Supplementary information

Formation of Well-Defined, Functional Nanotubes *via* Osmotically Induced Shape Transformation of Biodegradable Polymersomes

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Materials and Methods

Materials

All chemicals and enzymes were used as received unless otherwise stated. For the synthesis of the block copolymer, monomethoxy PEG_{44}-OH (2 kDa) and azido-PEG_{75}-OH (3.3 kDa) were purchased from Rapp Polymers whereas $mPEG_{22}$-OH (1 kDa) was purchased from Creative PEG Works – all polymers were used as supplied. D,L-Lactide was purchased from Acros Organics and used as supplied. Bicyclo[6.1.0]nonyne – lysine used in the preparation of GFP^{BCN} was purchased from Synaffix BV and used without further purification. All other chemicals were supplied by Sigma-Aldrich. Ultra pure MilliQ water obtained from Labconco Water Pro PS purification system (18.2 MΩ) was used for the polymersome self-assembly and their dialysis. Dialysis Membranes MWCO 12-14000 g mol^{-1} Spectra/Por® were used for dialysis during the tubes formation. Sodium chloride was purchased from Merck.

Methods

Nuclear Magnetic Resonance (NMR): Proton NMR spectra were recorded on a Bruker Avance 400MHz spectrometer with $CDCl_3$ as a solvent and TMS as internal standard.

Gel permeation chromatography (GPC): GPC was conducted using a Shimadzu Prominence GPC system with a PL gel 5 μm mixed D column (Polymer Laboratories) with a differential refractive index detector and THF used as an eluent with a flow rate of 1 mL min^{-1}.

Differential scanning calorimetry (DSC): DSC was conducted using a Mettler Toledo DSC822 calorimeter.

Dynamic light scattering measurements (DLS): DLS measurements were conducted using a Malvern Instruments Zetasizer (ZEN 1600), and Zetasizer Software (Malvern Instruments) was used for processing and analyzing the data.

Cryogenic transmission electron microscopy (cryo-TEM): Experiments were performed using a JEOL TEM 2100 microscope (JEOL, Japan). Analysis and processing of the data was performed using ImageJ, a program developed by the NIH and available as public domain software at http://rsbweb.nih.gov/ij/.

Asymmetric Flow Field-Flow Fractionation and multi-angle light scattering (AF4-MALS): The asymmetric flow field-flow fractionation – UV – Quels (AF4-UV-Quels) experiments were performed

on a Wyatt Eclipse AF4 instrument connected to a Shimadzu LC-20A Prominence system with Shimadzu CTO20A injector. The AF4 was further connected to the following detectors: a Shimadzu SPD20A UV detector, a Wyatt DAWN HELEOS II light scattering detector (MALS) installed at different angles (12.9 º, 20.6 º, 29.6 º, 37.4 º, 44.8 º, 53.0 º, 61.1 º, 70.1 º, 80.1 º, 90.0 º, 99.9 º, 109.9 º, 120.1 º, 130.5 º, 149.1 º, and 157.8 º) using a laser operating at 664.5 nm and a Wyatt Optilab Rex refractive index detector. Detectors were normalized using Bovine Serum Albumin and for enzyme molecular weight calculations, dn/dc of 0.1850 was used. The processing and analysis of the LS data and radius of gyration (R_G) calculations were performed on Astra 6.1.1 software (using the Berry model, which is recommended for particles of size > 50 nm). All AF4 fractionations were performed on an AF4 short channel with regenerated cellulose (RC) 10 KDa membrane (Millipore) and spacer of 350 μm.

Confocal microscopy: Confocal microscopy was performed using the Leica Microsystems SP8x system with a 40x objective and HyD detector, measuring at a speed of 1800 Hz. Images were obtained using time gating in order to nullify the contribution of scattering to the fluorescence signal. For imaging of GFP^{BCN}-modified nanotubes excitation at 480 nm and emission from 490-600 nm was used, along with a 2 airy pinhole. For imaging of DOX-loaded nanotubes excitation at 480 nm and emission from 490-700 nm was used, along with a 1 airy pinhole (and lower laser intensity).

Flow cytometry measurements on modified nanotubes: Flow cytometry measurements were performed using a Gallios™ flow cytometer (Beckman Coulter) with side scatter triggering and either 1 minute or 50,000 events of accumulation for each sample analysed. Separate fluorescent channels were used to detect GFP (490/520 nm) and doxorubicin (490/560 nm) fluorescence.

Nanotube purification via size exclusion chromatography (SEC): SEC purification of the modified nanotubes was conducted using a Shimadzu system (equipped with a SPD-M20A photodiode array) with a Superose 6 column (GE Healthcare) using an eluent identical to that of the nanotube solution.

Experimental Procedures

*Synthesis and characterization of poly(ethylene glycol) – poly(D,L-Lactide) block copolymers***:** The synthesis of PEG-PDLLA was performed according to a modified literature procedure.[1] Monomethoxy-PEG-OH macroinitiator was weighed into a round bottom flask along with D,L-Lactide (DLL) in order to obtain around 13 wt% PEG in the final copolymer. This means that in order to obtain a copolymer with composition PEG_{22}-$PDLLA_{45}$, 0.194 g (0.2 mmol) of mPEG and 1.3 g (9 mmol) of DLL were used with values being doubled when using $mPEG_{44}$. However, when using azido-PEG_{75}-OH, the stoichiometry was adjusted in order to achieve a PDLLA chain with 45 repeat units. In all cases, dry toluene (*ca*. 50 mL) was then added to the flask and the solvent evaporated in order to dry the contents before polymerization. The dried reagents were then re-dissolved in dry DCM (13 mL, [monomer] = 0.5M) and DBU was added (0.5 equiv. with respect to [initiator]; 0.1 mmol = 15 μL) under argon. The reaction was stored at RT for around 2 hours, until there was no evidence of the monomer from the ^{1}H-NMR spectra (**Figure S1**). After completion was confirmed by ^{1}H-NMR, the reaction mixture was diluted using DCM and washed twice with 1 M $KHSO_4$ and once with brine before drying with Na_2SO_4, filtering and evaporating most of the solvent. The concentrated copolymer solution (in DCM) was then precipitated into ice cold diethyl ether (100 mL) and the remaining wax was partially dried under nitrogen before dissolving in dioxane and lyophilisation to yield a white powder (75-85 % yield).

