Supplementary Information

Morphology Under Control: Engineering Biodegradable Stomatocytes

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Experimental Materials and Methods

Materials

All chemicals were used as received unless otherwise stated. For the synthesis of poly(ethylene glycol)-*b*-poly(D,L-lactide) (PEG-PDLLA), Poly(ethylene glycol) 1K and 2K were purchased from JenKem technology and freeze-dried before use. D,L-lactide was purchased from Acros and used as supplied. All other chemicals were supplied by Sigma-Aldrich and were used without any purification. Ultra pure MilliQ water obtained from Merck Millipore Q-Pod system (18.2 MΩ) with a 0.22 μm Millipore ® Express 40 filter 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 stomatocyte formation. Sodium chloride was purchased from Merck.

Methods

Nuclear Magnetic Resonance (NMR): Proton nuclear magnetic resonance measurements were performed on a Bruker 400 Ultrashield™ spectrometer equipped with a Bruker SampleCase autosampler, using $CDCl_3$ as a solvent and TMS as internal standard.

Gel permeation chromatography (GPC): GPC was conducted using a Shimadzu Prominence-*i* GPC system with a PL gel 5 μm mixed D and mixed C column (Polymer Laboratories) with PS standard and equipped with a Shimadzu RID-20A differential refractive index detector and THF used as an eluent with a flow rate of 1 mL min^{-1}.

Differential scanning calorimetry (DSC): DSC measurements were performed using TA Instruments Multicell DSC.

Dynamic Light Scattering (DLS): DLS measurements were performed on a Malvern instrument Zetasizer (model Nano ZSP) equipped with an autosampler. Zetasizer software was used to process and analyse the data.

Cryogenic transmission electron microscopy (cryo-TEM): Experiments were performed using a FEI Tecnai G2 Sphere (200 kV electron source) equipped with LaB_6 filament utilizing a cryoholder or a FEI Titan (300 kV electron source) equipped with autoloader station. Samples for cryo-TEM were prepared by treating the grids (Lacey carbon coated, R2/2, Cu, 200 mesh, EM sciences) in a Cressington 208 carbon coater for 40 seconds. Then, 3 μl of the polymersome solution was pipetted on the grid and blotted in a Vitrobot MARK III at 100% humidity. The grid was blotted for 3 seconds (offset -3) and directly plunged and frozen in liquid ethane. Processing of TEM images was performed

with ImageJ, a program developed by 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 Dualtec AF4 instrument connected to a Shimadzu LC-2030 Prominence-*i* system with Shimadzu LC-2030 autosampler. The AF4 was connected to 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 (BSA). The processing and analysis of the LS data and radius of gyration (R_G) calculations were performed on Astra 7 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.

Experimental Procedures

Ring opening polymerization (ROP) of poly(ethylene)glycol-poly(D,L-lactide) PEG-PDLLA block copolymers: PEG_n-$PDLLA_m$ was synthesized by ring opening polymerization starting from PEG-macro initiators and using DBU as a catalyst as described previously[1] yielding PEG_n-$PDLLA_m$ polymers with 80 % yield. Copolymer composition was calculated by comparing the protons of PEG (3.65-3.7 ppm) and its terminal methyl unit (singlet at 3.37 ppm) to PDLLA CH (multiplet at 1.4-1.8 and 5.11-5.28 ppm). All PDI values were calculated to be less than 1.2 using PS standards for calibration. Differential Scanning Calorimetry measurements were performed to determine glass transition temperatures (T_g) by scanning from -20 °C to 60 °C at a rate of 0.5 °C/min. and 3 heating/cooling steps. The average polymer compositions and values for PDI and T_g are given in Table S1.

Preparation of polymersomes: In a 15 mL vial, PEG_{22}-$PDLLA_{95}$ and PEG_{44}-$PDLLA_{95}$ block copolymers (1:1 w/w, 20 mg) were dissolved in 2 mL of THF and dioxane (1:4 v/v) and the vial was sealed with a rubber septum. The solution was stirred at 700 rpm for a minimum of 30 minutes prior to the addition of MilliQ (2 mL, 1 mL h^{-1}) *via* a syringe pump. A needle was inserted into the septum to release pressure. The resulted cloudy suspension was transferred into a prehydrated dialysis bag (SpectraPor, MWCO: 12-14 kDa, 2 mL/cm). Dialysis was performed against MilliQ at room temperature for max. 24 hours with a water change after 1 hour.

