Electronic Supplementary Material (ESI) for Chemical Communications.
This journal is © The Royal Society of Chemistry 2018

Light-sheet microscopy as a tool to understanding the behavior of polyelectrolyte-complexes for drug delivery

Fan Chen, Kecheng Li, Gene Hart-Smith, You Dan Xu, Yanyan Jiang, Hongxu Lu, Sandra Fok, Alexander Macmillian, Elvis Pandzic, Martina Stenzel

Methods

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.

Synthesis of block copolymers. In this paper, the block copolymers were synthesized using reversible addition fragmentation chain transfer (RAFT) polymerization. In brief, 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 polymerization at 60 °C for 2 h. The polymerization 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 8200 g mol^{-1} with a polydispersity of 1.17 using *N*,*N*-dimethylacetamide (DMAc) as the mobile phase and poly(methyl methacrylate) (PMMA) standards.

The PPEGMEA-*b*-PCEA was prepared by chain extension of PPEGMEA as a macro-RAFT agent. PPEGMEA (200 mg, 2.13 × 10^{-5} mol) was mixed with CEA (307 mg, 2.13 × 10^{-3} mol) and AIBN (0.70 mg, 4.26 × 10^{-6} mol in 1,4-dioxane (1.23 mL) in a feed ratio of 1:100:0.2. The solution was degassed by freeze-pump-thaw of three cycles. Polymerization was carried out at 60 °C in an oil bath 1.5 h. The polymerization was terminated by immersing the vial

into ice bath and exposure to air. The crude was dialyzed against Milli-Q water for two days followed by lyophilization. The number of repeating units of the CEA block was determined by ^{1}H NMR in DMSO-d6. The dispersity (Đ) of the polymer was obtained from SEC using DMAc as the mobile phase and PMMA standards.

Micellization. The block copolymer, PPEGMEA-*b*-PCEA was 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. The final molar ratios of polymer and HEWL are listed in **Table S2**.

Dynamic light scattering (DLS) measurement. The size, polydispersity index (PDI) and zeta potential of all the samples were determined by 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.

Cell viability assay. PIC micelles prepared by PPEGMEA-*b*-PCEA and HEWL at a molar ratio of 2 were selected for the cell viability assay. 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 (8000 cells/well) in 200 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 fresh 2x concentrated fresh DMEM media. The micelles were sterilized by UV irradiation for 15 min and then serially diluted (2x 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.

Labelling. The block copolymer, PPEGMEA-*b*-PCEA, was labelled with Cy5. The polymer was dissolved in DMSO (1.5 mL) at a concentration of 17.2 mg/mL (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 dialyzing against methanol for one day and Milli-Q water for another day, followed by lyophilization. HEWL was labelled with FITC as follows: HEWL (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.

Crosslinking with 1,8-diaminooctane. The crosslinking method can be found in a previous report of our group.[2] Briefly, 500 µL fluorescently labelled PIC micelles solutions (HEWL (FITC) 0.7 mg/mL, CEA (Cy5) polymer 1.6 mg/mL) were prepared at a polymer/protein molar ratio of 2. Stock solutions of 1,8-diaminooctane (8 mg/mL), EDC (1mg/mL) and NHS (2 mg/mL) were prepared in Milli-Q water. 22.5 µL of 1,8-diaminooctane, 400 µL of EDC and 200 µL of NHS were then added into the micelle solution and allowed stirring overnight in the dark. The mixture was dialyzed against Milli-Q followed by lyophilisation.

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 (0.3 mg HEWL, 0.75 mg CEA polymer, i.e. 0.1 mg/ mL HEWL and 0.25 mg/mL CEA polymer) for 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 cells 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 FlowJo® software.

Spheroids. MCF-7 breast cancer tumour spheroids were prepared using a liquid overlay method. MCF-7 cancer cells (0.2 mL, 1 x 10^4 cells/mL) were seeded into each well of a 96-well U-bottom microplates containing ultra-low attachment (ULA) coating and centrifuged for 5 min at 500 g. The cells were then incubated at 37 °C with 5% CO_2 for 4 days.

Light-sheet microscopy. The prepared cell spheroids were firstly incubated with 0.2 mL fresh DMEM culture media containing dual-colour labelled micelles (20 µg HEWL(FITC), 50 µg CEA(Cy5) polymer) at the same concentration as used in flow cytometry for 2 h before the spheroids were washed with Hank's buffer for 2 times. Each spheroid was then embedded in 1% low-melting-temperature agarose gel and mounted into a glass capillary. After gel

formation, the capillary was inserted into the sample chamber on the microscope (ZEISS light-sheet Z.1) for imaging. 1.3 NA, 20× detection objective and 10× excitation objectives were used for imaging. *z*-Stack images of each spheroid were acquired at 0° and 180° with an interval of 0.76 mm.

Co-localisation. 2 mL of micelles formed by FITC labelled HEWL and Cy5 labelled polymer solution was loaded into a 35 mm glass bottom FluoroDish (World Precision instruments). Co-localization measurements were carried out by laser scanning confocal microscope (CLSM, Zeiss LSM780) using a 63× 1.3 NA glycerol immersion objective. A 488 nm argon laser was used to excite FTIC labelled on HEWL and a 633 nm helium neon laser was used to excite Cy5 labelled on CEA polymer. 490-630 nm and >650 nm emission ranges were used to collect signals from FITC (green channel) and Cy5 (red channel) respectively. Time-lapse images were taken over 48.11 s.

Mass spectrometry. MCF-7 breast cancer cells (2000 cells/well) were firstly grown in Dulbecco's Modified Eagle Medium (DMEM, 1.5 mL) cell culture media for 4 days. The cells were then separately incubated with fresh pure DMEM culture media as well as culture media containing CEA/HEWL micelles (0.38 mg CEA polymer, 0.15 mg HEWL) and HEWL (0.15 mg) for 10 h. Supernatant (1 mL) was taken from each and was then subjected to trypsin digestion (400 µg) for 18 h. The digested peptides from each sample were analysed using standard proteomics techniques using a Q Exactive Plus (Thermo Fisher, Bremen, Germany). Briefly, proteins were identified from peptides via sequence database searches employing MaxQuant (version 1.5.8.0) and Andromeda, run using standard parameters. All sequences in the Swiss-Prot database (February 2017 release) were searched.

Table S1. Summary of RAFT polymerized macro-RAFT agent and block copolymer.