Copolymer composition was calculated by using the protons of PEG (3.65-3.7 ppm), terminal methyl unit (singlet at 3.40 ppm), lactide CH_3 (multiplet at 1.55-1.65 ppm) and CH (multiplet at 5.15-5.25 ppm) (**Figure S2**). All PDI values were calculated to be less than 1.1 using PEG standards for calibration (**Figure S3, table below**), demonstrating that the reaction conditions and base catalyst gave good control over polymerization. Differential scanning calorimetry (DSC) was performed by

scanning from -20 °C up to 80 °C at 5 °C/min, with T_g values taken from the second heating run. For PEG_{22}-$PDLLA_{45}$ and PEG_{42}-$PDLLA_{90}$ the T_g values were 26.2 and 30.4 °C, respectively (**Figure S4**).

Copolymer	DP (NMR)	Mw / kDa	GPC (PEG standards)		
			Mw / kDa	Mn / kDa	Đ
PEG_{22}-$PDLLA_x$	45	7.5	14.01	13.07	1.07
PEG_{44}-$PDLLA_x$	90	14.9	26.98	24.99	1.08
N_3-PEG_{75}-$PDLLA_x$	45	9.8	15.38	14.17	1.09

Preparation of polymersomes: In a 15 mL vial, block-copolymer PEG_{22}-$PDLLA_{45}$ (20 mg) was dissolved in 2 mL of organic solvent - a mixture of distilled THF and dioxane (4:1 v/v). A magnetic stirring bar was added and the vial was sealed with a rubber septum. The solution was let to stir for at least 30 minutes. Afterward, 2 mL of Milli-Q water was added via a syringe pump at a rate of 1 mL h^{-1}. After 2 hrs, the resulted cloudy suspension was transferred into a dialysis membrane (SpectraPor, molecular weight cut-off: 12,000-14,000 Da, flat width 25 mm), which was pre-hydrated. The polymersomes were dialyzed at 4 °C against precooled water (1 L) over 24 hrs with a water change after 1 hr.

Osmotically-induced shape transformation of spherical polymersomes into elongated nanotubes: In a 15 mL vial, block-copolymer PEG_{22}-$PDLLA_{45}$ (20 mg) was dissolved in 2 mL of organic solvent - a mixture of distilled THF and dioxane (4:1 v/v). A magnetic stirring bar was added and the vial was sealed with a rubber septum. The solution was let to stir for at least 30 minutes. Afterward, 2 mL of Milli-Q water was added via a syringe pump at a rate of 1 mL h^{-1}. After 2 hrs, the resulted cloudy suspension was transferred into a dialysis membrane (SpectraPor, molecular weight cut-off: 12,000-14,000 Da, flat width 25 mm), which was pre-hydrated. The polymersomes were dialyzed at 4 °C against precooled sodium chloride solution (1 L of 5, 10 or 50 mM) over 24 hrs with a solution change after 1 hr.

Loading the nanotubes with doxorubicin: Hydrophobic, free-base doxorubicin (DOX) was obtained by stirring the hydrochloride salt (DOX.HCl) with a stoichiometric amount of triethylamine overnight before extraction with DCM and lyophilisation. In a 15 mL vial, PEG_{22}-$PDLLA_{45}$ (20 mg) and DOX (0.4, 1.1 and 2.2 mg) were together dissolved in 2 mL of THF : dioxane (4 : 1 v/v). The solution was let to stir for 30 minutes followed by addition of 2 mL of 10 mM EPPS buffer solution (pH 9) with a rate of 1 mL h^{-1}. The resulting dispersion was transferred into a prehydrated dialysis membrane (SpectraPor, molecular weight cut-off: 12,000-14,000 Da, flat width 25 mm) and dialyzed at 4 °C against 50 mM precooled sodium chloride solution (1 L) over 24 hrs with a solution change after 1 hr. Nanotubes encapsulating DOX were purified using size exclusion chromatography.

Formation of azide functional nanotubes (N_3-nanotubes): Block copolymers PEG_{22}-$PDLLA_{45}$ and N_3-PEG_{75}-$PDLLA_{45}$ were combined in either 2 or 5 wt% mixtures prior to polymersome formation. 20 mg of the polymer mixture was dissolved in 2 mL of THF:dioxane 4 : 1 v/v. The vial was sealed with a rubber septum and the solution was left to stir for at least 30 min. Afterward, 2 mL of Milli-Q water was added via a syringe pump at a rate of 1 mL h^{-1}. After 2 hrs, the resulting cloudy suspension was transferred into a pre-hydrated dialysis membrane (SpectraPor, molecular weight cut-off: 12,000-14,000 Da, flat width 25 mm). The polymersomes were dialyzed at 4 °C against 50 mM precooled sodium chloride solution (1 L) over 24 hrs with a solution change after 1 hr.