Preparation of discs: Polymersomes were prepared according to the abovementioned procedure. Afterwards, dialysis was performed against MilliQ at 5 °C for max. 24 hours with a water change after 1 hour.

General method for stomatocytes preparation: First, a batch of polymersomes was prepared following the previously mentioned procedure. Polymersomes were dialyzed against pre-cooled salt solution (50 mM NaCl, unless stated otherwise) at 5 °C for max. 24 hours.

Reverse engineering of stomatocytes into polymersomes: Stomatocytes were prepared according to above described procedure. Stomatocytes were transferred into a prehydrated dialysis bag. Stomatocytes were dialyzed against 50 vol % organic solvent (THF/dioxane (1:4)). Aliquots of 250 µL were withdrawn every hour and quenched with water to a total volume of 2 mL. Samples were directly used for cryo-TEM imaging and DLS.

Supplementary Figures and Tables

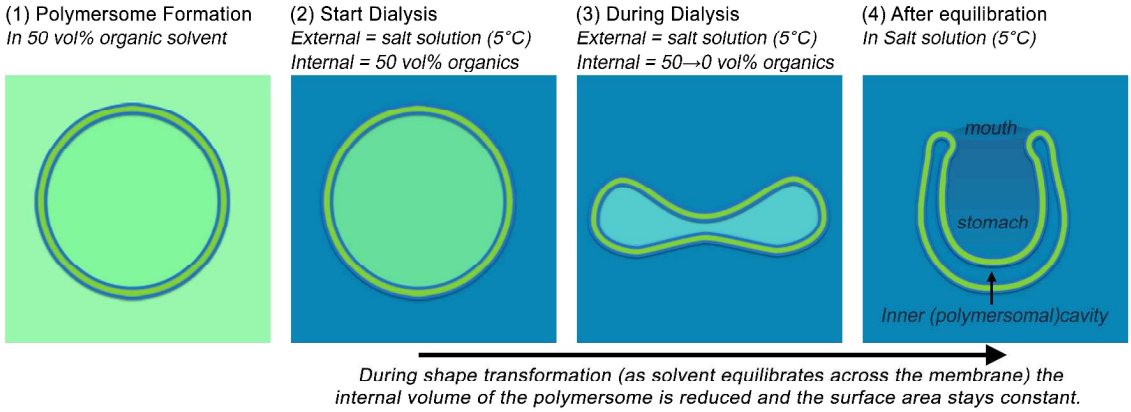

Figure S1: Description of the shape transformation process utilized in this work, whereby polymersomes (having been fabricated in 50 vol% of organic solvent) are dialyzed against salt solution at 5 °C. During the early stages of dialysis, as organic solvent re-equilibrates across the polymersomal membrane, shape transformation occurs as a result of osmotically-induced volume reduction and consequent folding of the membrane. The color scheme here is intended to highlight the solvent composition inside and outside of the polymersome *inner* cavity during this process. Shape transformation of spherical into bowl-shaped (stomatocytes) polymersomes proceeds *via* an oblate pathway as a result of induced *negative* C_0.[2]

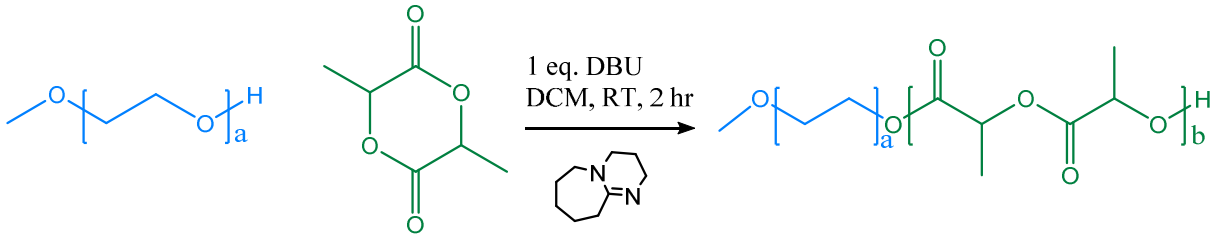

Figure S2: Organocatalyzed synthesis of block copolymer PEG_a-$PDLLA_b$ with a=22/44 and b=45/70/95/120.