Polymer	**Polymerisation conditions**		M_n (g mol^{-1})[b]	M_n (g mol^{-1})[c]	$Đ$[c]	Conversion[b] (%)
	[M]:[R]:[I][a]	**Time (h.)**				
$PPEGMEA_{18}$	50:1:0.1	2	8912	10862	1.12	36
$PPEGMEA_{18}$-*b*-$PCEA_{52}$	100:1:0.2	1.5	16400	31850	1.07	52

[a] [M]:[R]:[I] = [monomer]:[BSPA]:[AIBN]. [b] Results from ^{1}H NMR. [c] Results from size exclusion chromatography (SEC) using *N,N*-dimethylacetamide (DMAc) as eluent. PPEGMEA was polymerized at 60°C in toluene; PPEGMEA-*b*-PCEA was polymerized at 60°C in 1,4-dioxane.

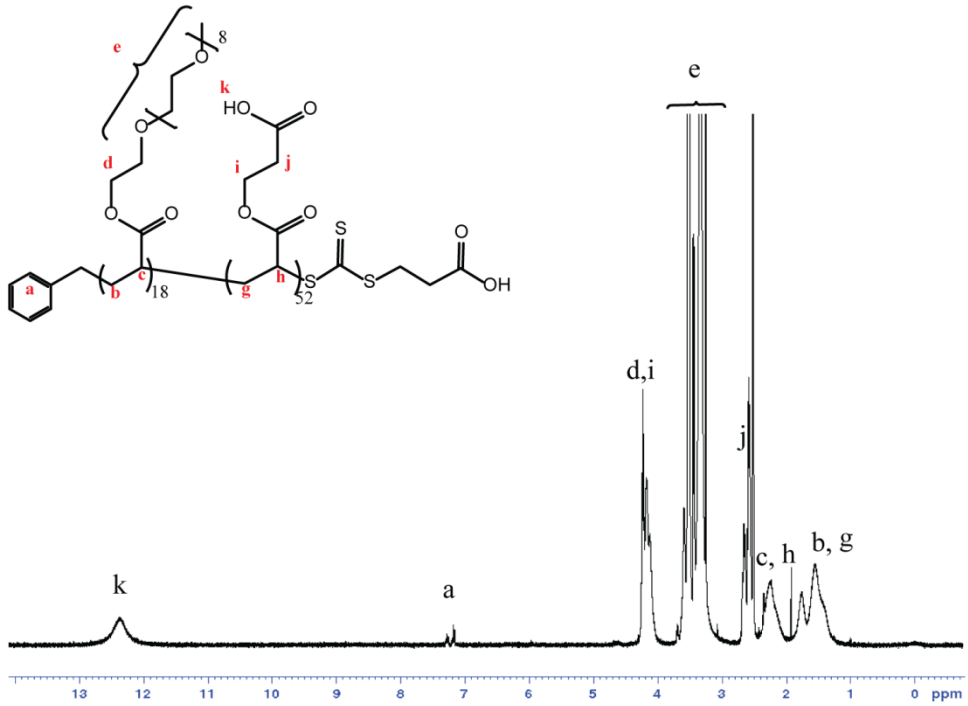

Figure S1. ^{1}H NMR spectrum of PPEGMEA$_{18}$-*b*-PCEA$_{52}$ block copolymer in dimethyl sulfoxide-d6.

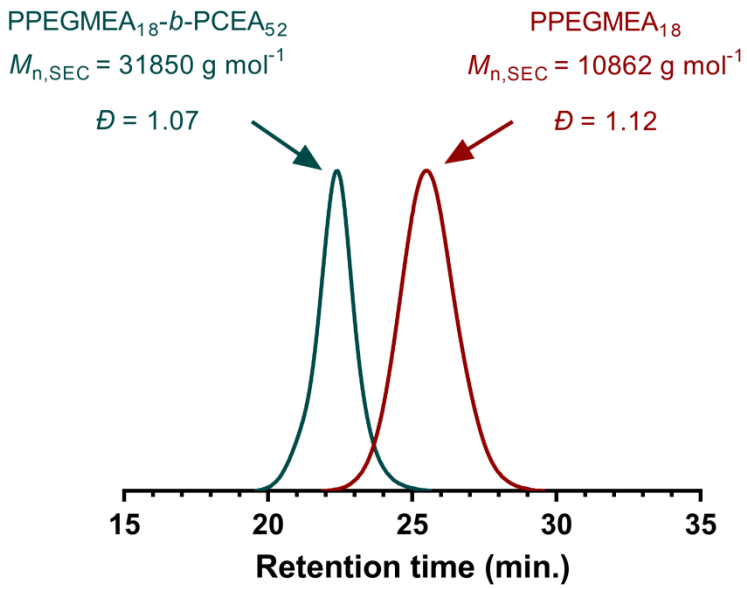

Figure S2. Size exclusion chromatography (SEC) traces of PPEGMEA$_{18}$ and PPEGMEA$_{18}$-*b*-PCEA$_{52}$ block copolymer using *N*,*N*-dimethylacetamide (DMAc) as eluent.

Table S2. Summary of polyion complex (PIC) micelles formed with different polymer to lysozyme (HEWL) molar ratios (the concentration of HEWL was kept constant to be 0.1 mM).

Micelle	Polymer/protein molar ratio	Size (nm)[a]	PDI[a]	Zeta potential (mV)[a]	Median FITC[b]	Relative cellular uptake (%)[c]
Control	-	-	-	-	193 ± 5	18 ± 1
Positive	0.5:1	52.73 ± 0.33	0.115 ± 0.008	9.82 ± 0.95	1070 ± 144	100 ± 13
Neutral	2:1	43.89 ± 0.35	0.060 ± 0.007	-1.57 ± 0.21	912 ± 59	85 ± 5
Negative	4:1	45.86 ± 0.10	0.098 ± 0.008	-7.96 ± 0.38	995 ± 22	93 ± 2

Note: Data are obtained from [a]DLS and [b]flow cytometry, data are expressed as mean ± SD (n = 3); [c] positive micelle was regarded as 100% cellular uptake.