Expression & Characterization of eGFP^{BCN}: The construction of the plasmids encoding eGFP^{Y39TAG} and the $tRNA_{pyr}$/pylRS pair was described previously.[2] The plasmids were transformed into *E.coli* TOP10 (Invitrogen) cells and grown on agar plates containing 100 µg/mL ampicillin and 25 µg/mL chloramphenicol. One colony was grown in LB medium overnight at 30 °C 200 rpm. The cells were pelleted and resuspended in 800 mL TB medium containing 100 µg/mL ampicillin and 25 µg/mL chloramphenicol to an OD_{600} of 0.05 and grown at 37 °C, 200 rpm. Protein expression was induced by adding 0.02 w/v % L-arabinose at an OD_{600} of 0.6. Twenty minutes before induction, bicyclo [6.1.0] nonyne – lysine was dissolved in 0.2 M NaOH, 15 v/v % DMSO and added to a final concentration of 1 mM. Expression was allowed to continue for 16 hours at 37 °C, 200 rpm. Cells were collected by centrifugation (20 minutes, 4 °C, 3000 g) and resuspended in resuspension buffer (0.55 M NaCl, 0.01 M KCl, 0.04 M Na_2HPO_4, 0.007 M KH_2PO_4, 0.1 mM PMSF, 10 mM imidazole). Cells were lysed by sonication and the cell debris was removed by centrifugation (30 minutes, 4 °C, 15000 g). Ni-NTA beads were added to the supernatant and incubated for 2 hours at 4 °C. Ni-NTA beads were collected by gravity flow and the beads were washed with resuspension buffer containing 20 mM imidazole. eGFP^{BCN} was eluted by increasing imidazole concentration to 200 mM. The obtained fraction was concentrated with centrifugal dialysis membranes (4 kDa MWCO) and purified on an Agilent Bio-Inert HPLC with an Agilent BIO-SEC 5 150 Å column (flow 2 mL/min, mobile phase phosphate buffered saline pH 7.4). Fractions containing eGFP^{BCN} were pooled and analyzed on a 12% SDS-PAGE gel. The purity was sufficient for the sought application, concentration of active eGFP^{BCN} was determined by measuring absorbance at 488 nm (ε 55000 $cm^{-1}M^{-1}$).[3] Total yield was ~ 6 mg. Approximately 15 µg were dialyzed to MQ with centrifugal dialysis membranes (4 kDa MWCO); mass spectra were recorded by ESI on a JEOL-AccuTOF. The obtained mass of 28853 Da is in good agreement with the theoretical mass of 28854 Da. Mass spectrum, SEC trace and SDS-PAGE gel for the eGFP^{BCN} are included in Figure S10.

Covalent tethering of eGFP^{BCN} to N_3-nanotubes: The concentration of eGFP^{BCN} (in PBS) after purification was calculated to be 73 µM (calculated by UV-Vis). The concentration of functional groups on the surface of nanotubes comprising 2 and 5 wt% azido copolymer were calculated to be *ca*. 3 and 7 µM, respectively, in the dialysed nanotube solution. For each reaction mixture, 500 µL of N_3-nanotubes were mixed with 100 µL of diluted eGFP^{BCN} solution in order to give either an equimolar amount or 3-fold excess of the protein with respect to surface azides. The N_3-nanotube + eGFP^{BCN} mixtures were shaken at 4 °C for 24 hours before purification by SEC. Cryo-TEM was used to confirm the tubular morphology before further characterisation was performed.