Polymer	Average polymer composition (^{1}H-NMR)	PDI (GPC)	T_g (DSC)
PEG_{22}-$PDLLA_{95}$	PEG_{22}-$PDLLA_{98}$	1.17	29 °C
PEG_{44}-$PDLLA_{95}$	PEG_{44}-$PDLLA_{95}$	1.11	26 °C
PEG_{22}-$PDLLA_{70}$	PEG_{22}-$PDLLA_{72}$	1.09	32 °C
PEG_{44}-$PDLLA_{70}$	PEG_{44}-$PDLLA_{68}$	1.15	36 °C
PEG_{22}-$PDLLA_{120}$	PEG_{22}-$PDLLA_{125}$	1.06	35 °C
PEG_{44}-$PDLLA_{120}$	PEG_{44}-$PDLLA_{121}$	1.07	21 °C

Table S1: Characterization of PEG_a-$PDLLA_b$ block copolymers.

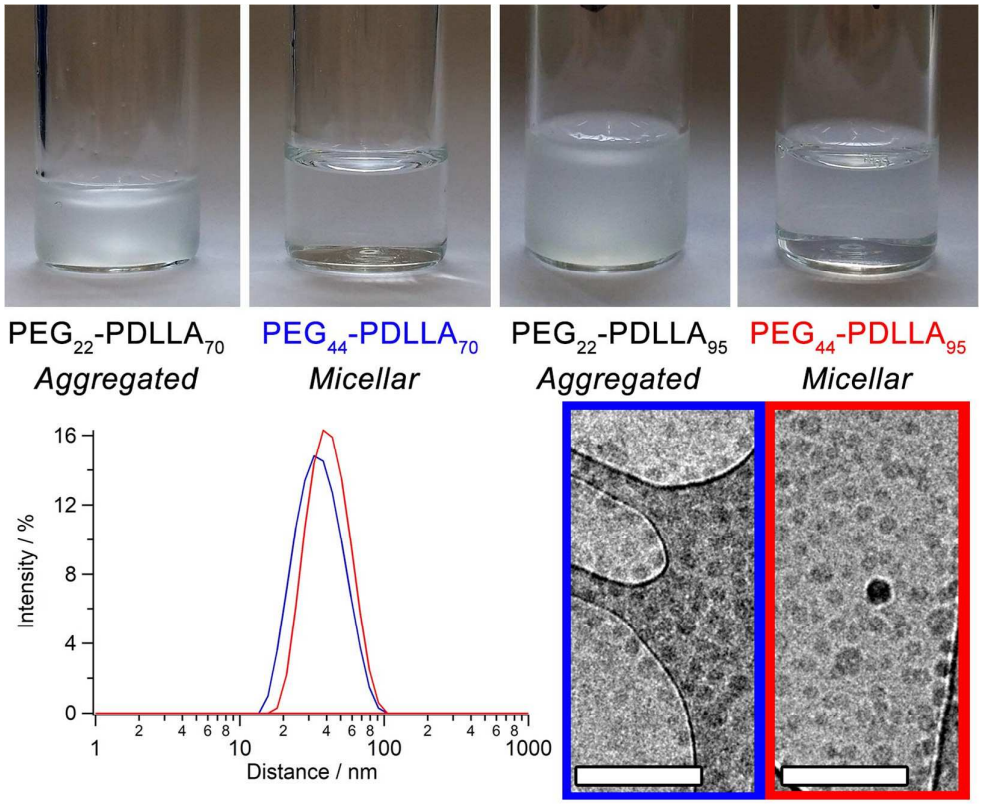

Figure S3: (Top) Photographs showing the outcome of the self-assembly of $PDLLA_{70}$ and $PDLLA_{95}$ based copolymers. (Bottom) DLS intensity profile and cryo-TEM images confirming micelles formation from PEG_{44} -$PDLLA_{70}$ (blue) and PEG_{44} -$PDLLA_{95}$ (red) with average hydrodynamic diameters (D_h) of 39 and 47 nm, respectively. Scale bars correspond to 200 nm.