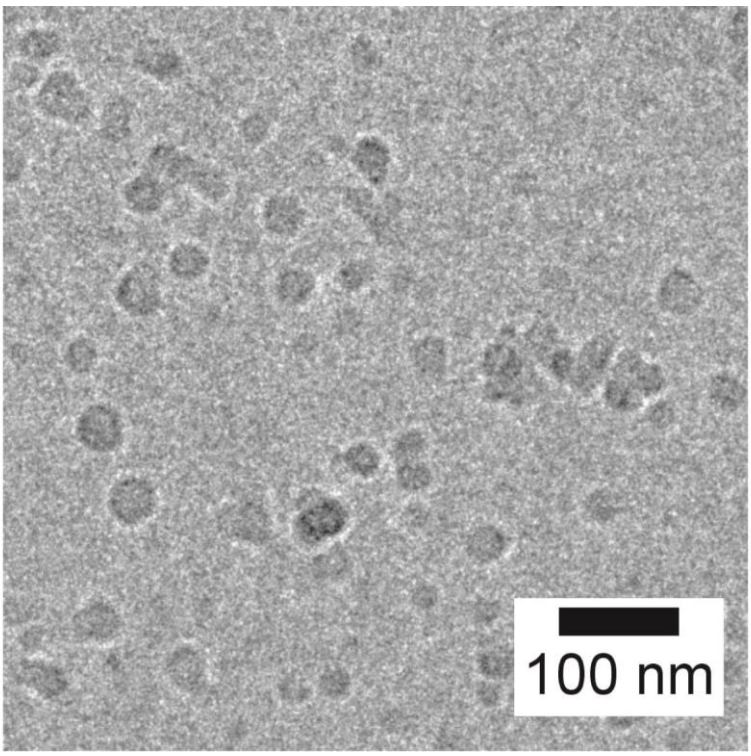

Figure S3. Cryo-TEM image of CEA/HEWL micelles at a molar ratio of 2. The image was taken at a slight defocus to improve image contrast.

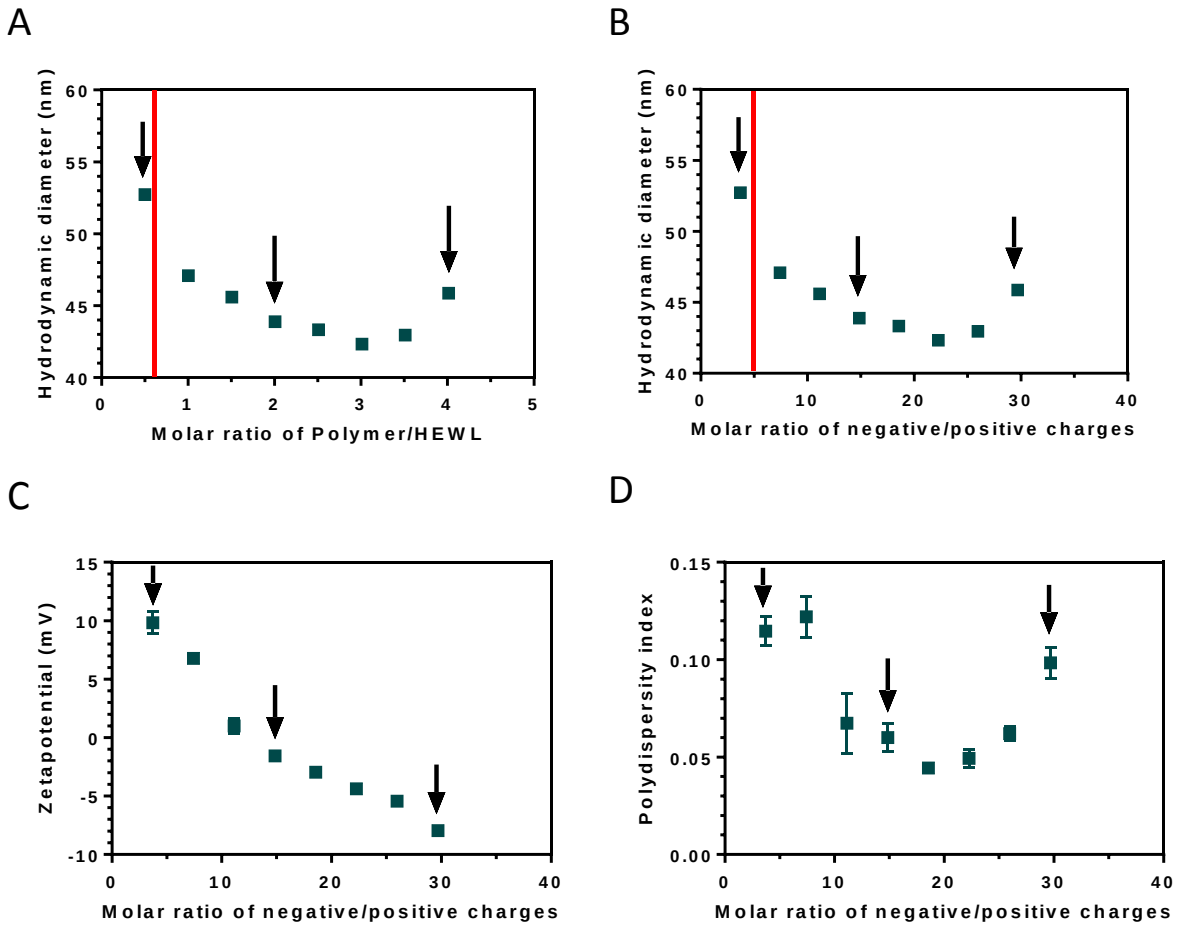

Figure S4. A) *z*-Averaged hydrodynamic diameters of polyion complex (PIC) micelles formed by $PPEGMEA_{18}$-*b*-$PCEA_{51}$ with Lysozyme (HEWL, 0.05 mM) in correlation to the molar ratio of polymer to protein B) in correlation to the employed molar ratio of negative (52 COO^- charges per polymer according to a pKa value of 5.1) to positive (positive net surface charge is 7 according to Kuehner et al *J. Phys. Chem. B* **1999,** *103*, 1368-1374) (C) zetapotential and (D) polydispersity in correlation to charge ratio. It needs to be noted here that the measured amount present an average value as depending on the amount of both ingredients, polymers or proteins can exist in excess forming bimodal distributions.

The arrow indicates the ratios used in this study expressed as molar ratios of polymer and protein or as molar ratio between negative and positive charges. Although an equimolar ratio of both charges should lead to full surface coverage, the uneven distribution of charges on the protein surface as well as the ability of the polymer to stretch between two positive charges require more polymers for coverage. Isothermal calorimetry titration reveals a binding stoichiometry of 1 mol polymer to 1.73 mols of protein, which is indicated above as red line. The equilibrium constant K is 4.04×10^5 M^{-1}.