Supplementary Figures and Tables

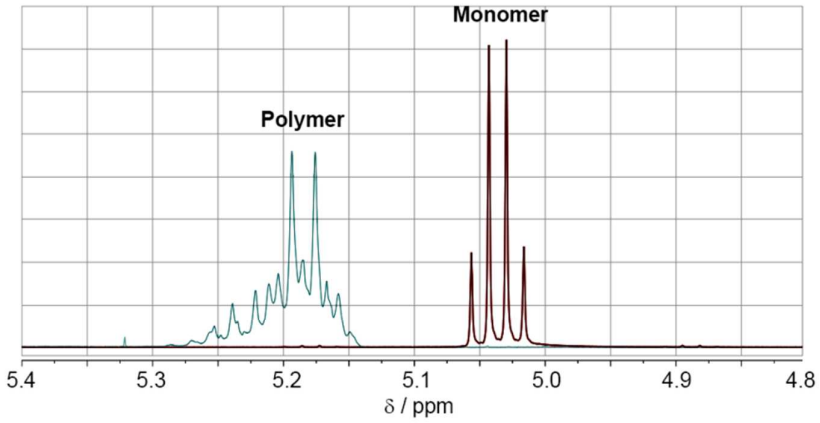

Figure S1. Comparison of the -CH- peaks for monomeric and polymeric DL-lactide

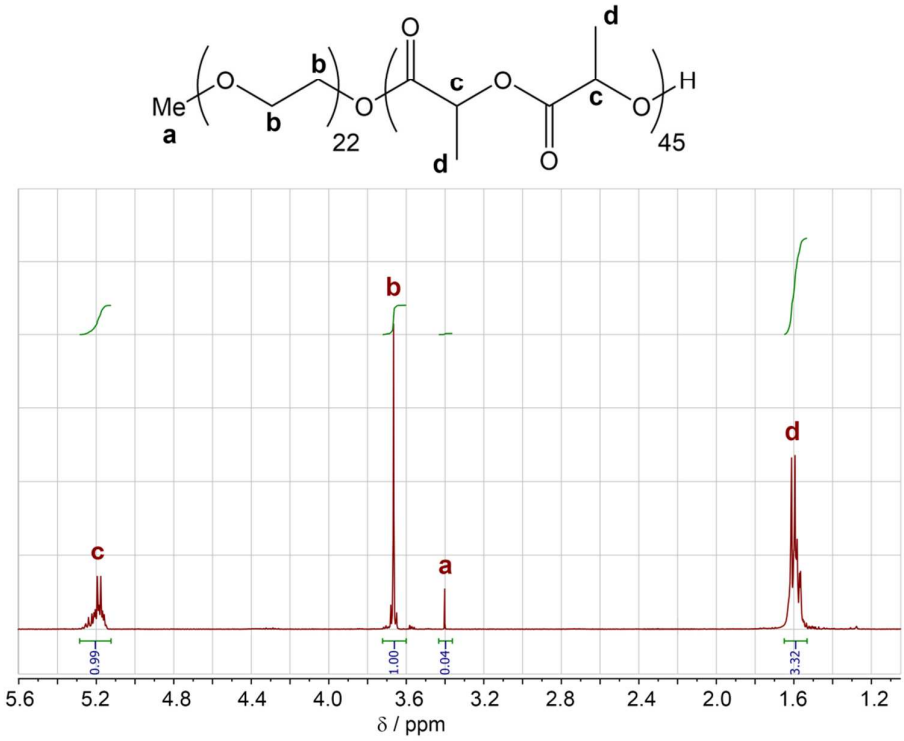

Figure S2. ^{1}H-NMR spectrum of PEG_{22}-$PDLLA_{45}$ copolymer

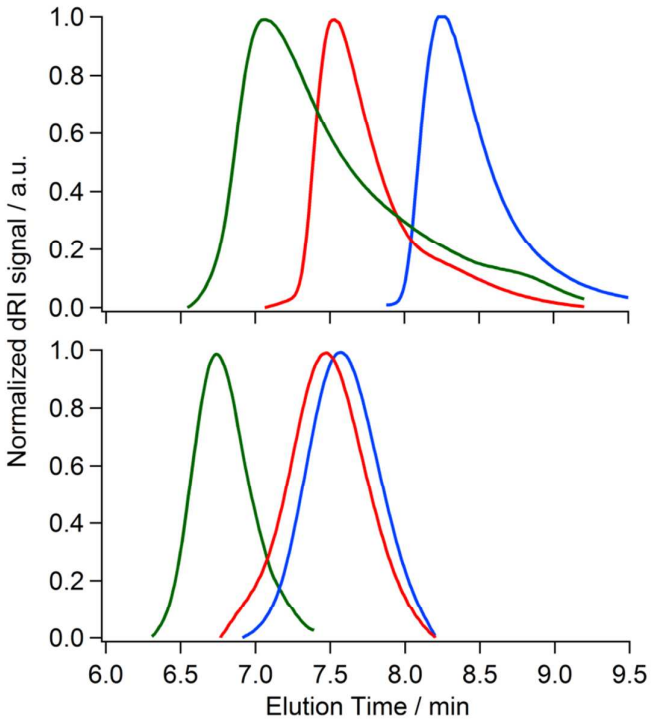

Figure S3. [Upper] GPC traces of low polydispersity PEG standards of 6.45 (blue), 11.84 (red) and 22.8 kDa (green). [Lower] GPC traces of copolymers: PEG_{22}-$PDLLA_{45}$ (blue), N_3-PEG_{75}-$PDLLA_{45}$ (red) and PEG_{44}-$PDLLA_{90}$ (green).

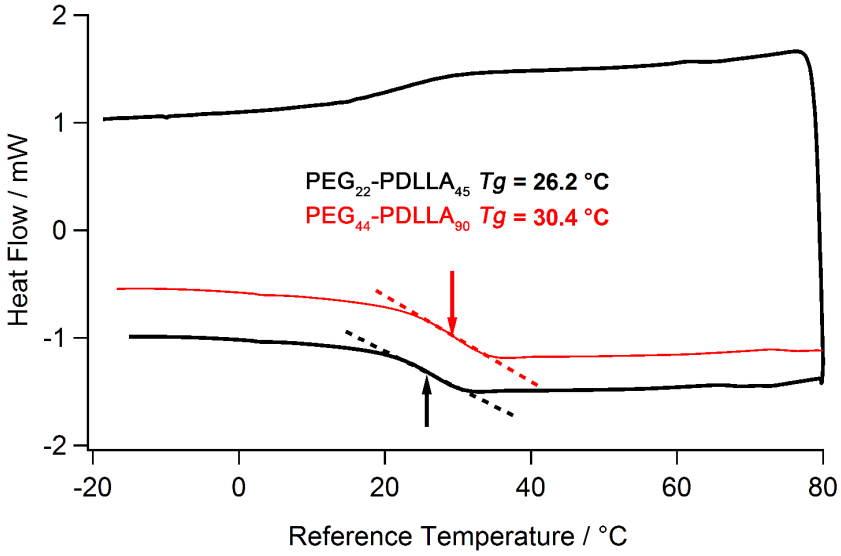

Figure S4. DSC curves for PEG_{22}-$PDLLA_{45}$ (black trace) and PEG_{44}-$PDLLA_{90}$ (red trace) copolymers (cooling run for PEG_{22}-$PDLLA_{45}$ displayed for comparison).