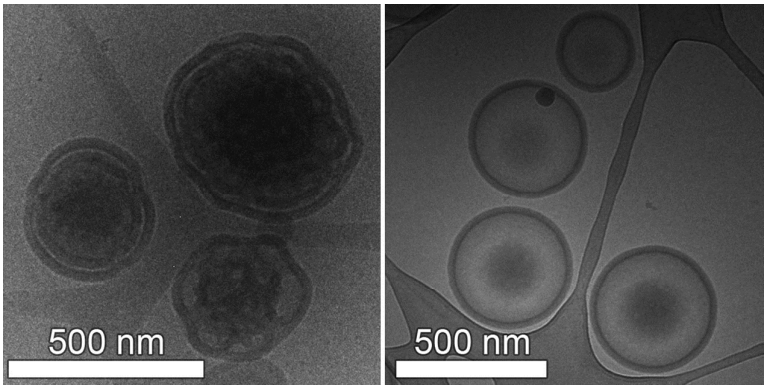

Figure S4: Cryo-TEM images of blended $PEG_{22/44}$-$PDLLA_{120}$ and non-blended PEG_{44}-$PDLLA_{120}$ prepared via dialysis against 50 mM NaCl at 5 °C, displaying sponge-phase particles (left) and polymersomes (right).

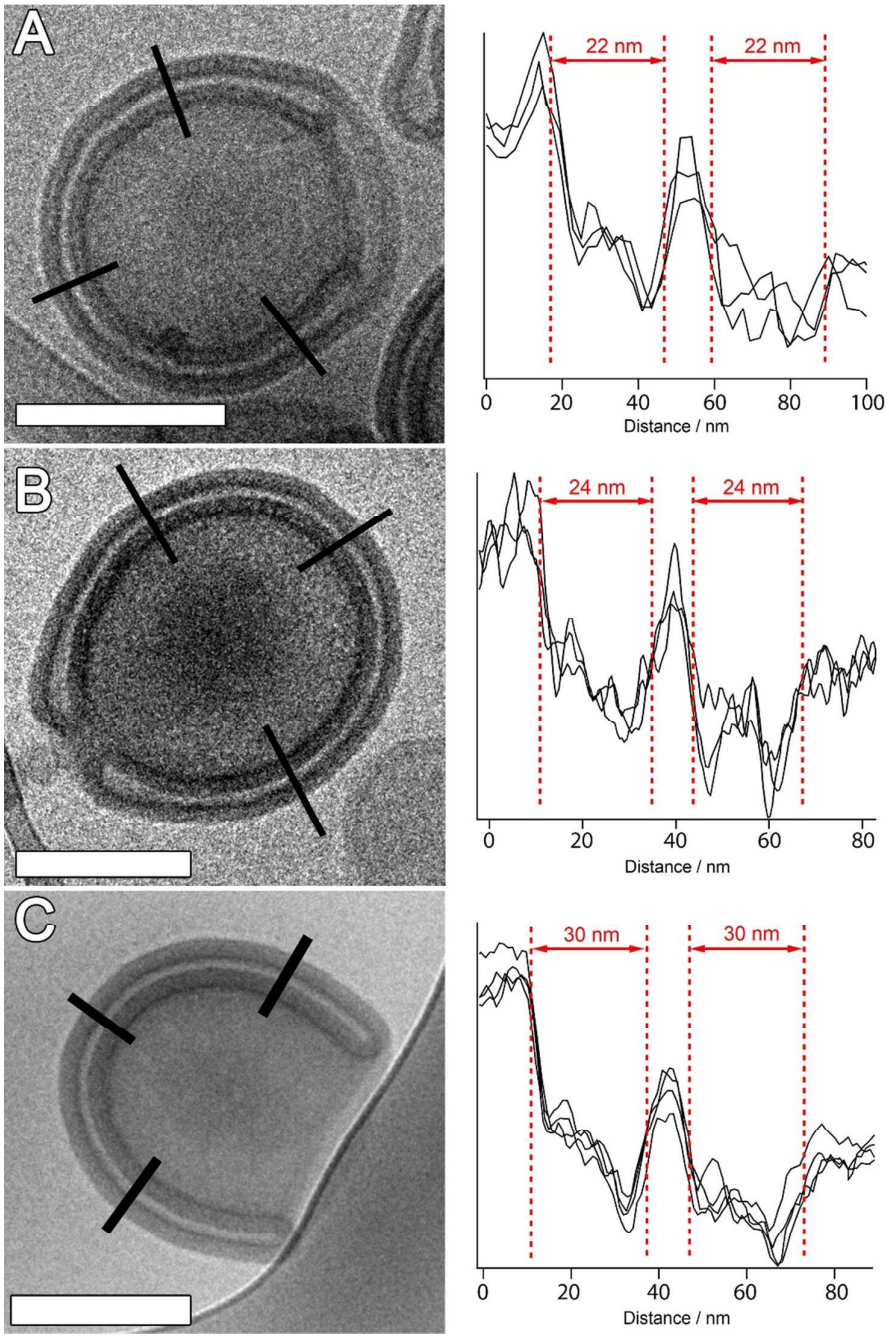

Figure S5: Cryo-TEM images of stomatocytes with varying membrane thickness and corresponding line profile: (A) $PEG_{22/44}$-$PDLLA_{70}$ stomatocytes, (B) $PEG_{22/44}$-$PDLLA_{95}$ stomatocytes and (C) $PEG_{22/44}$-$PDLLA_{120}$ sponge-phase particles. All scale bars correspond to 200 nm.

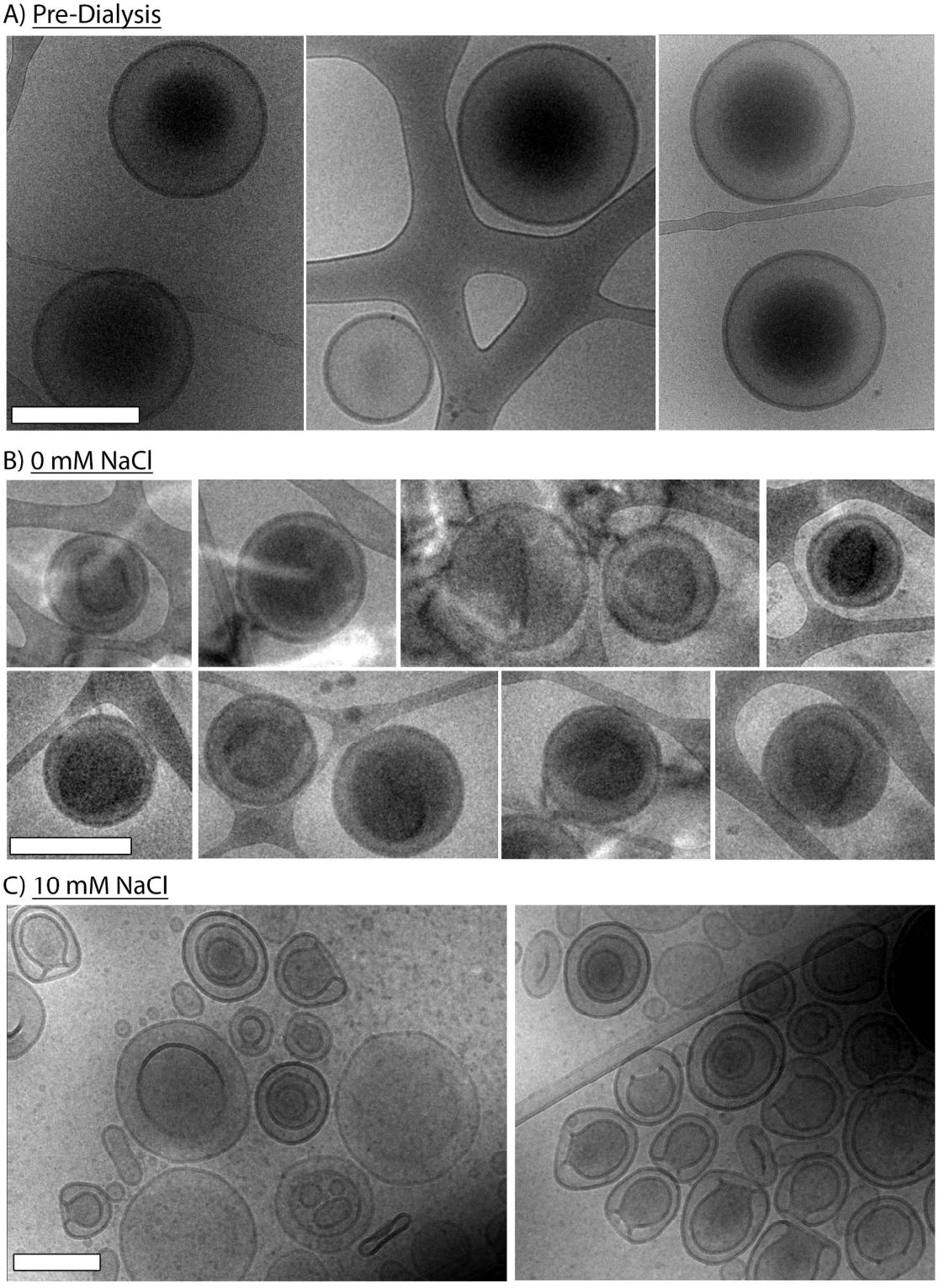

Figure S6: Cryo-TEM images of a) $PEG_{22/44}$-$PDLLA_{95}$ polymersomes before dialysis. Shape transformation of polymersomes (A) after dialysis against MilliQ at 5 °C (B) and against 10 mM NaCl (C) at 5 °C. All scale bars correspond to 500 nm.

