixture was then stirred in the dark at room temperature for 1 h. Unreacted FITC was removed by extensive dialysis against Milli-Q water for 2 days at 4 °C, followed by lyophilisation.

PIC micelles were then prepared using FITC labelled Lys. All the samples were prepared at a polymer/Lys molar ratio of 2. The fluorescently labelled PIC micelles were then incubated with MCF-7 cancer cells for 2 h. The fluorophore was excited by a 488 nm laser and fluorescence emission was collected using a 530/30 nm band-pass filter.

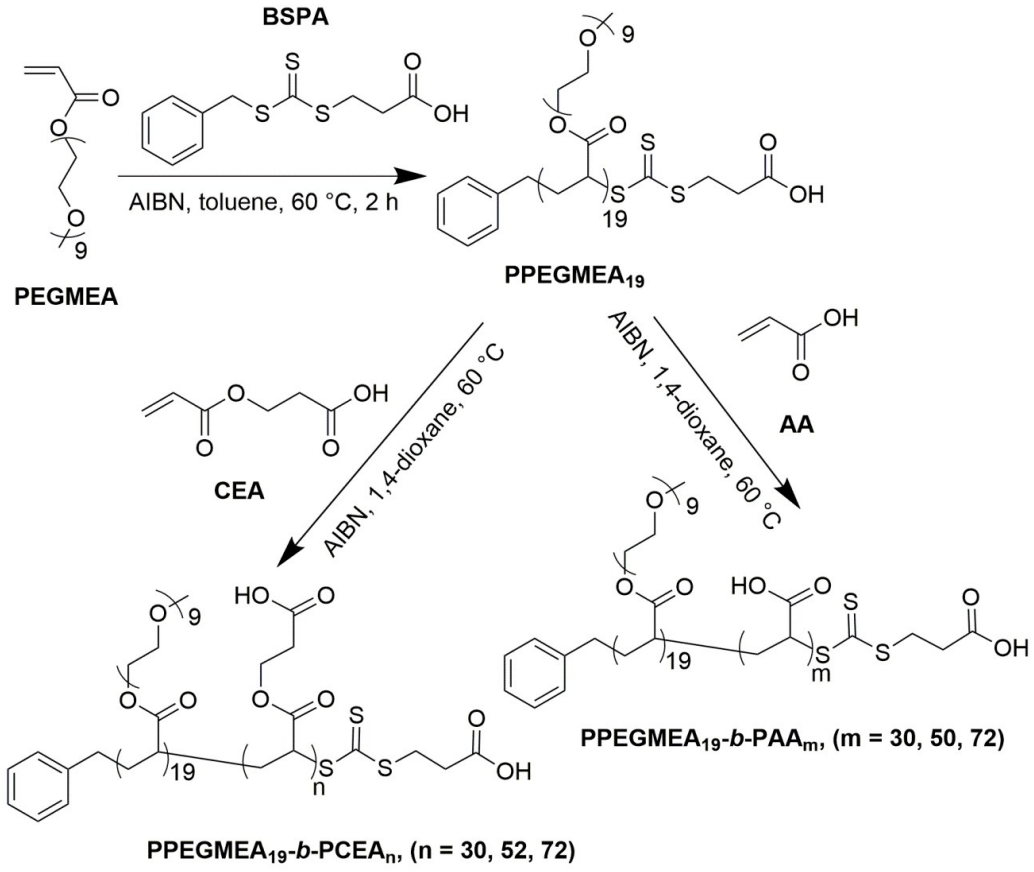

Figure S1. Synthesis scheme of RAFT polymerised PPEGMEA-*b*-PCEA and PPEGMEA-*b*-PAA with various lengths of the charged blocks.

Table S1. Characterisation of macroRAFT agent and block copolymers.

Polymer	Polymerisation conditions		M_n (g mol^{-1})[b]	M_n (g mol^{-1})[c]	$Đ$[c]	Conversion[b] (%)
	[M]:[C]:[I][a]	Time (h)				
$PPEGMEA_{19}$	50:1:0.1	2	9400	11000	1.13	38
$PPEGMEA_{19}$-*b*-$PCEA_{30}$	100:1:0.2	1	14000	15000	1.17	30
$PPEGMEA_{19}$-*b*-$PCEA_{52}$	100:1:0.2	1.3	17000	31000	1.19	52
$PPEGMEA_{19}$-*b*-$PCEA_{72}$	100:1:0.2	2	20000	34000	1.23	72
$PPEGMEA_{19}$-*b*-PAA_{30}	100:1:0.2	1	12000	25000	1.17	30
$PPEGMEA_{19}$-*b*-PAA_{50}	100:1:0.2	1.3	13000	28000	1.21	50
$PPEGMEA_{19}$-*b*-PAA_{72}	100:1:0.2	2	22000	31000	1.26	72

[a] [M]:[C]:[I] = [monomer]:[CTA]:[AIBN]. [b] Results from ^{1}H NMR. [c] Results from SEC using DMAc as eluent and PMMA standard. PPEGMEA-*b*-PCEA and PPEGMEA-*b*-PAA was polymerised at 60°C in 1,4-dioxane.