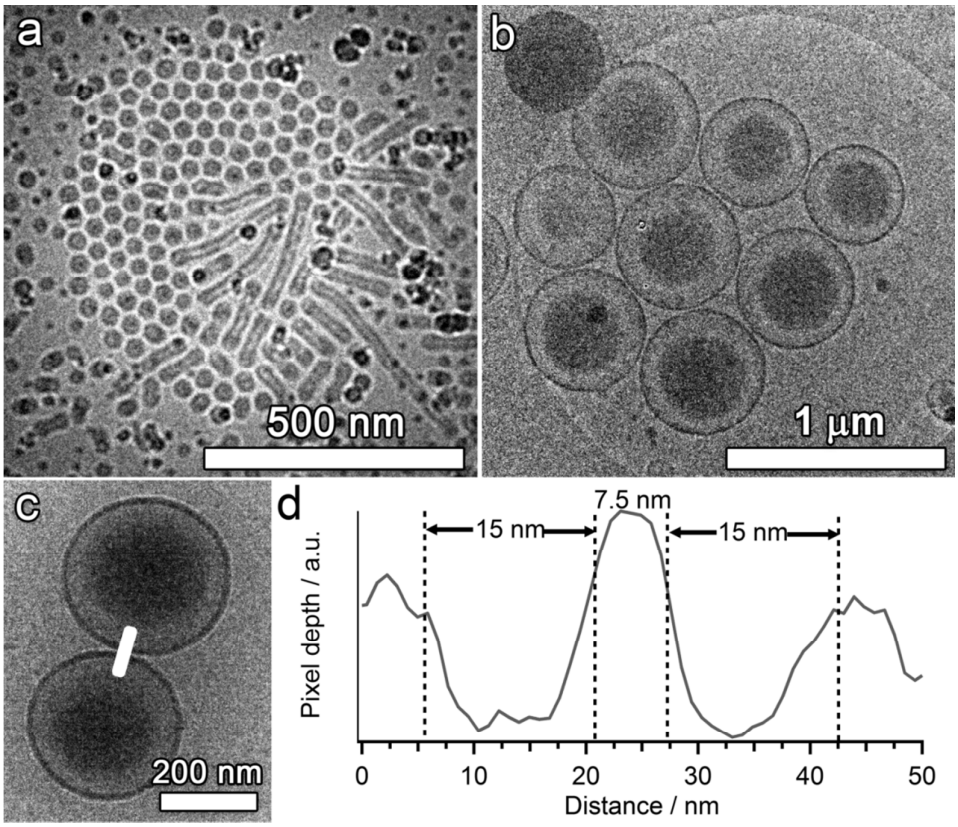

Figure S5. Cryo-TEM images of (a) PEG_{42}-$PDLLA_{90}$ micelles and (b,c) PEG_{22}-$PDLLA_{45}$ polymersomes. (d) Line profile of the polymersome membrane as indicated in (c).

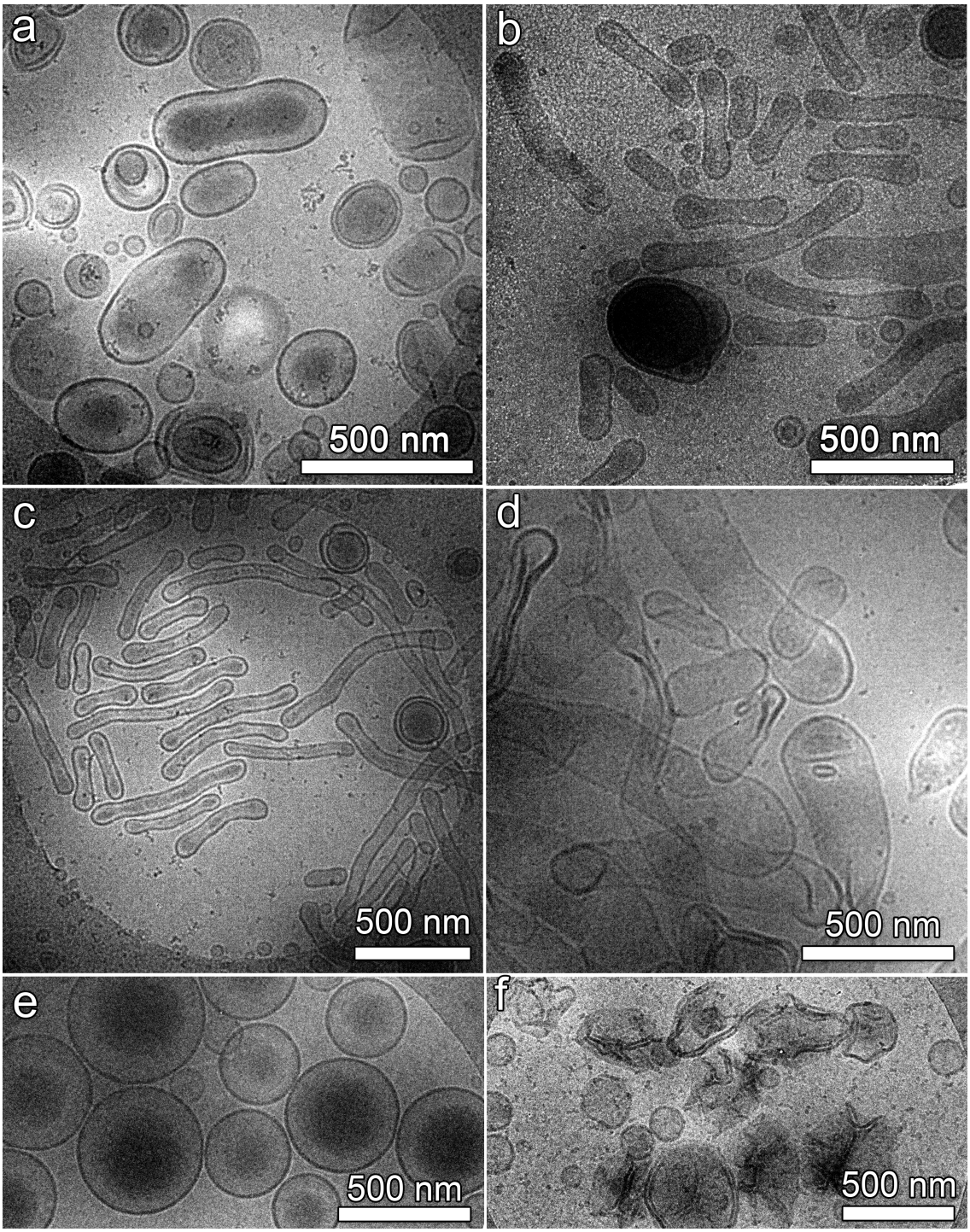

Figure S6. Cryo-TEM images of polymersomes dialyzed against (a) 5 mM, (b) 10 mM, (c) 50 mM and (d) 100 mM NaCl at 4°C. Polymersomes dialyzed against 50 mM NaCl at (e) 25 and (f) 30 °C.