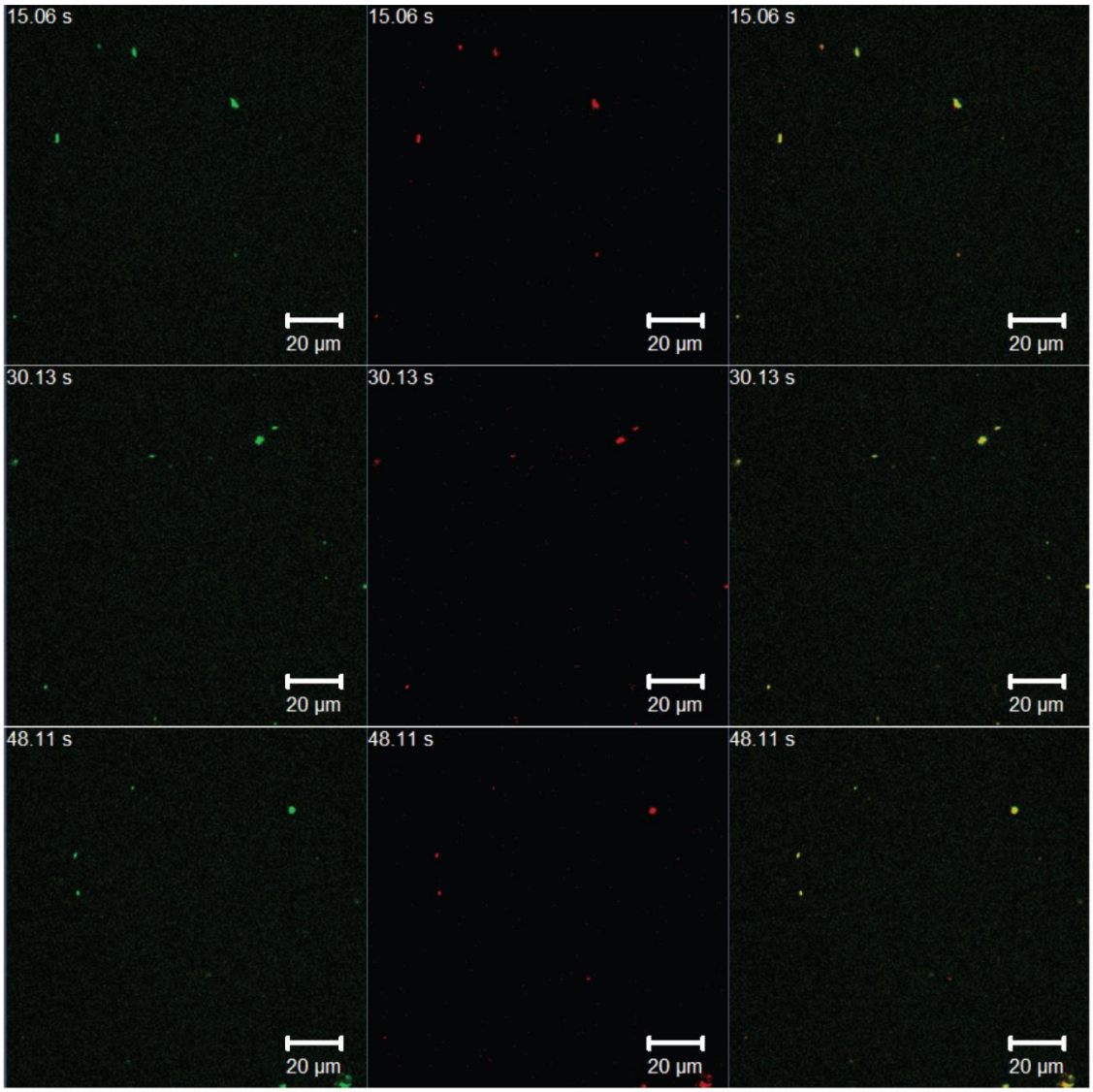

Figure S5. Observation of CEA polymer and lysozyme co-localisation in DMEM cell culture media using confocal laser scanning microscopy (CLSM). The red colour represents Cy5 labelled CEA polymer, green is FITC labelled lysozyme and yellow is the overlay of CEA polymer and lysozyme.

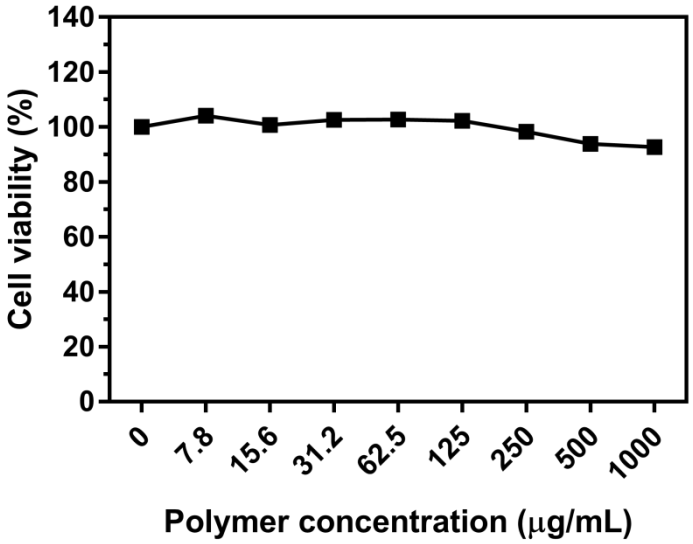

Figure S6. Cell viability of MCF-7 human breast cancer cells after incubation with Dulbecco's Modified Eagle Medium (DMEM) cell culture media containing different concentrations of $PPEGMEA_{18}$-*b*-$PCEA_{52}$ block copolymer for 3 days.

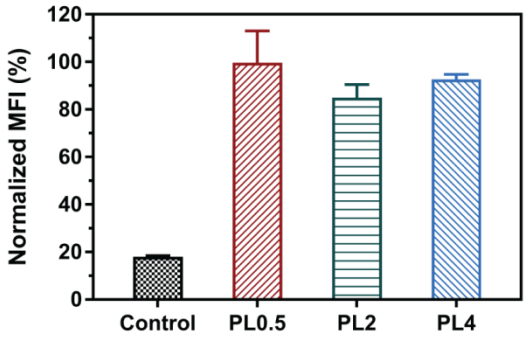

Figure S7. Flow cytometry analysis of PIC micelles with various polymer/protein molar ratios, i.e. polymer/lysozyme molar ratio of 0.5 (PL0.5, 10 mV), 2 (PL2, -1.5 mV) and 4 (PL4, -8 mV) on MCF-7 breast cancer cells. The median fluorescence intensity (MFI) of FITC conjugated lysozyme was measured after an incubation of 2 h (n = 3) using an excitation of 488 nm. The emission was collected with a 530/30 band-pass filter. The fluorescence intensity of PL0.5 micelles was regarded as 100% for normalization.