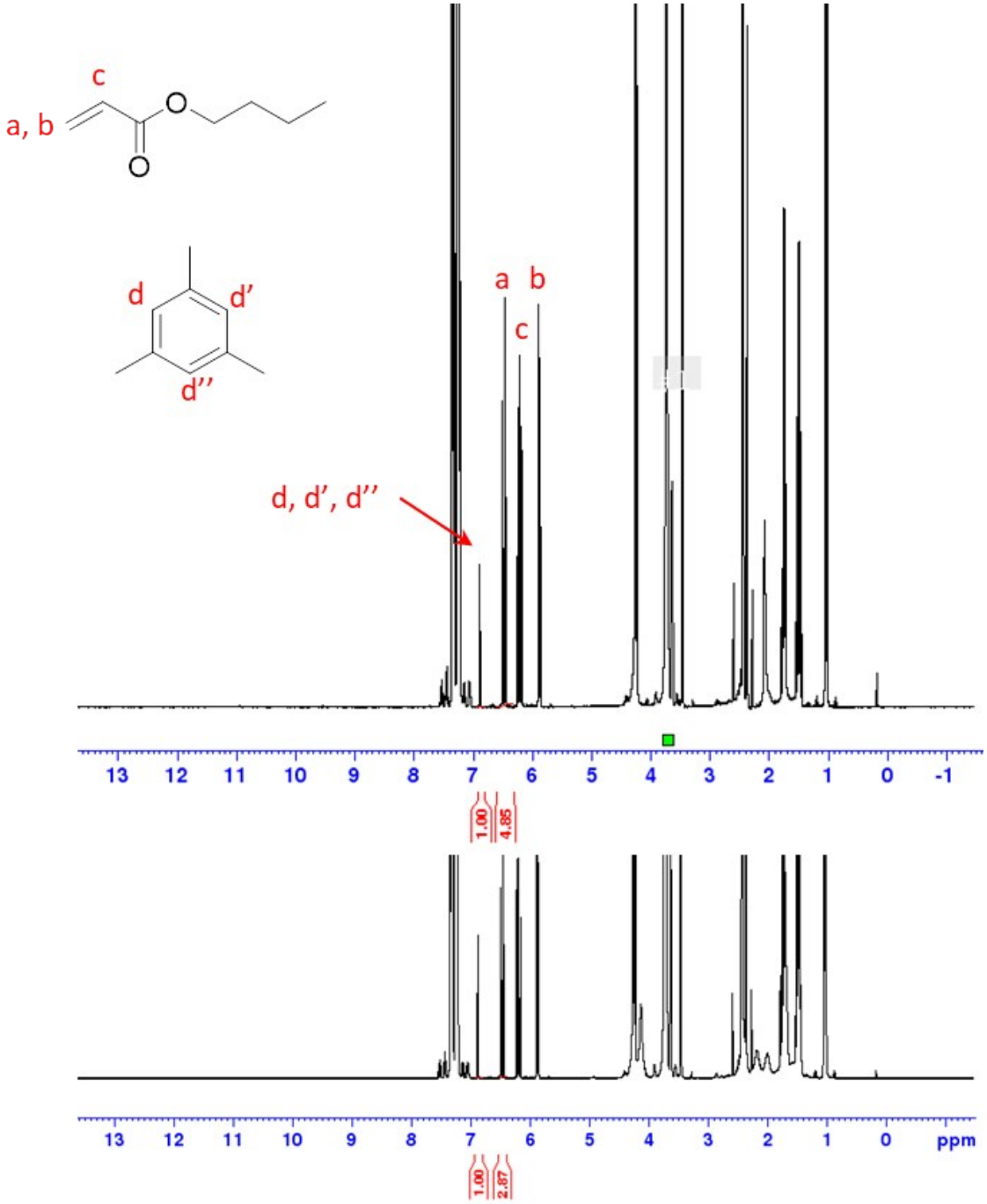

Figure S2. ^{1}H NMR spectra for determining the conversion of *n*-butyl acrylate.

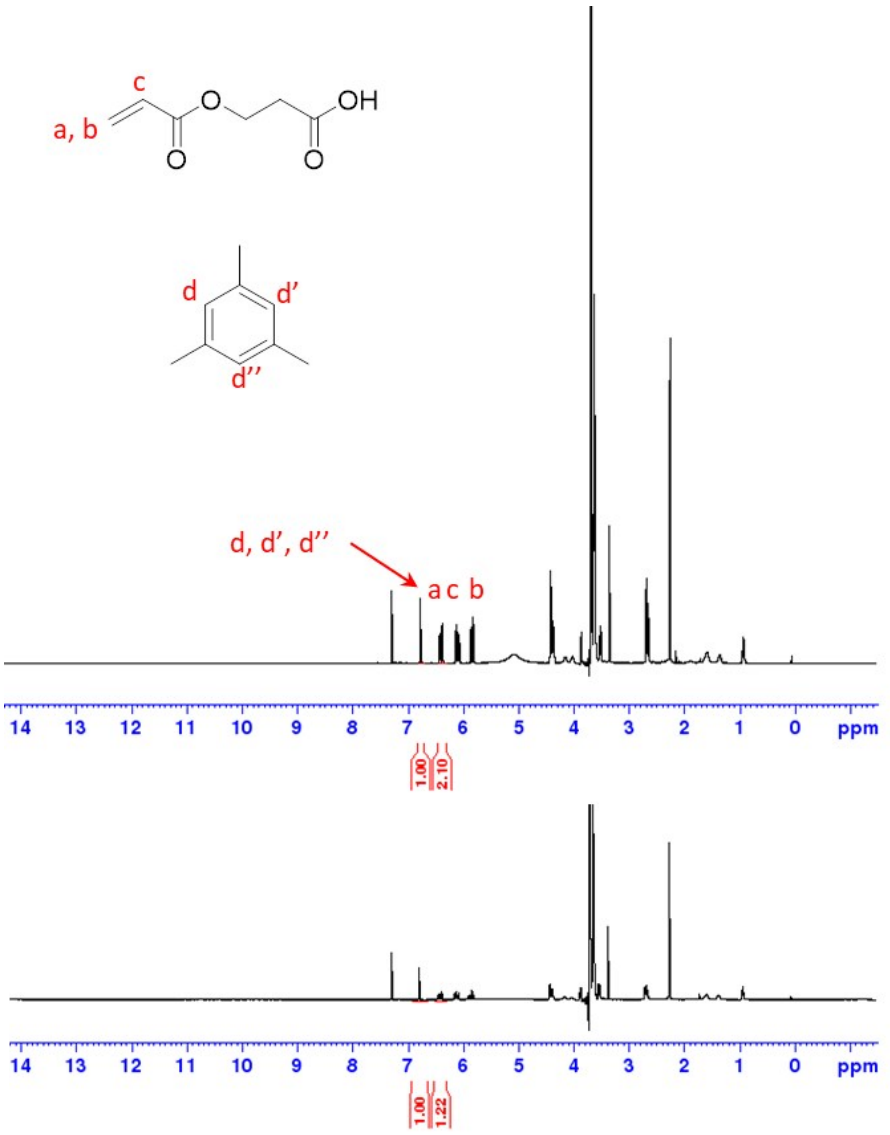

Figure S3. [1]H NMR spectra for determining the conversion of 2-carboxyethyl acrylate.

The repeating units (RU) of *n*-butyl acrylate (BA) and 2-carboxyethyl acrylate (CEA) were calculated as following:

$$Conversion_{BA} = \frac{4.85 - 2.87}{4.85} = 40\%$$

$$RU_{BA} = 50 \times 40\% = 20$$

$$Conversion_{CEA} = \frac{2.10 - 1.22}{2.10} = 42\%$$

$$RU_{CEA} = 100 \times 42\% = 42$$

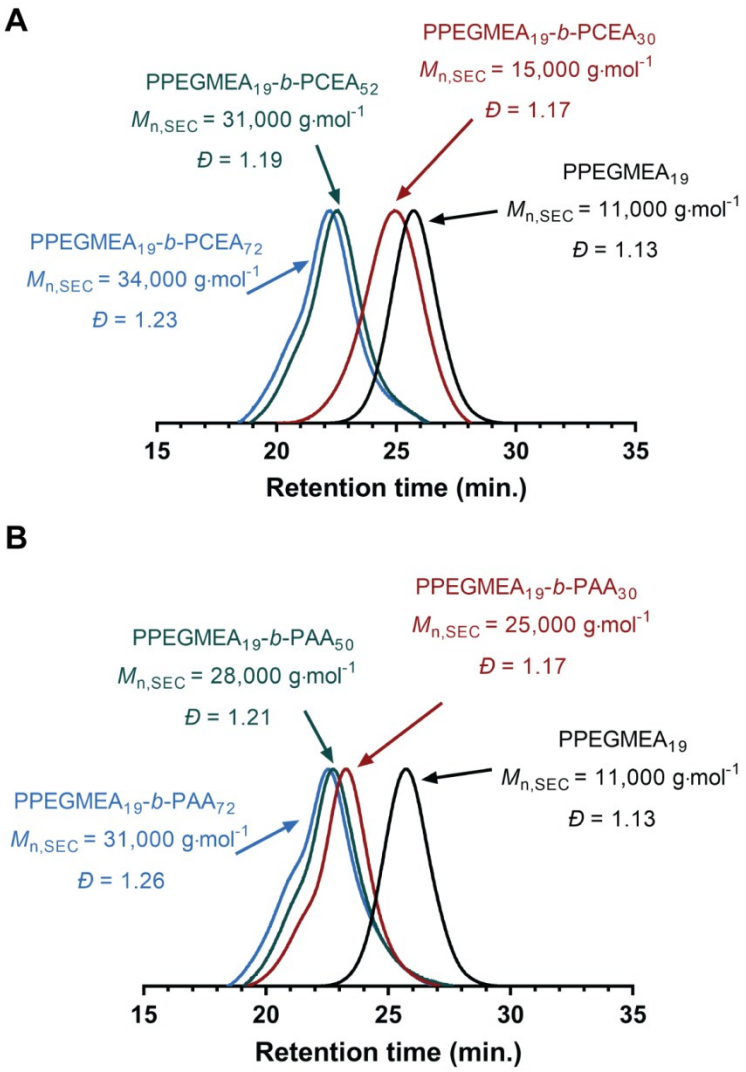

Figure S4. DMAc SEC traces of **(A)** PPEGMEA$_{19}$-*b*-PCEA series and **(B)** PPEGMEA$_{19}$-*b*-PAA series.

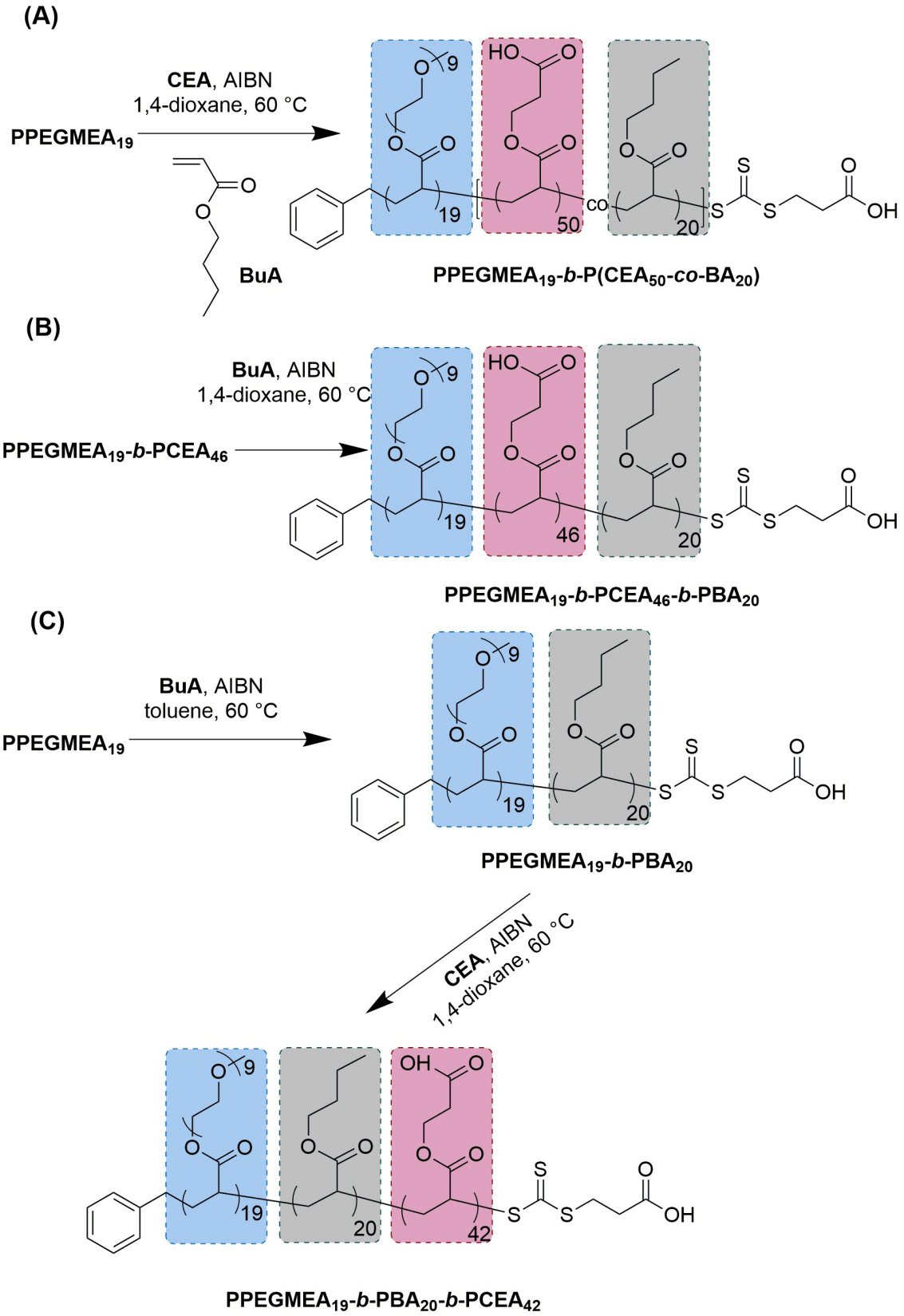

Figure S5. Synthetic routes of (A) PPEGMEA$_{19}$-*b*-(PCEA$_{50}$-*co*-PBA$_{20}$), (B) PPEGMEA$_{19}$-*b*-PCEA$_{46}$-*b*-PBA$_{20}$ and (C) PPEGMEA$_{19}$-*b*-PBA$_{20}$-*b*-PCEA$_{42}$.

Table S2. Conditions used to prepare polymers *via* RAFT polymerisation to study the effect of hydrophobic units (butyl acrylate) on micelles' stability.

Polymer	Polymerisation conditions			$M_{n,\,NMR}$ (g mol^{-1})[c]	$M_{n,\,SEC}$ (g mol^{-1})[d]	$Đ$
	[M]:[C]:[I][a]	Time (h)	Temperature (°C)			
$PPEGMEA_{19}$	50:1:0.1	2	60	9392	11000	1.13
P(C-co-B)	50::20:1:0.2[b]	16	60	19152	34000	1.14
PC	100:1:0.2	1.5	60	16016	25000	1.13
PCB	50:1:0.2	4.67	60	18576	31000	1.12
PB	50:1:0.2	1.75	60	11952	13000	1.12
PBC	100:1:0.2	2.17	60	18000	26000	1.20

[a][M]:[C]:[I] = [monomer]:[CTA]:[AIBN]. [b][CEA]:[BA]:[CTA]:[I] = 50:20:1:0.2. [c]Molecular weights calculated from ^{1}H NMR. [d]Molecular weights obtained from DMAc SEC using PMMA standards. $PPEGMEA_{19}$, PCB and PB were polymerised in toluene. P(C-*co*-B), PC, PBC were polymerised in 1,4-dioxane. P(C-*co*-B) = $PPEGMEA_{19}$-*b*-($PCEA_{50}$-*co*-PBA_{20}); PC = $PPEGMEA_{19}$-*b*-$PCEA_{46}$; PCB = $PPEGMEA_{19}$-*b*-$PCEA_{46}$-*b*-PBA_{20}; PB = $PPEGMEA_{19}$-*b*-PBA_{20}; PBC = $PPEGMEA_{19}$-*b*-PBA_{20}-*b*-$PCEA_{42}$. The monomer concentration for all polymerisations was 1 M.