Please note: Numerical analysis of cryo-TEM images was performed in order to approximate the percentage of stomatosomal or nested structures, with samples prepared by dialysis against 10 mM and 50 mM NaCl; values of ~ 10 % and ~ 4% were determined respectively. Such analysis was not appropriate for the samples dialyzed against 5 mM NaCl due to the incipient morphological transformation that occurred under these conditions, resulting in a heterogeneous mixture.

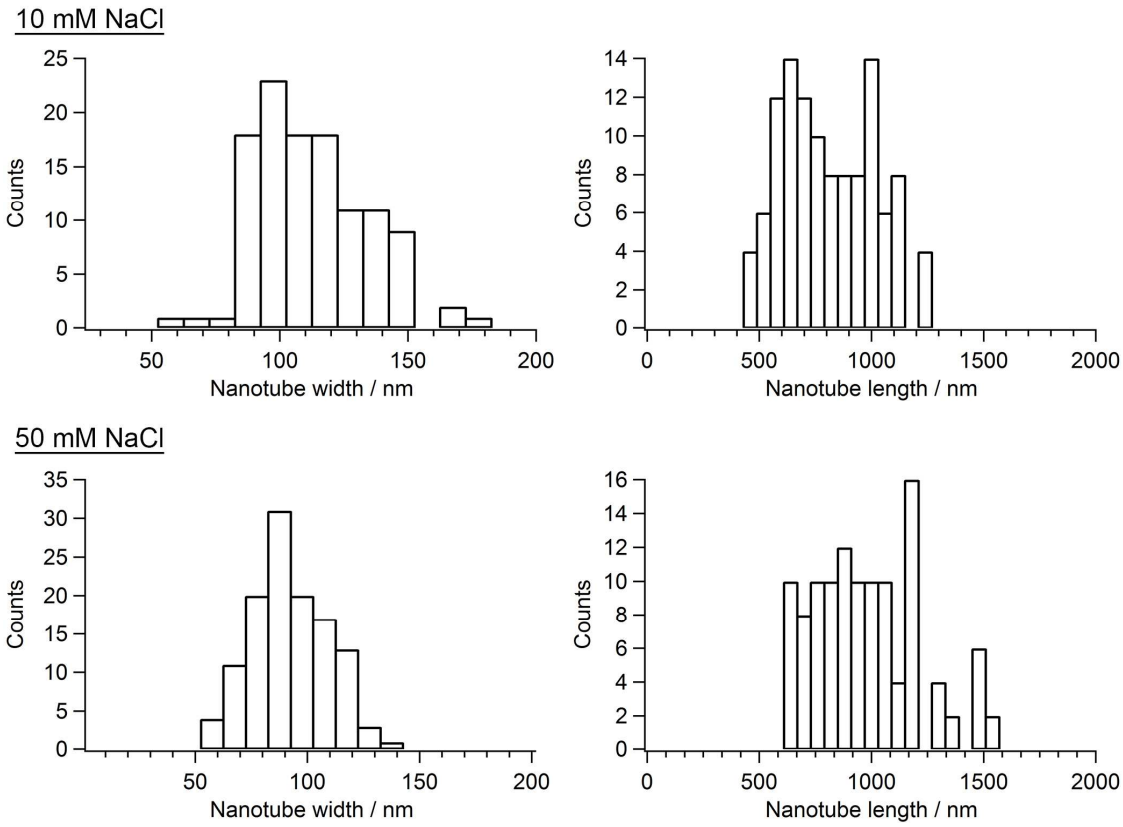

Figure S7. Histogram analysis of cryo-TEM data processing of nanotubes formed *via* dialysis with NaCl concentration of 10 and 50 mM (statistics given in Table S1).

Sample	Average Diameter* (nm)	Average width (nm)	Average length (nm)	Surface Area (nm^2)	Internal Volume (nm^3)	Volume Reduction (%)
Polymersomes	333	-	-	$3.48x10^5$	$19.3x10^6$	-
Dialysed against 10 mM NaCl	298	110 (+/- 20)	800 (+/- 200)	$2.87x10^5$	$8.5x10^6$	39
Dialysed against 50 mM NaCl	304	90 (+/- 20)	1040 (+/- 370)	$2.99x10^5$	$7.2x10^6$	51

* Average diameter of sphere or equivalent sphere with same surface area.

Table S1: Comparisons of the dimensions of PEG-PDLLA polymersomes and nanotubes formed under varying conditions.