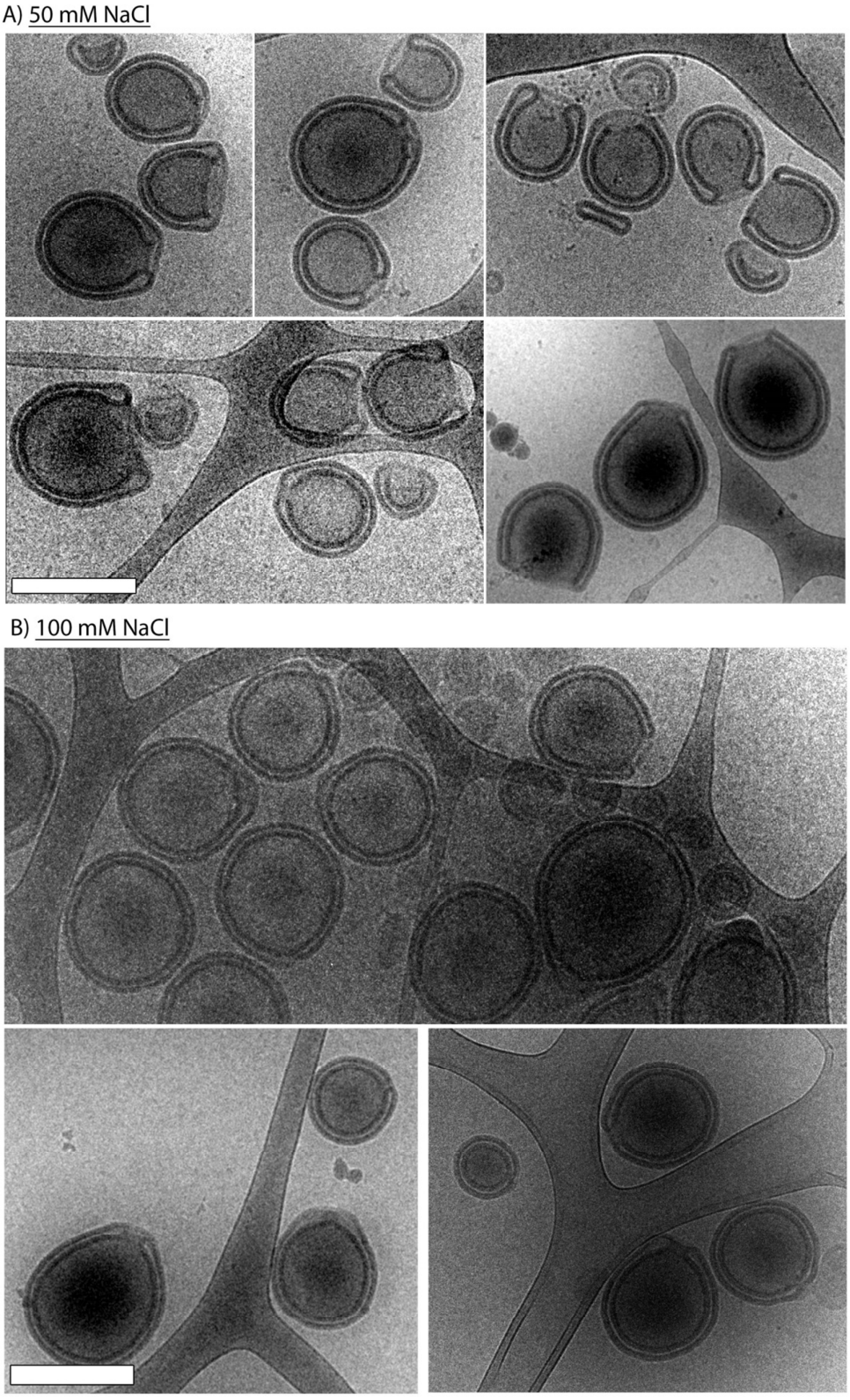

Figure S7: Cryo-TEM images of stomatocytes prepared from $PEG_{22/44}$-$PDLLA_{95}$ obtained *via* dialysis against 50 mM NaCl (A) and 100 mM NaCl (B) at 5 °C. Please note decreasing neck size at higher salt concentrations. All scale bars correspond to 500 nm.