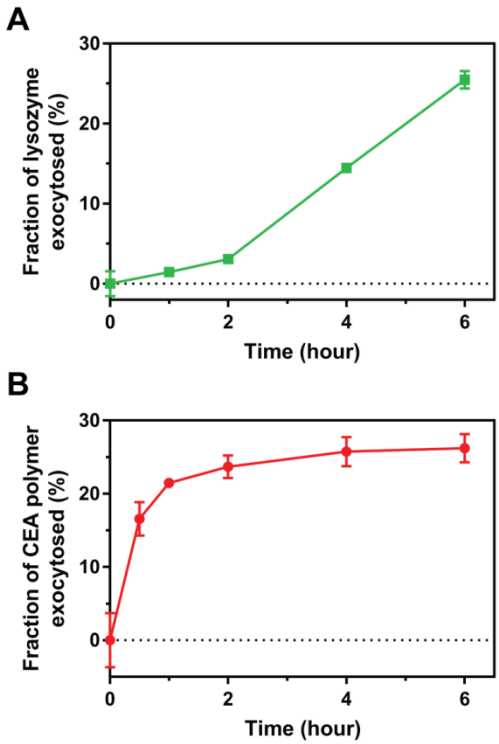

Figure S8. A flow cytometry study of PIC micelles, i.e. lysozyme **(A)** and CEA polymer **(B).** The PIC micelles with a molar ratio of 2 were incubated with MCF-7 monolayers (not 3D models) for 6 h. The PIC micelle containing cell growth media was replaced with fresh media to allow exocytosis. The cells were analysed using flow cytometry and the content of polymer and lysozyme was compared to the sample at t=0 (replacement of media) using flow cytometry.

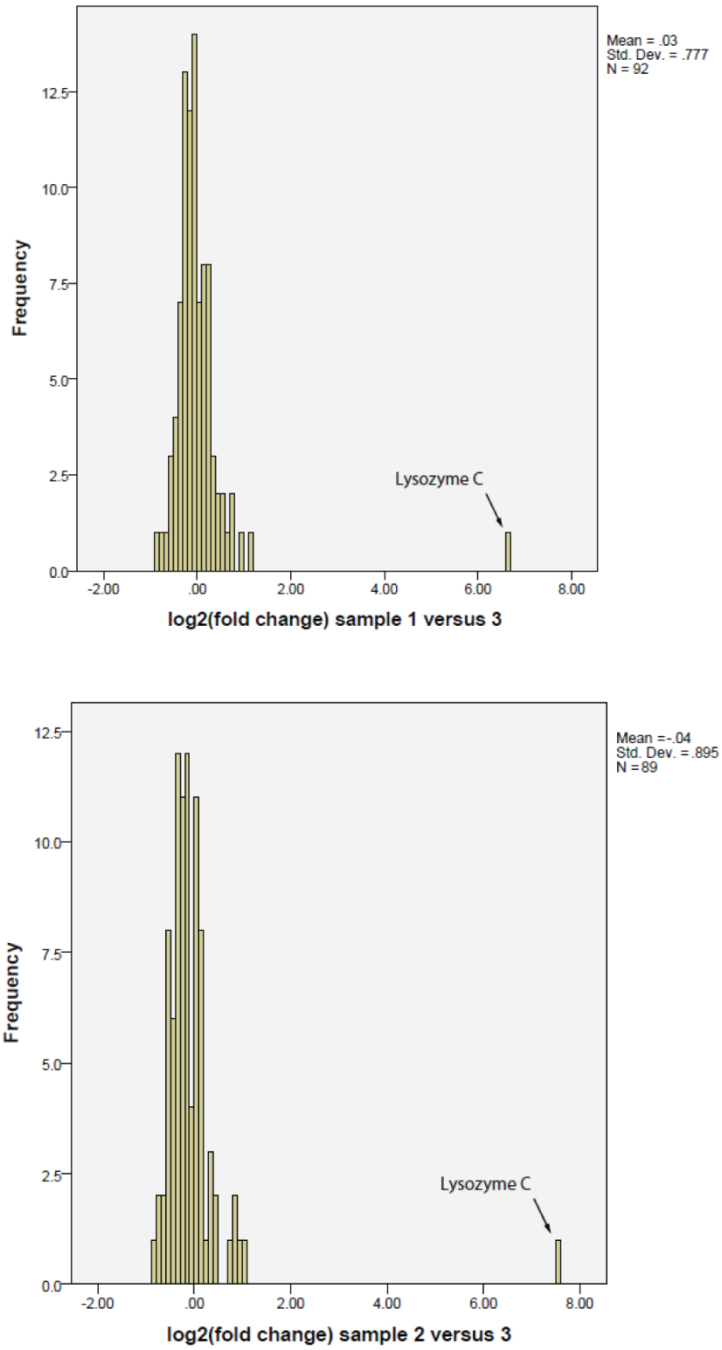

Figure S9. Fold changes of lysozyme from chicken egg white (lysozyme C) in sample: 1. Polymer/lysozyme; 2. Lysozyme; 3. Cell culture media incubated with MCF-7 human breast cancer cells for 18 h respectively.

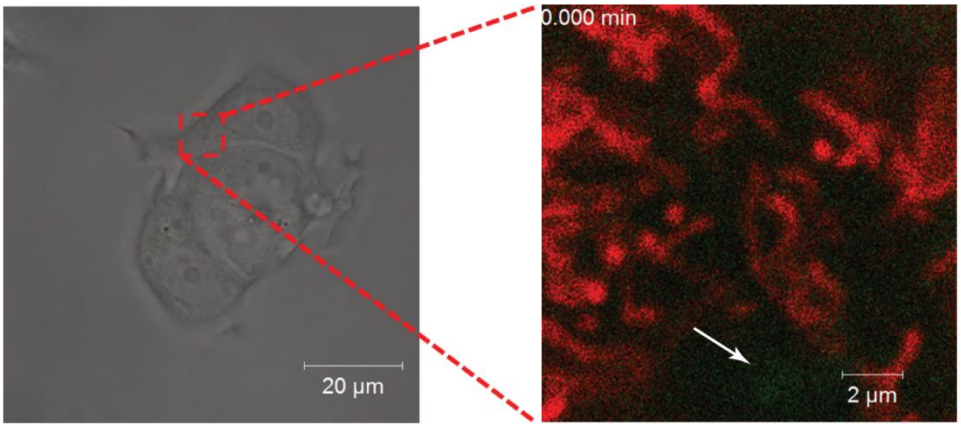

Figure S10. Confocal images of (left) MCF-7 human breast cancer cells, and (right) separation of CEA polymer (red) and lysozyme (green) inside the cell. The arrow points released lysozyme to highlight the faint green area. The images were taken by confocal laser scanning microscopy.