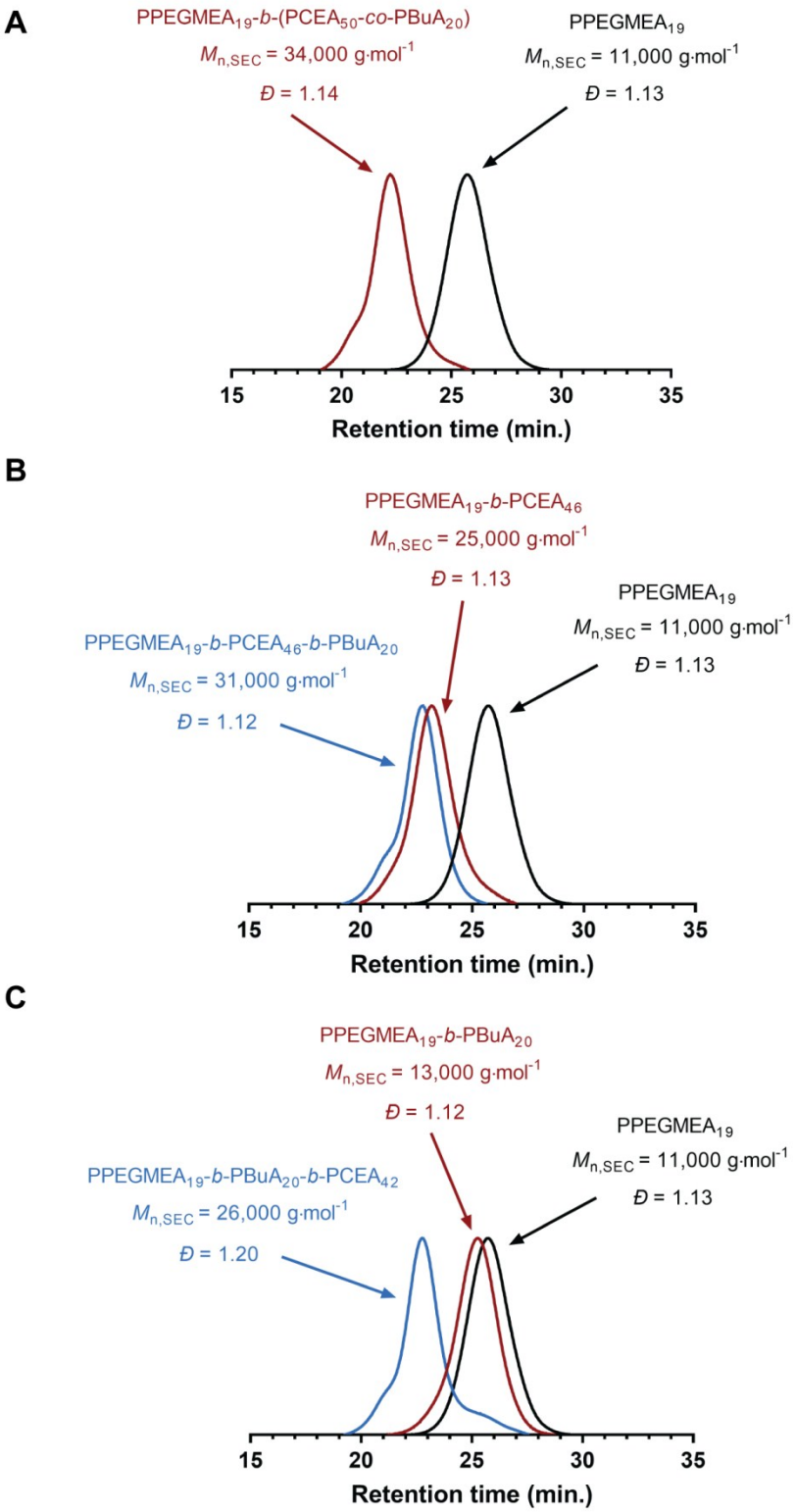

Figure S6. *N,N*-dimethylacetamide size exclusion chromatography (DMAc SEC) traces of polymers used for synthesis of (A) PPEGMEA$_{19}$-*b*-(PCEA$_{50}$-*co*-PBA$_{20}$), (B) PPEGMEA$_{19}$-*b*-PCEA$_{46}$-*b*-PBA$_{20}$ and (C) PPEGMEA$_{19}$-*b*-PBA$_{20}$-*b*-PCEA$_{42}$.

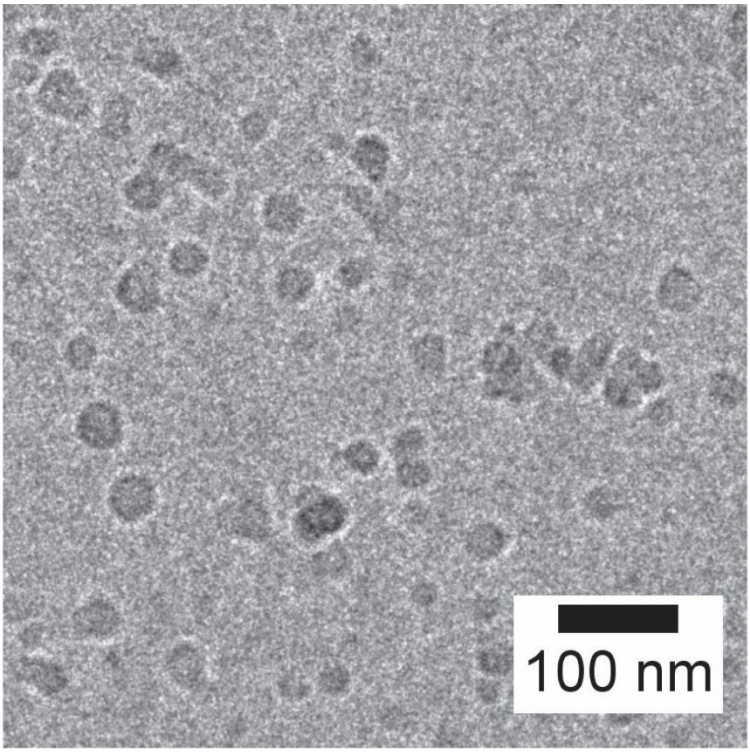

Figure S7. Cryo-TEM image of PPEGMEA$_{19}$-*b*-PCEA$_{52}$/lysozyme micelles at a molar ratio of 2. The image was taken at a slight defocus to improve image contrast.

