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Supplementary Information

Probing membrane asymmetry of ABC polymersomes

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Materials. The following chemicals were obtained from Sigma-Aldrich and used as received: *p*-toluenesulfonyl chloride (TsCl), anhydrous CH_2Cl_2, anhydrous pyridine, NaN_3, *N*,*N*-dimethylformamide (DMF), DMF-d_7, EtOH absolute, $CuSO_4 \cdot 5H_2O$, sodium ascorbate (NaAsc), dibenzocyclooctyne-PEO_4-biotin conjugate (DBCO-biotin), azide-PEO_3-biotin conjugate (N_3-biotin), Cy5-streptavidin. Bodipy 630/650 NHS ester was purchased from Thermo Fisher Scientific Inc. For dialysis technical solvents (CH_2Cl_2 and tetrahydrofuran (THF)) were purified via rotary evaporation. Milli-Q water with a resistivity of 15 MΩ·cm was used from a Purelab Option-R 7/15 system (ELGA). Polyethylene oxide monomethyl ether (PEO, 2000 $g \cdot mol^{-1}$, Sigma-Aldrich) and bifunctional HC≡C-CH_2-O-PEO-OH (alkyne-PEO, 2000 $g \cdot mol^{-1}$, Laysan Bio) were dissolved in Milli-Q water (~0.1 $g \cdot mL^{-1}$) and then lyophilized. ε-Caprolactone (ε-CL, Sigma-Aldrich) was dried over CaH_2 for 12 h and distilled under reduced pressure prior to use. Toluene (Sigma-Aldrich) was dried over CaH_2 for at least 12 h and distilled under argon atmosphere prior to use. Tin(II) 2-ethylhexanoate ($SnOct_2$, Sigma-Aldrich) was distilled under reduced pressure just before use due to its fast degradation after purification.[1] 2-Methyl-2-oxazoline (MOXA, Acros Organics) was dried over CaH_2 for 12 h and distilled under argon atmosphere prior to use. Sulfolane was dried over CaH_2 for 24 h under reduced pressure at 35 °C, distilled under reduced pressure, and stored in the glove box.

Microwave-assisted Synthesis. Microwave synthesis was performed on Biotage Initiator System equipped with Robot Eight. The microwave synthesizer operated at a constant set temperature monitored by the IR-sensor.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H NMR spectra were recorded in $CDCl_3$ (0.05 % tetramethylsilane) or DMF-d_7 on a Bruker Avance III NMR spectrometer (400.13 MHz). Chemical shifts are reported in ppm. Spectra were processed with MestReNova software.

Gel Permeation Chromatography (GPC). GPC traces were analyzed and recorded in WinGPC (v 8.20 build 4815, PSS systems) software. The DMF (20 mM LiBr) GPC system was equipped with 3 PSS GRAM columns (one 30 Å, two 1000 Å, each 30 cm long, 10 μm particles, 0.8 cm diameter) and Viscotek TDA 305 detector system with refractive index (RI), triple UV-Vis operating at different wavelengths (189-506 nm), light scattering at 90° (670 nm), and viscosity detectors. The samples were measured at 60 °C with the flow rate of 1 $mL \cdot min^{-1}$. The

system was calibrated against narrowly distributed poly(methyl methacrylate) (PMMA) standards.

Laser Scanning Microscopy (LSM). LSM images were recorded on a Zeiss LSM880 using a 63× 1.4 Oil Plan-Apochromat DIC M27 objective. Bodipy 630/650 and Cy5 were excited by the 633 nm He–Ne laser line. The excitation light was passed through a HFT UV 488/543/633 beam splitter. The emission light was passed through a BP 638−759 and recorded on PMT detector. The pinhole was set to 1 airy unit. 16 bit images of 1280 × 1280 pixels were recorded at a scan speed of 13.11 μs per pixel. All measurements were performed at 20 °C. 5 μL of a stained sample were placed onto a glass 22 mm × 50 mm cover slip and covered with a round (Ø 13 mm) cover slip. To prevent sample evaporation, the samples were sealed with nail polish. The images were processed with Fiji ImageJ software (ver. 1.50B). The average diameter of polymersomes was calculated from area ($d = 2(s/\pi)^{1/2}$) determined for each individual particle using "Analyze Particles" option with "Watershed" function used to separate touching polymersomes. All calculations were based on at least 3 different images recorded at different spots. The images used for calculations contained at least 150 objects in total.

Fluorescence Correlation Spectroscopy (FCS). FCS was performed on Zeiss LSM510 META/ConfoCor 2 FCS microscope using a Zeiss Apochromat 40×/1.2 water immersion objective. Bodipy 630/650 and Cy5 were excited by the 633 nm He–Ne laser line (5 % output). The excitation light was passed through a HFT UV 488/543/633 beam splitter. The emission light was passed through a LP 650 long pass filter. The fluorescence signal was recorded with an avalanche photodiode (APD). The pinhole was set to 90 μm. All measurements were performed at 20 °C. 10 μL of a freshly prepared sample was placed onto a glass slide. The surface of a glass slide was found via LSM, and the measurements were always performed at 200 μm above the surface. Measurement series of 30 × 10 s were recorded for each sample. Due to fast sedimentation of a sample and aggregation of polymersomes, typically only 20 out of 30 curves were suitable for analysis in case of Cy5 containing samples (i.e., polymersomes with Cy5-streptavidin). Higher number of measurements of the same sample did not improve the quality of the data, because over measurement time the amount of events decreased significantly. Therefore, in order to determine the mean diameter value D_h, measurements were performed 3 times on 3 different droplets. Such limitations were not the case for Bodipy 630/650 containing samples; the measurement was performed one time, and typically all 30 curves were suitable for

analysis. The data was processed using Origin software. The measured data was normalized and fitted using one-component or two-component functions:

$$G(\tau) = \frac{1}{N}\left(1 + \frac{T}{1-T}e^{-\frac{\tau}{\tau_{trip}}}\right)\left(\frac{1}{\left(1+\frac{\tau}{\tau_D}\right)\sqrt{1+s^2\frac{\tau}{\tau_D}}}\right) \qquad (1)$$

where N is the number of particles, T is the fraction of fluorophore in triplet state with the corresponding triplet time τ_{trip}, τ_D is the diffusion time.