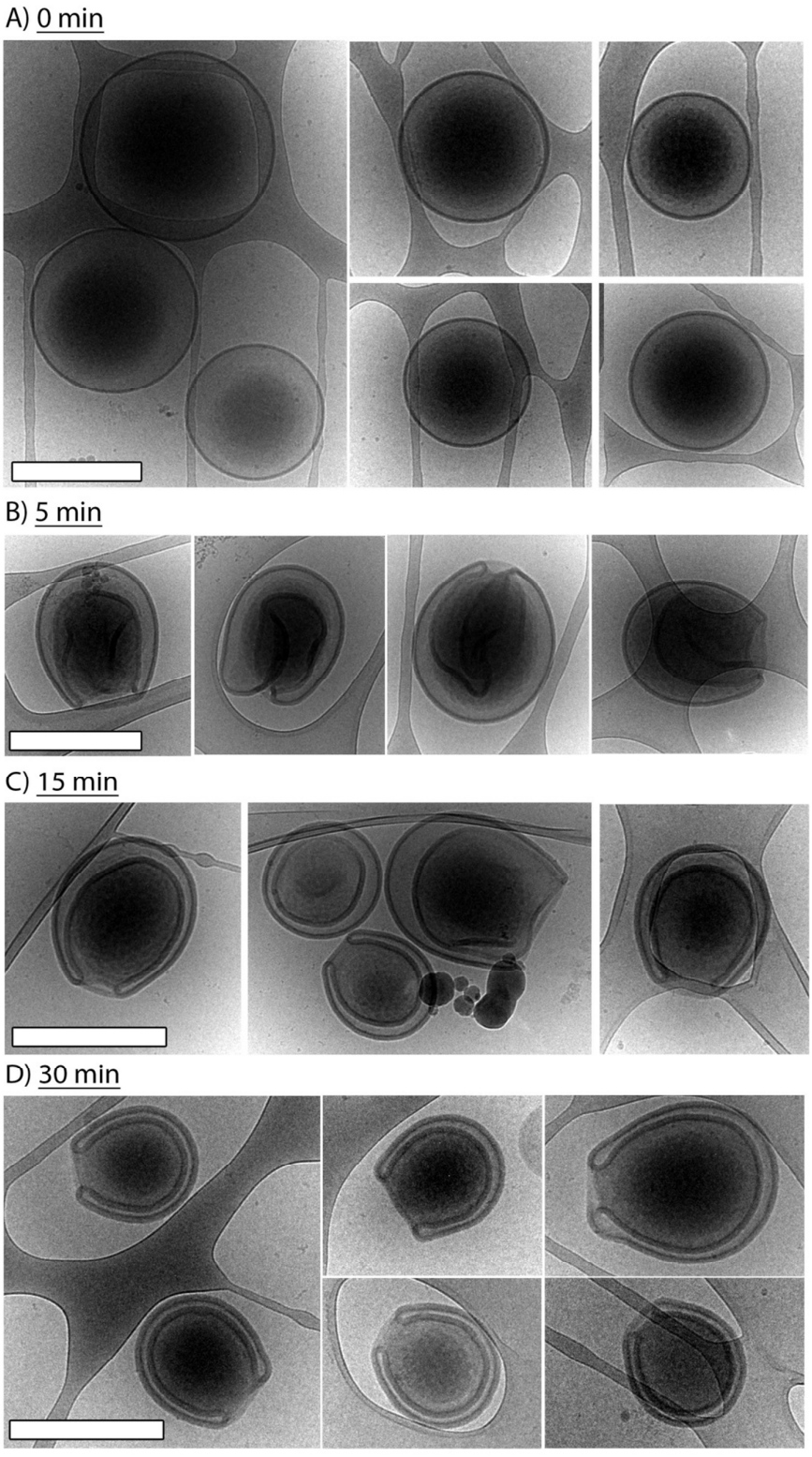

Figure S8: Cryo-TEM images of the shape transformation of polymersomes towards stomatocytes at different time intervals. Stomatocytes were obtained via dialysis of polymersomes of $PEG_{22/44}$-$PDLLA_{95}$ against 50 mM NaCl at 5 °C. All scale bars correspond to 500 nm.