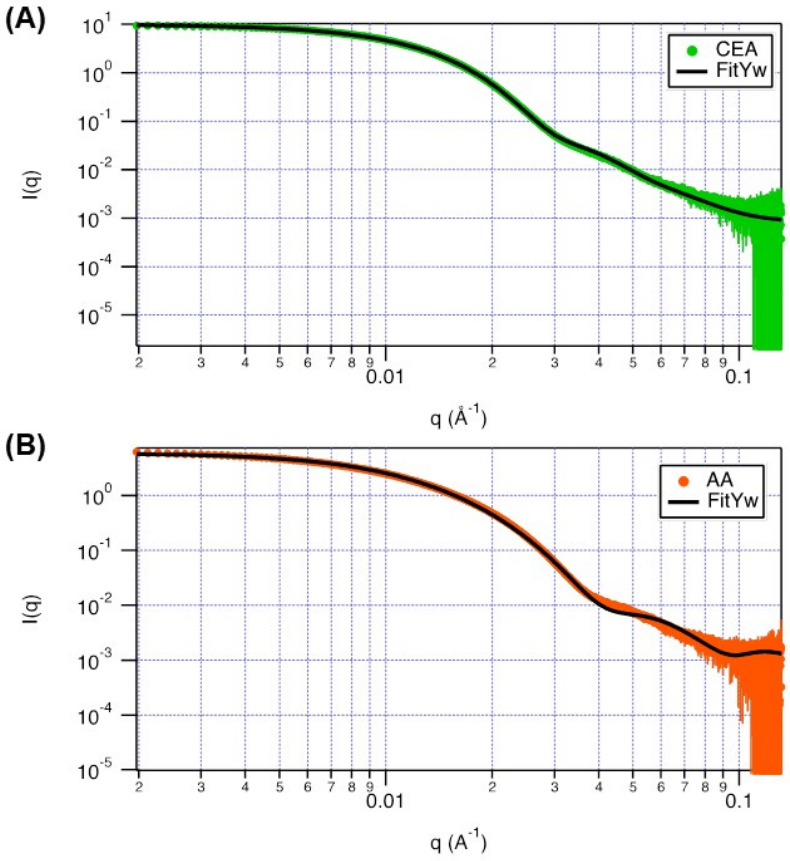

Figure S8. SAXS analysis of **(A)** PPEGMEA$_{19}$-*b*-PCEA$_{52}$/lysozyme micelles and **(B)** PPEGMEA$_{19}$-*b*-PAA$_{50}$/lysozyme micelles at a molar ratio of 2 in Milli-Q water.

Table S3. Fitting parameters of SAXS data.

Parameters	CEA$_{52}$/LYS*	AA$_{50}$/LYS*
core radius (nm)	6.29	7.92
Core Polydispersity(0,1)	0.47	0.43
Core SLD (A^{-2})	1.09*10^{-5}	1.09*10^{-5}
Shell 1 thickness (nm)	4.56	4.56
Shell 1 SLD (A^{-2})	1.08*10^{-5}	1.06*10^{-5}
Shell 2 thickness (nm)	3.56	3.57
Shell 2 SLD (A^{-2})	1.08*10^{-5}	9.34*10^{-6}

*CEA$_{52}$/LYS = PPEGMEA$_{19}$-*b*-PCEA$_{52}$/lysozyme micelles, AA$_{50}$/LYS = PPEGMEA$_{19}$-*b*-PAA$_{50}$/lysozyme, molar ratio of polymer to protein was 2.

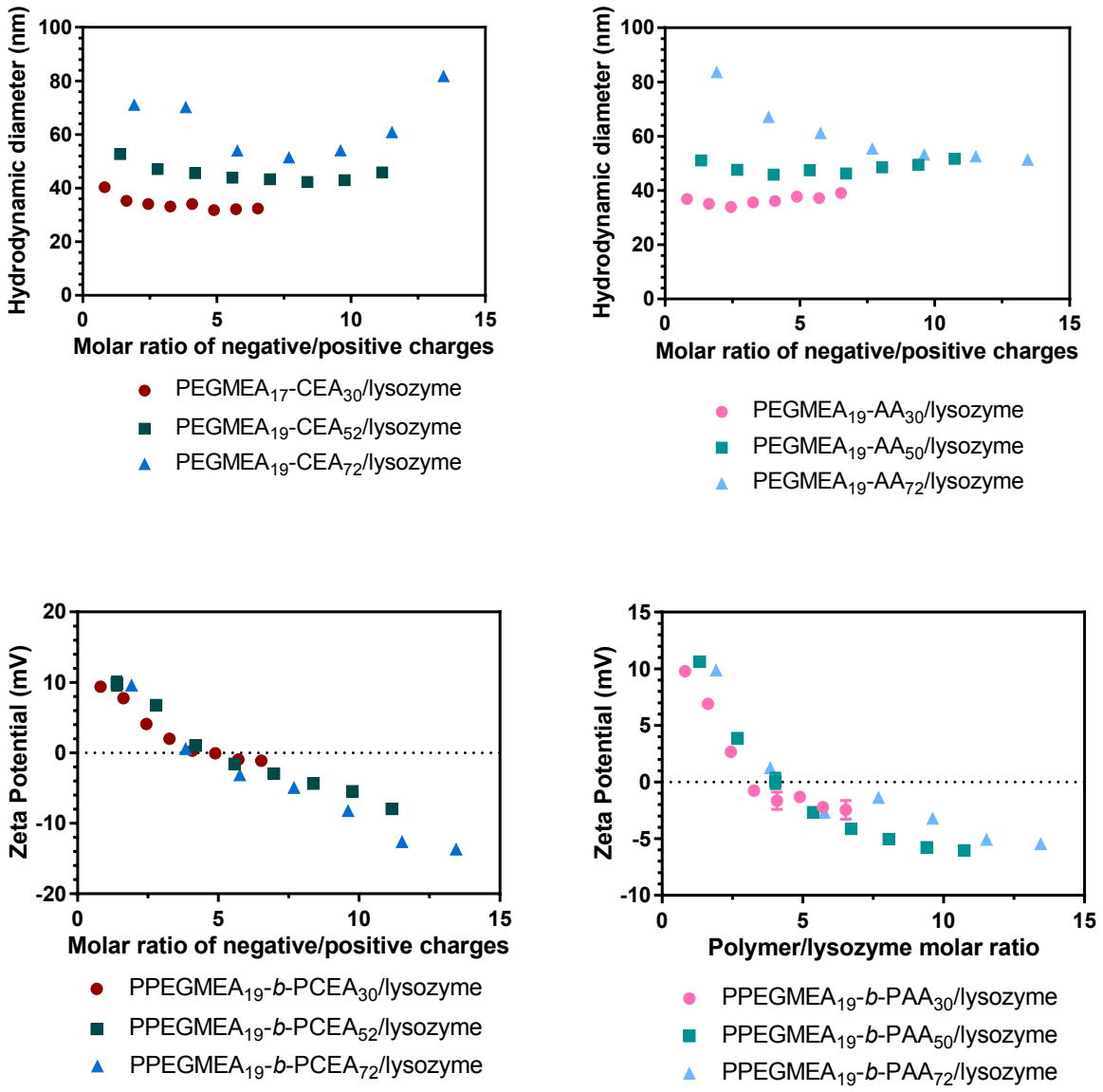

Figure S9. *z*-Averaged hydrodynamic diameters of polyion complex (PIC) micelles formed by (A) PPEGMEA-*b*-PCEA (CEA) and (B) PPEGMEA-*b*-PAA (AA) with Lysozyme (LYS, 0.05 mM) respectively at various molar ratios of negative charges to positive charges Zeta potential of (C) CEA/LYS PIC micelles and (D) AA/LYS PIC micelles. Data was averaged from three measurements.