Start (min)	End (min)	Mode	Cross flow start (mL min^{-1})	Cross flow end (mL min^{-1})
0	1	Elution	3.00	3.00
1	2	Focus	-	-
2	3	Focus + inject	-	-
3	4	Focus	-	-
4	6	Elution	3.00	1.17
6	8	Elution	1.17	0.70
8	10	Elution	0.70	0.40
10	15	Elution	0.40	0.10
15	32	Elution	0.10	0.10
32	33	Elution	0.00	0.00
33	34	Elution + inject	0.00	0.00
34	39	Elution	0.00	0.00

Table S2: The flow conditions applied for the FFF separation were: 1.50 mL min^{-1} detector flow, 2.00 mL min^{-1} focus flow and 0.20 mL min^{-1} injection flow

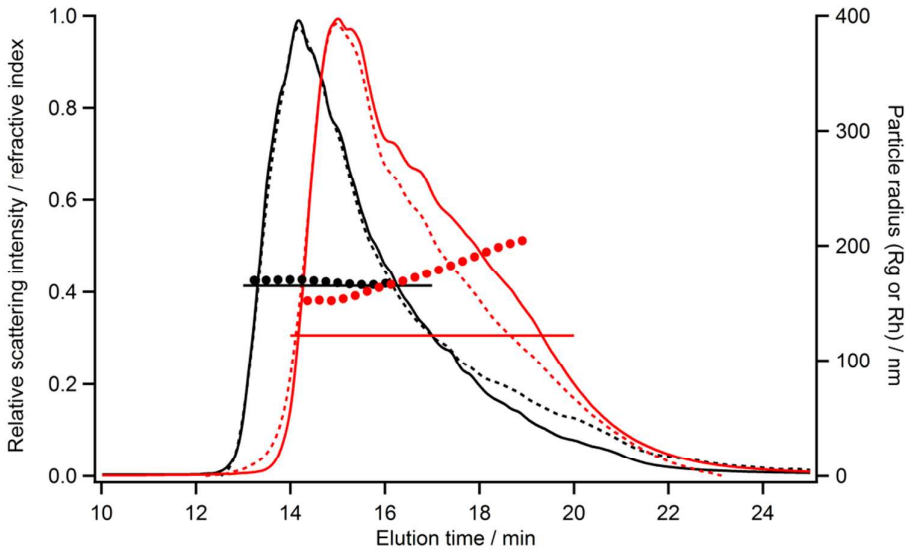

Figure S8. AF4 fractograms for samples of polymersomes (black) and nanotubes (red) with light scattering (solid lines) and differential refractive index (dashed lines) given for sample elution plotted alongside values for the radius of gyration as measured using MALS (solid spheres). Horizontal lines represent values obtained for the hydrodynamic radius using DLS.

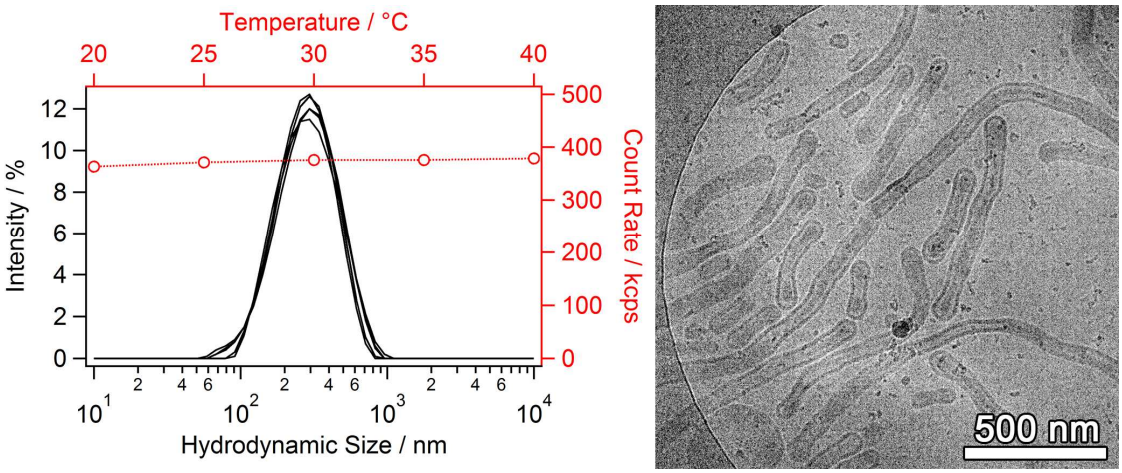

Figure S9. DLS size distribution and count rates for thermal scan of nanotube stability from 20-40 °C alongside corresponding cryo-TEM image (after heat cycle).

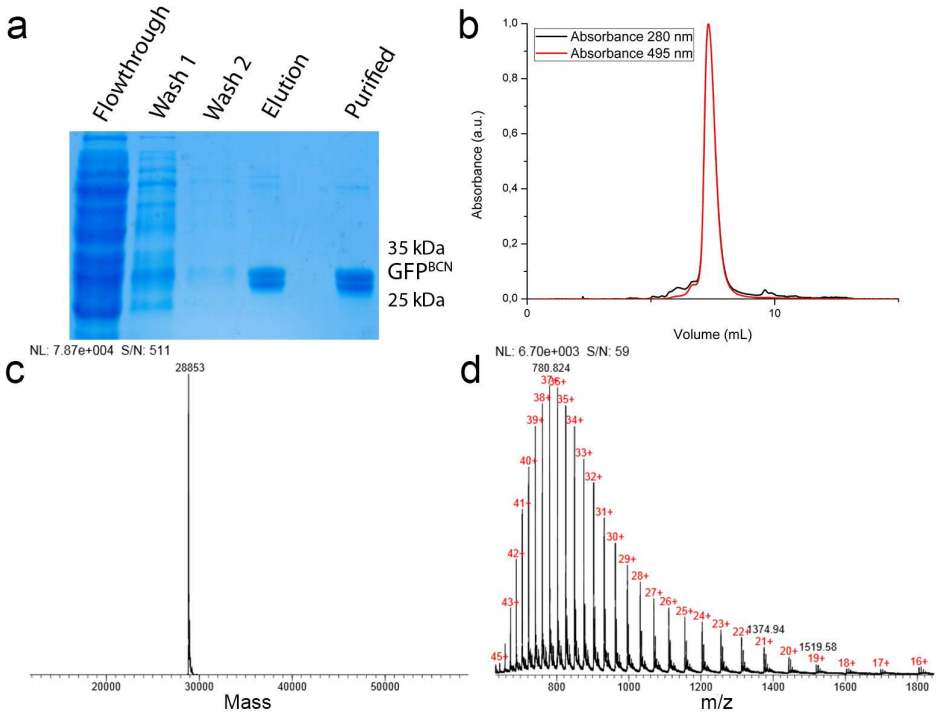

Figure S10. Characterization of eGFP^{BCN} using (a) SDS-PAGE gel, (b) SEC and (c,d) ESI-TOF mass spectrometry.