Start (min)	End (min)	Mode	Cross flow start (mL min^{-1})	Cross flow end (mL min^{-1})
0	2	Elution	0.20	0.20
2	3	Focus	-	-
3	5	Focus + inject	-	-
5	6	Focus	-	-
6	36	Elution	0.20	0.10
36	43	Elution	0.10	0.00
43	44	Elution	0.00	0.00
44	45	Elution + inject	0.00	0.00
45	50	Elution	0.00	0.00

Table S2: General method for AF4 fractionation. 20 µL Aliquots of polymersome samples were injected into the AF4 short channel and the conditions were programmed as follows: detector flow (1.5 mL min^{-1}), focus flow (0.70 mL min^{-1}) and injection flow (0.2 mL min^{-1})

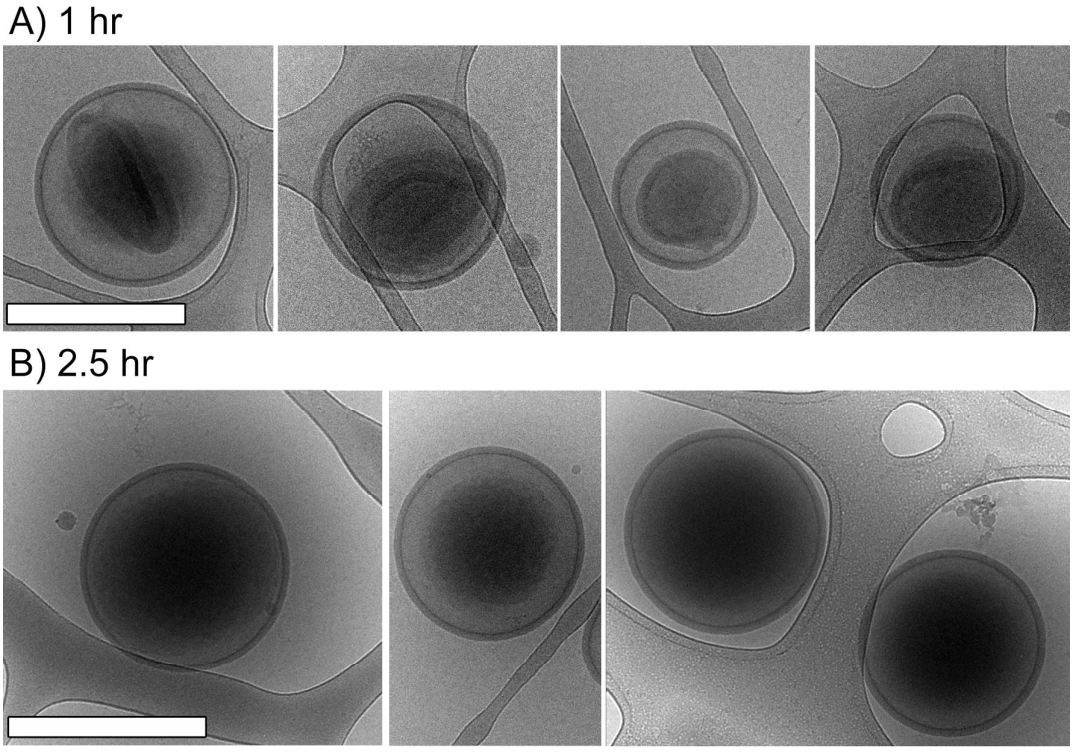

Figure S9: Cryo-TEM images tracking the reverse dialysis process as stomatocytes are reverse engineered to spherical polymersomes after dialysis against 50 vol% organic solvent. All scale bars correspond to 500 nm.

References

1. Abdelmohsen, L. K. E. A. *et al.* Formation of Well-Defined, Functional Nanotubes via Osmotically Induced Shape Transformation of Biodegradable Polymersomes. *J. Am. Chem. Soc.* **138,** 9353–9356 (2016).

2. Rikken, R. S. M. *et al.* Shaping Polymersomes into Predictable Morphologies via Out-of-Equilibrium Self-assembly. *Nat. Commun.* **7,** (2016)