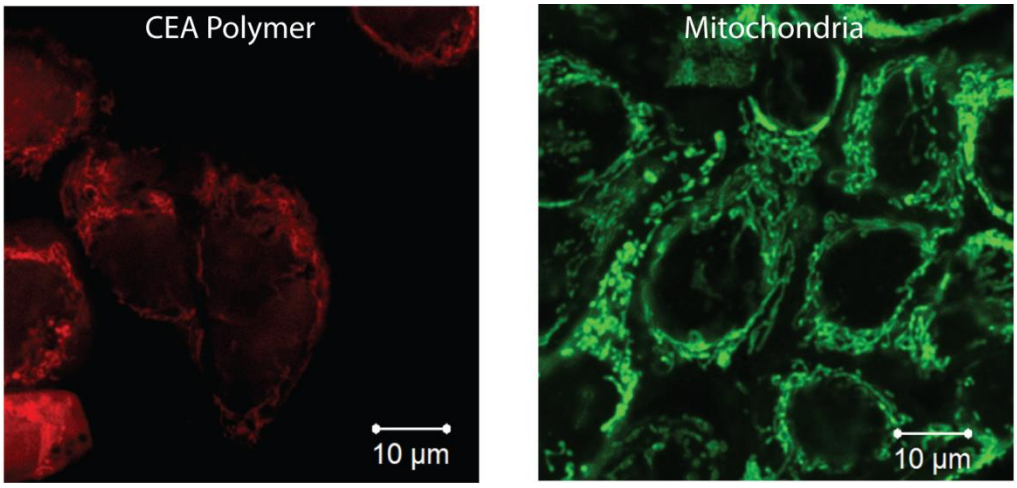

Figure S11. A shape comparison of (left) fluorescein labelled CEA polymer in MCF-7 human breast cancer cells and (right) mitochondria labelled with mito tracker deep red after 40 min incubation with cells. The images were taken by confocal laser scanning microscopy.

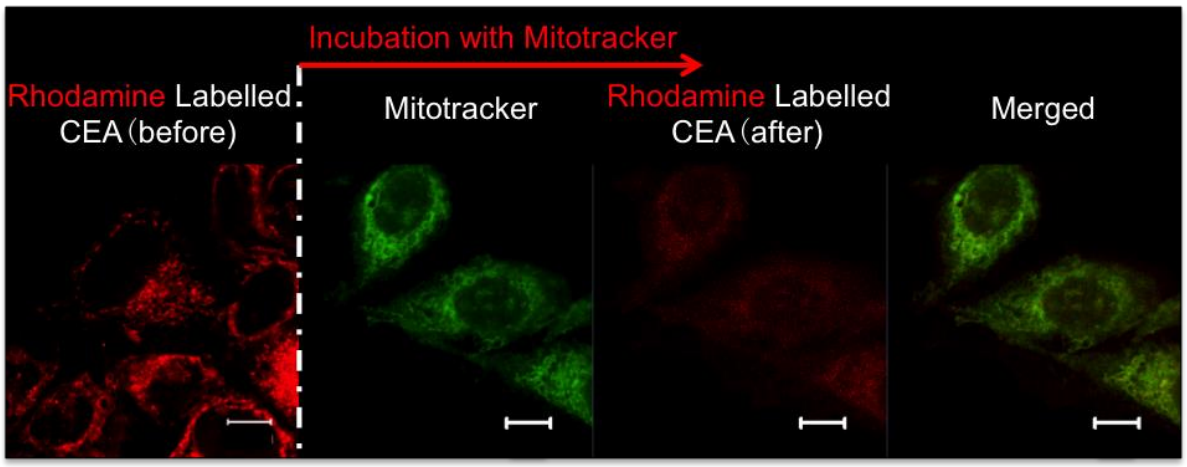

Figure S12. Confocal images of PL2 micelles (i.e. formed with a polymer/lysozyme (non-labelled) molar ratio of 2) with rhodamine B labelled on CEA polymers in MCF-7 breast cancer cells after incubation for two hours (left) CEA (red) before mitochondria labelling (right) after mitochondria labelling with mitotracker (green) (procedure from Cell Signaling Technology) showing staining of the mitochondria by the mitotracker and the disappearance of the tubular shape caused by the polymer suggesting that the mitotracker competes with the CEA polymer for the mitochondria surface. The mitotracker was incubated with cells for 40 min before images were taken. Scale bar: 10 μm.