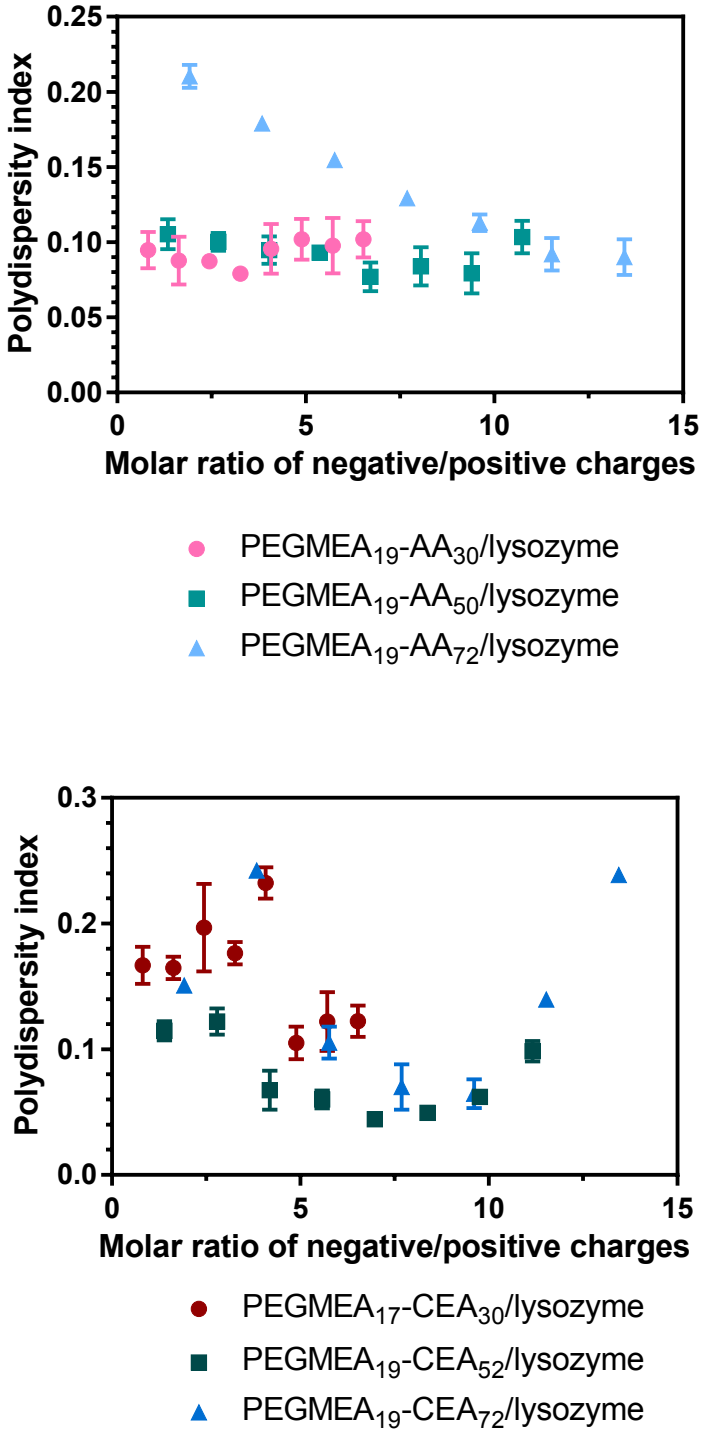

Figure S10. PDI of the DLS distribution of polyion complex (PIC) micelles formed by (A) PPEGMEA-*b*-PCEA (CEA) and (B) PPEGMEA-*b*-PAA (AA) with Lysozyme (LYS, 0.05 mM) respectively at various molar ratios of negative charges to positive charges

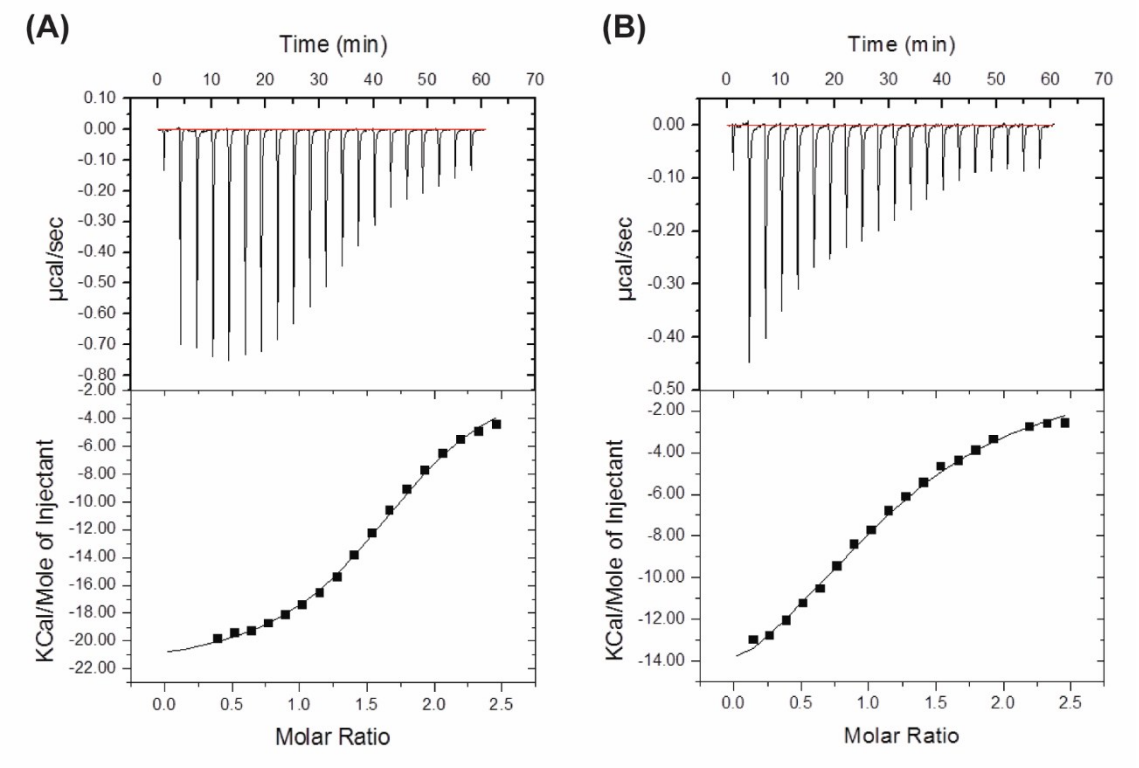

Figure S11. Isothermal titration calorimetry (ITC) profiles of titrating lysozyme (Lys) into **(A)** PPEGMEA$_{19}$-*b*-PCEA$_{52}$ and **(B)** PPEGMEA$_{19}$-*b*-PAA$_{50}$ at 25 °C.