Note: Although 2 bands are visible in the gel, there is only one species present in the SEC and MS traces, suggesting that the apparent band splitting in SDS-page is insignificant.

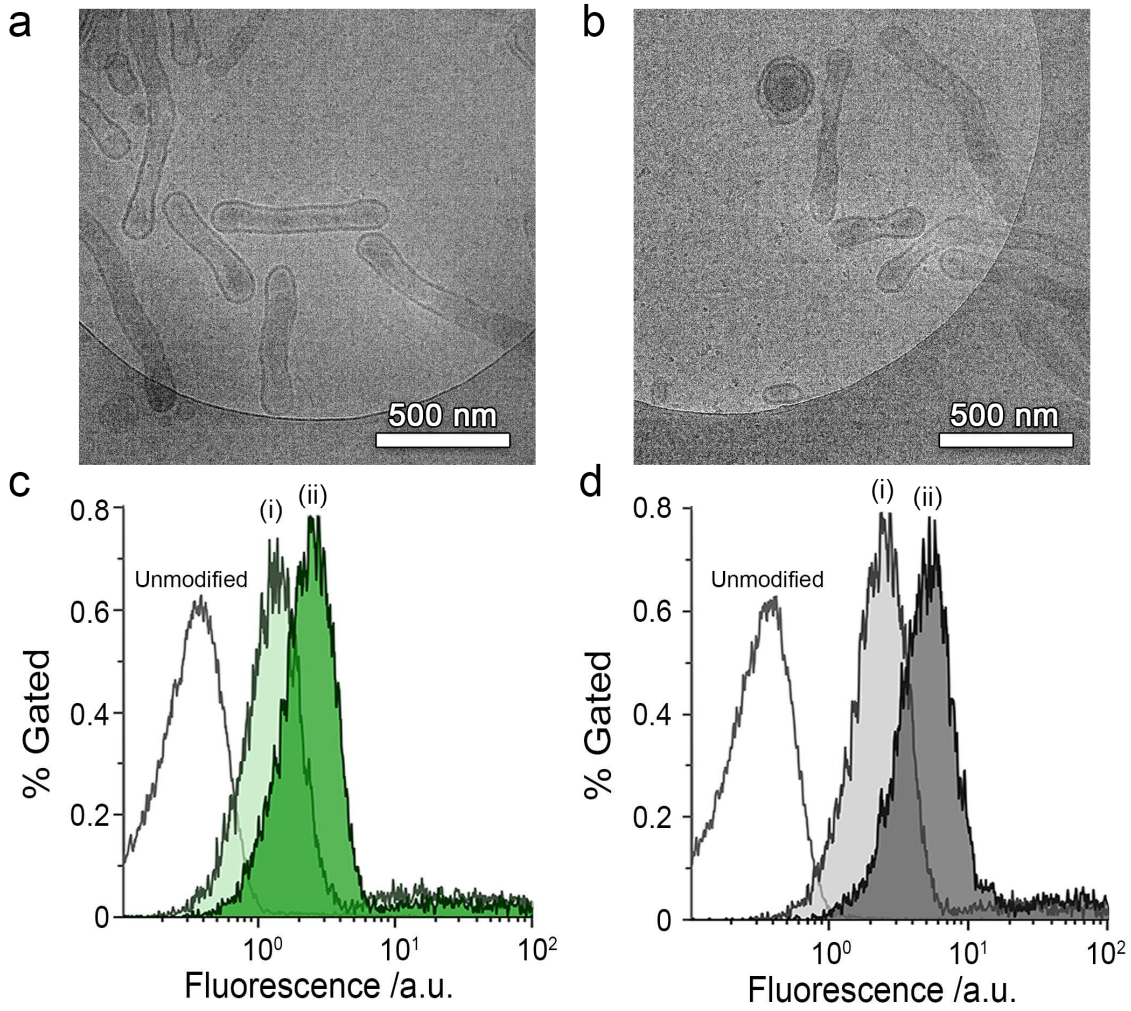

Figure S11. Cryo-TEM images showing the stability of nanotubes after (a) 5 wt% azide-modification and eGFP conjugation and (b) 5 wt% doxorubicin loading. Flow cytometry data comparing (c) increasing eGFP modification of 2 wt% N_3-nanotubes using either (i) an equimolar or (ii) 3-fold excess amount of $eGFP^{BCN}$ and (d) increasing eGFP modification with (i) 2 and (ii) 5 wt% N_3-nanotubes.

References

(1) Lohmeijer, B. G. G.; Pratt, R. C.; Leibfarth, F.; Logan, J. W.; Long, D. A.; Dove, A. P.; Nederberg, F.; Choi, J.; Wade, C.; Waymouth, R. M.; Hedrick, J. L., *Macromolecules* **2006**, *39*, 8574.
(2) Borrmann, A.; Milles, S.; Plass, T.; Dommerholt, J.; Verkade, J. M. M.; Wiessler, M.; Schultz, C.; van Hest, J. C. M.; van Delft, F. L.; Lemke, E. A., *Chembiochem* **2012**, *13*, 2094.
(3) McRae, S. R.; Brown, C. L.; Bushell, G. R., *Protein Expres. Purif.* **2005**, *41*, 121.