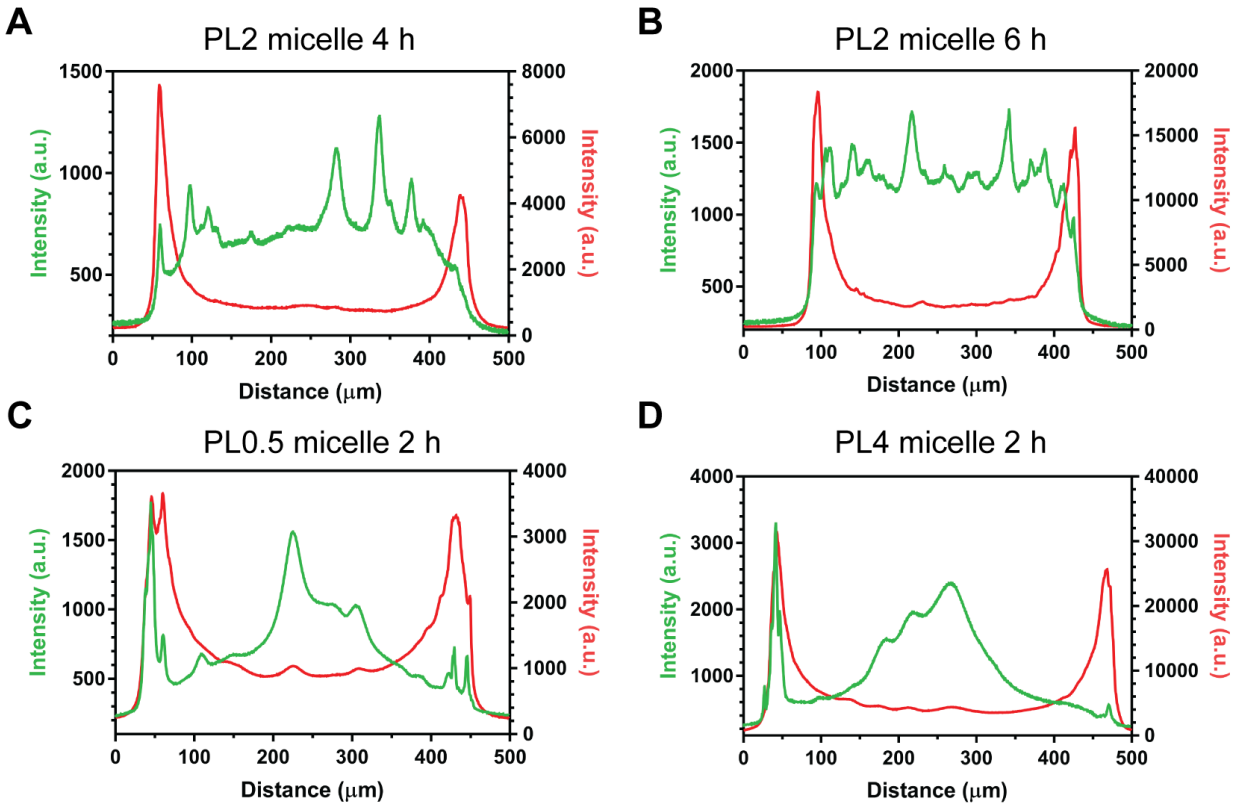

Figure S13. Distributions of CEA polymer (red) and lysozyme (green) in MCF-7 multicellular cell spheroids (MCTS). PL2 micelles (i.e. polymer to lysozyme molar ratio of 2, -1.5 mV) incubated with MCTS after **(A)** 4 h and **(B)** 6 h, **(C)** PL0.5 micelles (i.e. polymer to protein molar ratio of 0.5, 10 mV) and **(D)** PL4 micelles (i.e. polymer to protein molar ratio of 4, -8 mV) incubated with MCF-7 breast cancer MCTS after 2 h.

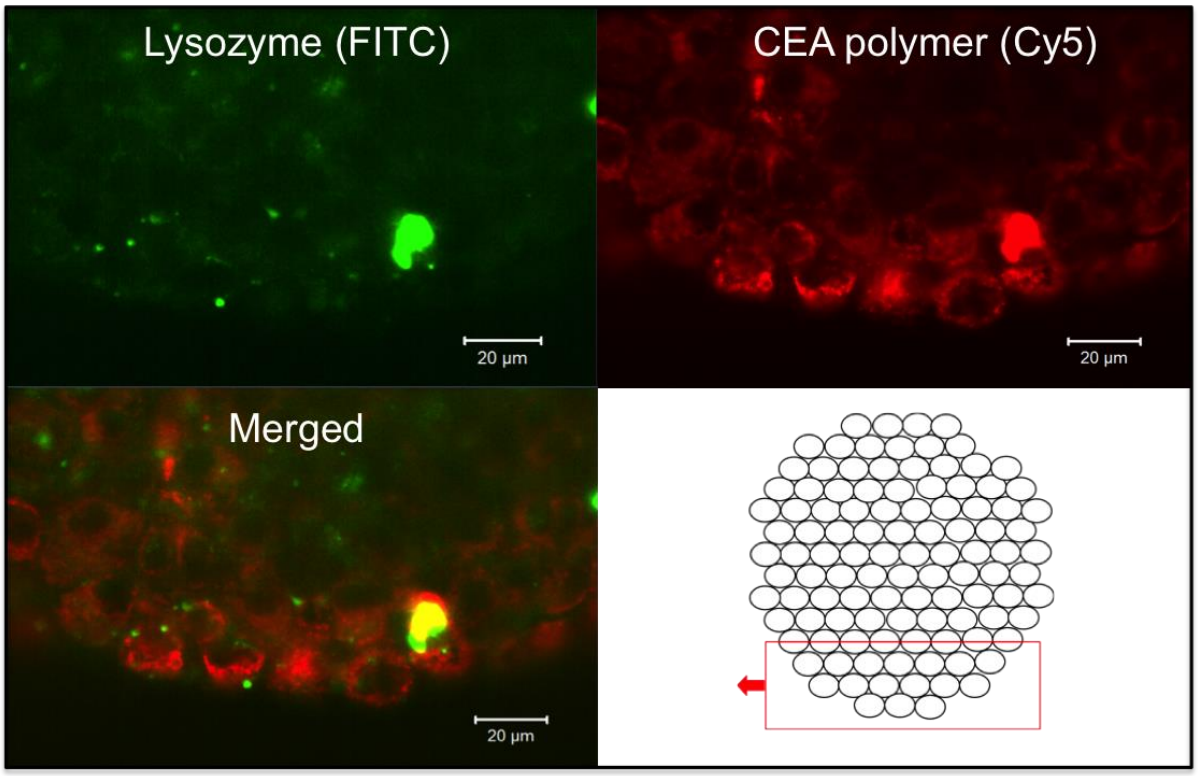

Figure S14. Distribution of polymer (red) and protein (green) in MCF-7 breast cancer multicellular cell spheroids (MCTS) after an incubation of 2 h. Scale bar: 20 µm. The images were taken by light-sheet fluorescence microscopy. The shown slice is at a depth of 30 µm from top to bottom of the spheroid.

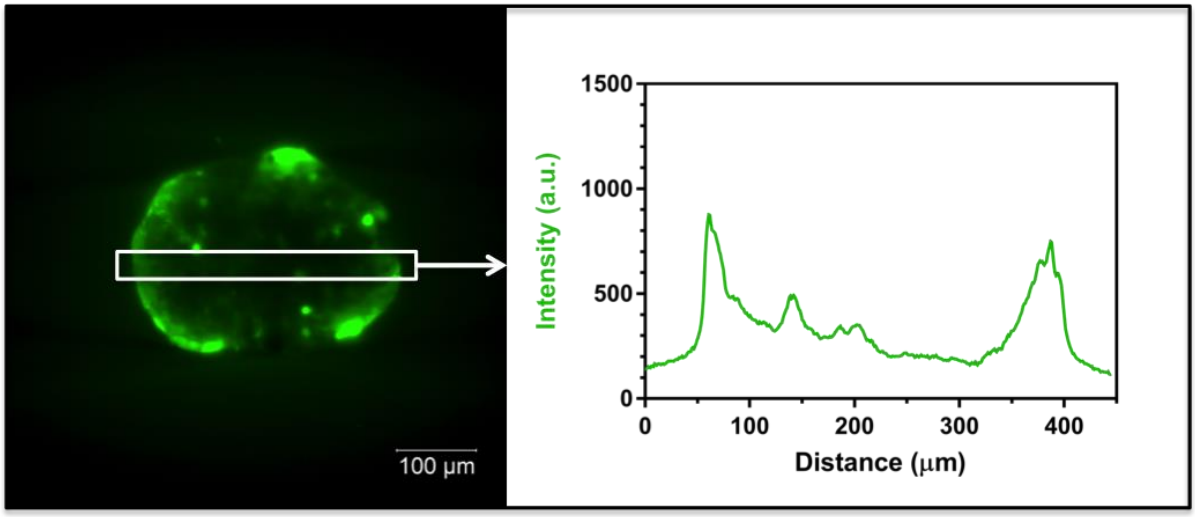

Figure S15. Distribution of the protein lysozyme in MCF-7 breast cancer multicellular cell spheroids (MCTS) after an incubation of 4 h. The images were taken by light-sheet fluorescence microscopy.