Table S4. Thermodynamic parameters obtained from isothermal titration calorimetry (ITC) measurements.

Sample	***N***	K_a (× 10^5 M^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	ΔG^c (kcal mol^{-1})
CEA$_{52}$[a]	1.73 ± 0.01	4.04 ± 0.26	-22.22 ± 0.27	-48.8	-7.67
AA$_{50}$[b]	1.16 ± 0.03	1.13 ± 0.10	-19.14 ± 0.74	-41.1	-12.25

[*]Titrations of lysozyme (LYS) into [a]PPEGMEA$_{19}$-*b*-PCEA$_{52}$ and [a]PPEGMEA$_{19}$-*b*-PAA$_{50}$. *N*, binding stoichiometry; K_a, binding affinity; ΔH, ΔS and ΔG, changes in enthalpy, entropy and Gibbs free energy respectively. [a]The ΔG value was calculated using Gibbs free energy equation: $\Delta G = \Delta H - T\Delta S$.

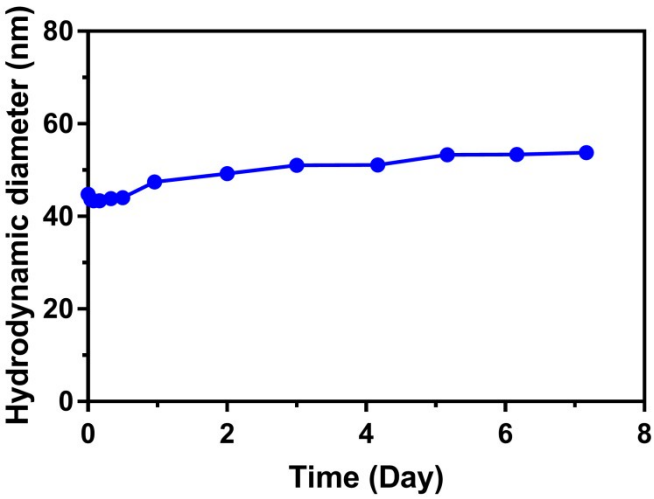

Figure S12. The size changes of PPEGMEA$_{19}$-*b*-PCEA$_{52}$/lysozyme micelles in buffer (HBSS) over a period of 7 days as determined by DLS. The concentrations of polymer and lysozyme are 7.14 and 3.57 μM, respectively.

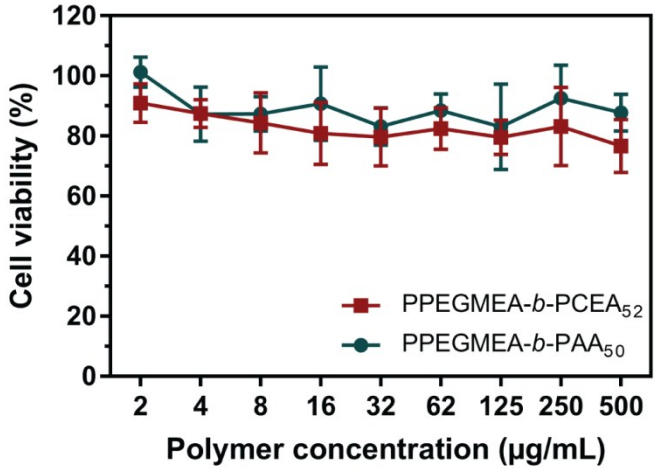

Figure S13. Evaluation of cytotoxicity of PPEGMEA-*b*-PCEA and PPEGMEA-*b*-PAA at concentrations up to 500 μg/mL against MCF-7 human breast cancer cells after incubation of 72 h.

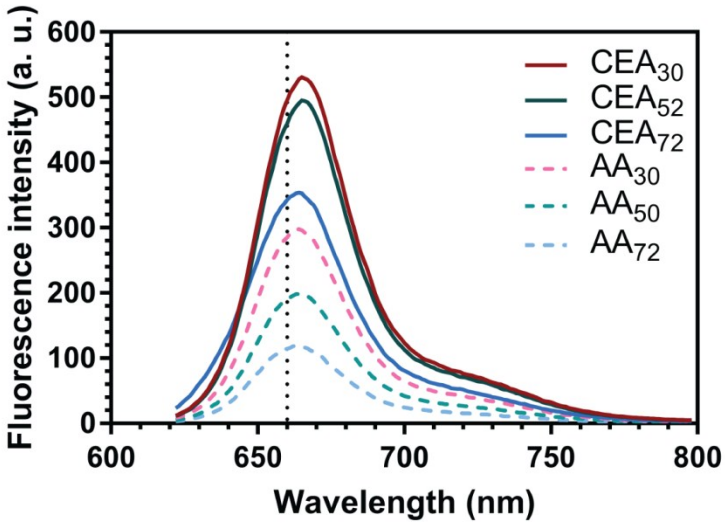

Figure S14. Fluorescence spectra of cy5 labelled PPEGMEA$_{19}$-*b*-PCEA$_n$ (n = 30, 52, 72) and PPEGMEA$_{19}$-*b*-PAA$_m$ (m = 30, 50, 72) in aqueous solutions (0.1 mg/mL). Excitation wavelength = 600 nm. The dotted line shows wavelength of the filter (660 nm longpass) used on LSFM.

Table S5. Fluorescence intensity of polymers used for light-sheet fluorescence microscopy (LSFM).

Polymer	Fluorescence intensity at 660 nm (a. u.)
PPEGMEA$_{19}$-*b*-PCEA$_{30}$	496
PPEGMEA$_{19}$-*b*-PCEA$_{52}$	460
PPEGMEA$_{19}$-*b*-PCEA$_{72}$	343
PPEGMEA$_{19}$-*b*-PAA$_{30}$	285
PPEGMEA$_{19}$-*b*-PAA$_{50}$	190
PPEGMEA$_{19}$-*b*-PAA$_{72}$	114

Polymer concentration was 0.1 mg/mL for all measurements. Excitation wavelength was 600 nm.

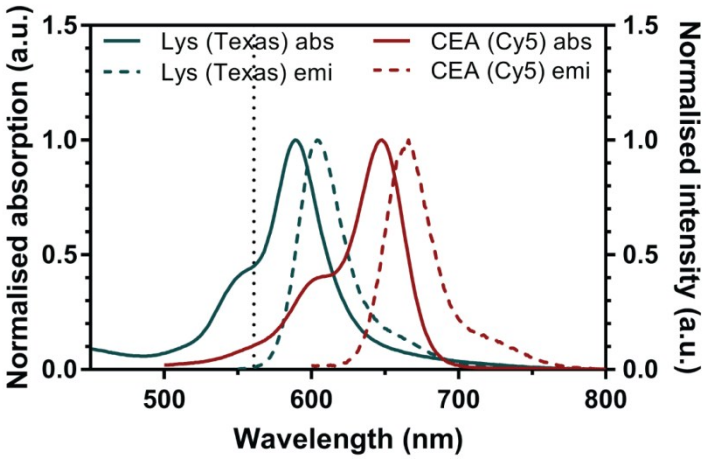

Figure S15. Normalised UV-Vis absorption (abs) spectra and fluorescence emission (emi) spectra of Texas Red labelled lysozyme and cy5 labelled CEA polymer in Milli-Q water. The block dotted line shows the wavelength of the laser subsequently used in confocal laser scanning microscopy and light sheet microscopy.