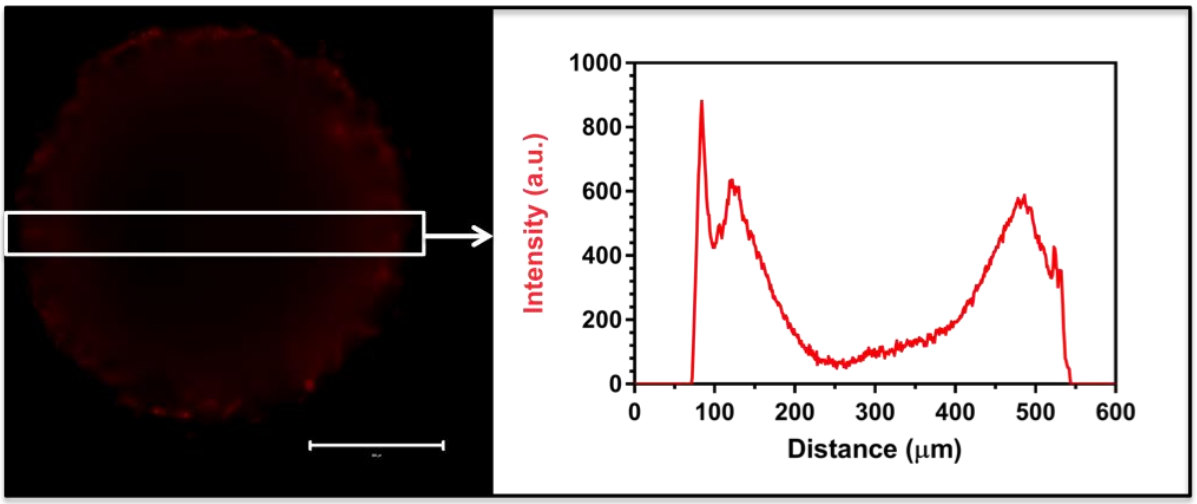

Figure S16. Distribution of Cy5 labelled free CEA polymer in MCF-7 breast cancer multicellular cell spheroids (MCTS) after an incubation of 2 h. Scale bar: 200 μm. Although the spheroid appears fluorescent throughout indicating some penetration of the polymer, the overall fluorescent intensity is very low. The images were taken by light-sheet fluorescence microscopy.

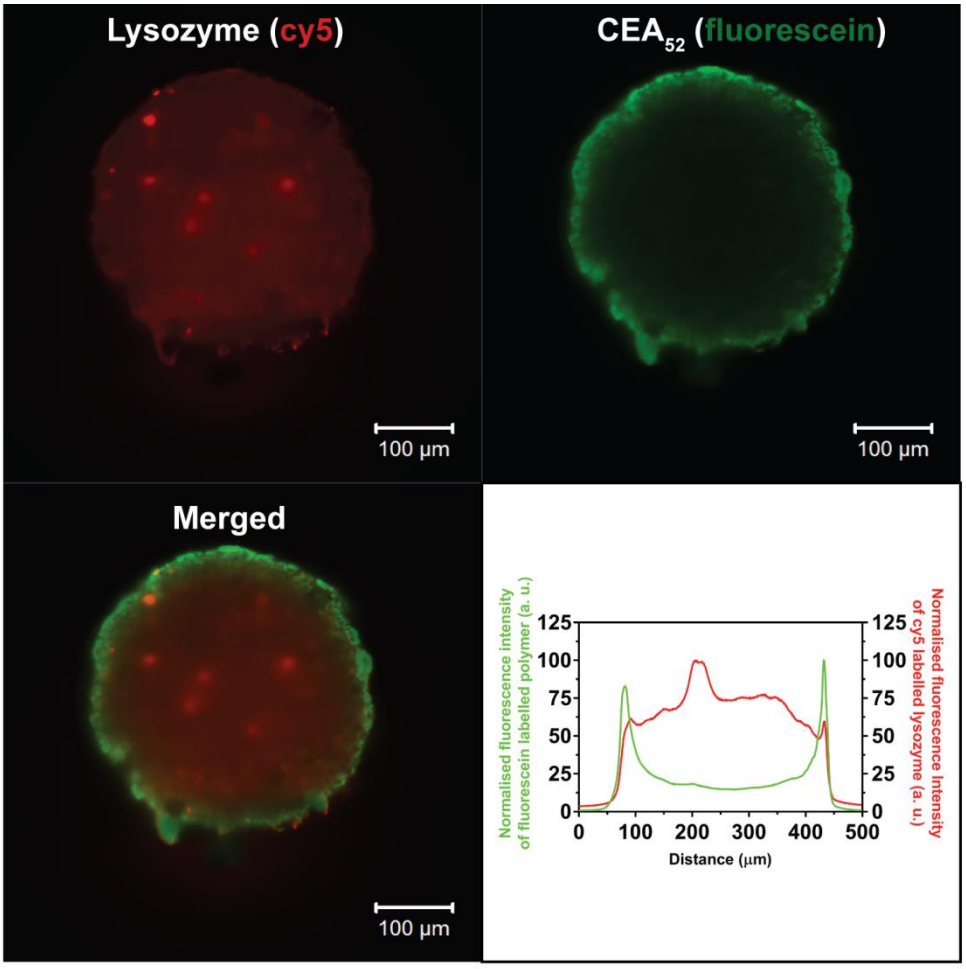

Figure S17. Analysis of penetration ability of PL2 micelles under light-sheet microscopy after an incubation time of 2 h. **(A)** Cy5 labelled lysozyme (red), **(B)** FITC labelled CEA polymer (green), **(C)** Merged image of CEA polymer and lysozyme, and **(D)** fluorescence intensity profiles. The images were taken by light-sheet fluorescence microscopy.

1. Dag, A.; Zhao, J.; Stenzel, M. H., Origami with ABC Triblock Terpolymers Based on Glycopolymers: Creation of Virus-Like Morphologies. *ACS Macro Letters* **2015,** *4*, 579-583.
2. Du, A. W.; Lu, H.; Stenzel, M. H., Core-Cross-Linking Accelerates Antitumor Activities of Paclitaxel–Conjugate Micelles to Prostate Multicellular Tumor Spheroids: A Comparison of 2D and 3D Models. *Biomacromolecules* **2015,** *16*, 1470-1479.