Table S6. Contents of PIC micelles for FRET studying.

Sample	Concentration (μM)		
	Lysozyme (Texas Red)	CEA (no label)[a]	CEA (cy 5)[a]
8:2	2.5	4	1
6:4	2.5	3	2
4:6	2.5	2	3
2:8	2.5	1	4
2:8 (control)	0	1	4

[a]CEA = $PPEGMEA_{39}$-*b*-$PCEA_{35}$. All samples were prepared in 2 mL Milli-Q water.

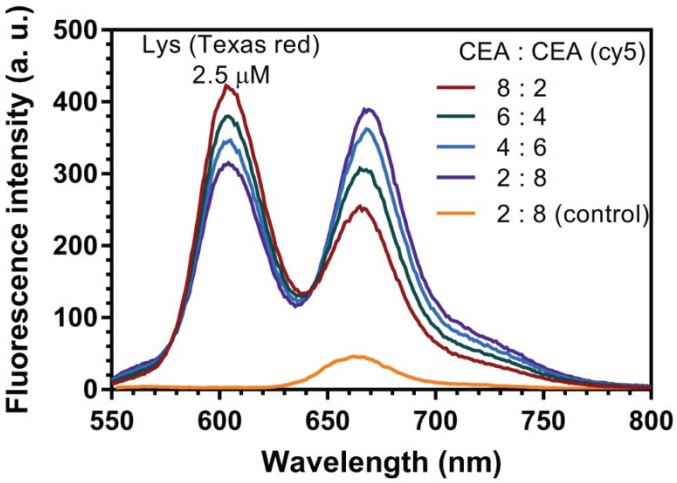

Figure S16. Fluorescence spectra of the mixture of CEA, CEA (cy5) and lysozyme (Texas Red) with increasing amount of CEA (cy5) polymer (from 1 to 4 μM) in 2 mL Milli-Q water. The concentration of Lys (Texas Red) was kept constant (2.5 μM) in all samples. A wavelength of 530 nm laser was used to excite fluorophores for all measurements. The control is CEA (cy5) only without Lys. Ratios are shown in moles. CEA = $PPEGMEA_{39}$-*b*-$PCEA_{35}$.

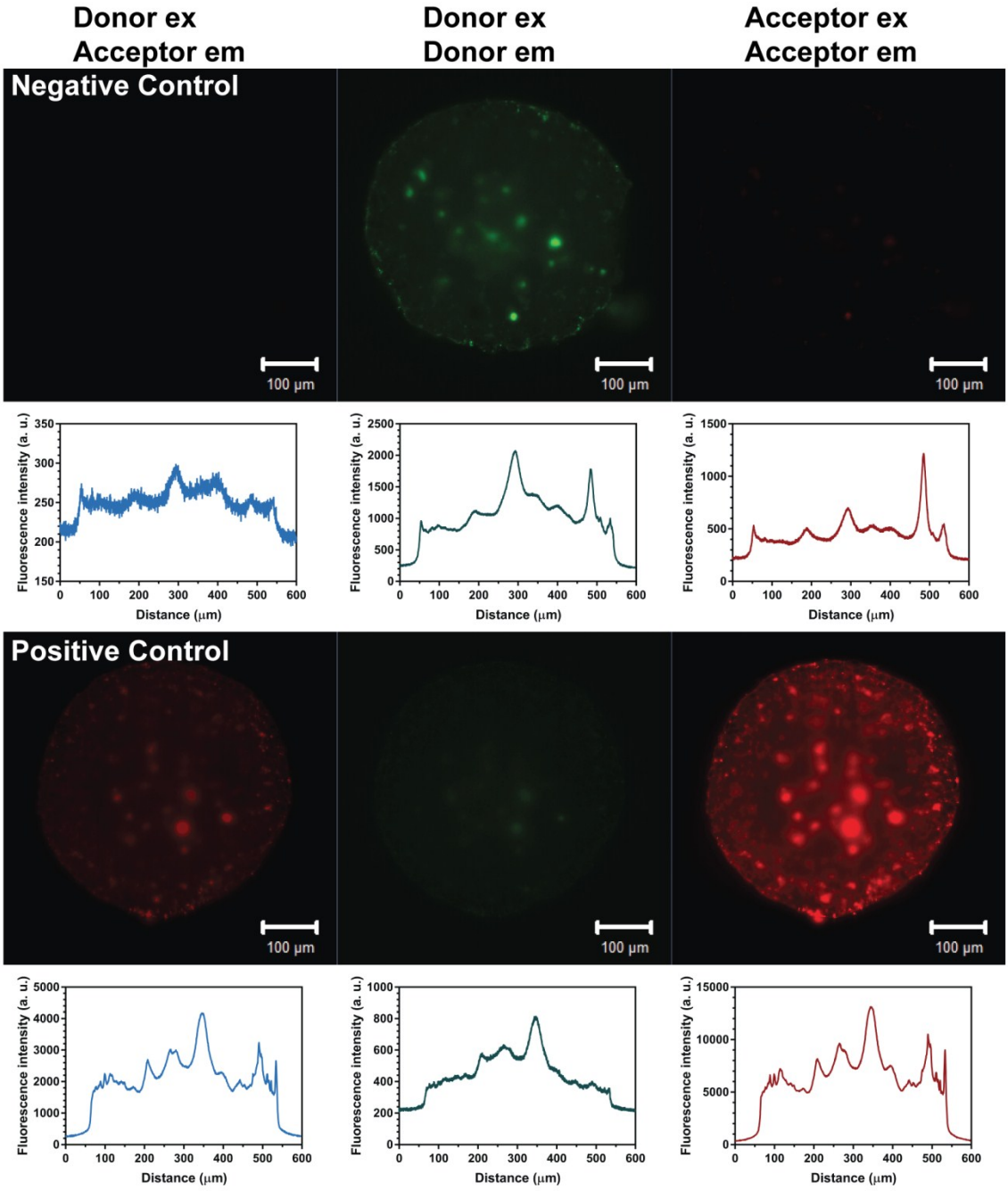

Figure S17. Images of MCF-7 breast cancer cell spheroids obtained using light-sheet fluorescence microscopy (LSFM**)** showing the fluorescence intensity of (negative control) PIC micelles of unlabelled CEA polymer/LYS (Texas Red), (positive control) PIC micelles of unlabelled CEA polymer/LYS (labelled with both Texas Red and cy5) after an incubation period of 2 h. The donor fluorophore (Texas Red) was excited using a 561 nm laser and the acceptor fluorophore (cy5) was exited at 638 nm. Fluorescence emission was collected with a 595/20 nm bandpass filter for the donor and a 660 nm longpass filter for the acceptor.