$$G(\tau) = \frac{1}{N}\left(1 + \frac{T}{1-T}e^{-\frac{\tau}{\tau_{trip}}}\right)\left(\frac{f}{\left(1+\frac{\tau}{\tau_{D1}}\right)\sqrt{1+s^2\frac{\tau}{\tau_{D1}}}} + \frac{1-f}{\left(1+\frac{\tau}{\tau_{D2}}\right)\sqrt{1+s^2\frac{\tau}{\tau_{D2}}}}\right) \qquad (2)$$

where f is the fraction of the component 1 with the diffusion time τ_{D1}, and τ_{D2} is the diffusion time of the second component in the mixture.

Synthesis of PEO_{45}-*b*-PCL_{110}-*b*-$PMOXA_4$ and PEO_{45}-*b*-PCL_{103}-*b*-$PMOXA_4$-biotin. PEO-*b*-PCL-*b*-PMOXA polymers were synthesized via coordination-insertion ring opening polymerization of ε-CL catalyzed by $SnOct_2$ followed by tosylation and cationic ring-opening polymerization of MOXA in microwave reactor.[1-2] Briefly, PEO was mixed with ε-CL in toluene, and then toluene solution of $SnOct_2$ was added. The polymerization was carried out at 110 °C under conventional heating. Purified PEO-*b*-PCL was tosylated using TsCl at 22 °C in anhydrous CH_2Cl_2 in the presence of pyridine. Modified polymer, PEO-*b*-PCL-OTs, was purified and mixed with MOXA and sulfolane in the glove box, and the polymerization was carried out at 100 °C under microwave irradiation. To obtain PEO_{45}-*b*-PCL_{110}-*b*-$PMOXA_4$, polymerization of MOXA was quenched with aqueous solution of pyridine (10 v/v %), and to obtain PEO_{45}-*b*-PCL_{103}-*b*-$PMOXA_4$-N_3, polymerization was quenched using saturated solution of NaN_3 in DMF. After quenching the polymerization mixture was placed into a regenerated cellulose dialysis membrane (MWCO 3.5-5 kDa, SpectraPor) and dialyzed against THF/H_2O (9/1) mixture for 2 days and THF/CH_2Cl_2 (9/1) mixture for 1 day (solution was exchanged 7 times). PEO_{45}-*b*-PCL_{103}-*b*-$PMOXA_4$-biotin was obtained via copper-free click coupling. 20 mg (1 eq., 1.42 μmol) of PEO_{45}-*b*-PCL_{103}-*b*-$PMOXA_4$-N_3 was mixed with 5 mg (4.7 eq., 6.67 μmol) of DBCO-biotin and dissolved in 1 mL of 1/1 EtOH/CH_2Cl_2 mixture. The reaction mixture was stirred under argon atmosphere for 48 h at 22 °C. Then the mixture was placed into a regenerated

cellulose dialysis membrane (MWCO 3.5-5 kDa, SpectraPor) and dialyzed against THF for 3 days (solution was exchanged 7 times). ^{1}H NMR PEO_{45}-*b*-PCL_{103}-*b*-$PMOXA_4$-biotin (400.13 MHz, δ (ppm), DMF-d_7): 1.30−2.10 (-X-CH_2-CH_2-CH_2-CH_2-CH_2-Y-, where X / Y = C(O) / O / biotin), 2.14−2.41 (-N(C(O)CH_3)-, -NH-C(O)-CH_2-), 2.53 (t, *J* = 7.3 Hz, -(O)C-CH_2-CH_2-), 3.25−3.29 (-C(CH_2)*H*-S-, -CH_2-S-), 3.34−3.42 (-CH_2-NH-C(O)-), 3.48 (s, CH_3-O-), 3.59 (m, -N(C(O)CH_3)-CH_2-CH_2-), 3.77 (s, -O-CH_2-CH_2-O-), 4.25 (t, *J* = 6.4 Hz, -(O)C-CH_2-CH_2-CH_2-CH_2-CH_2-O-), 4.37−4.78 (-CH_2-C(CH-)*H*-N(H)-C(O)-, Ar-CH_2-N-, -CH_2-triazole-), 6.19−6.95 (-N*H*-), 7.50−8.01 (Ar-*H*).

Synthesis of biotin-PEO_{45}-*b*-PCL_{100}-*b*-$PMOXA_4$. To obtain biotin-PEO_{45}-*b*-PCL_{100}-*b*-$PMOXA_4$, copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction was used. Alkyne-PEO_{45}-*b*-PCL_{100}-*b*-$PMOXA_4$ was synthesized in a similar manner as described for PEO-*b*-PCL-*b*-PMOXA, but alkyne-PEO was used instead of PEO. Alkyne-PEO_{45}-*b*-PCL_{100}-*b*-$PMOXA_4$ (1 eq., 5.27 mg, 0.38 μmol) was dissolved in 1.7 mL of anhydrous DMF in a microwave vial. N_3-biotin (2 eq., 0.34 mg, 0.76 μmol), $CuSO_4 \cdot 5H_2O$ (18 eq., 1.72 mg, 6.89 μmol), and NaAsc (56 eq., 4.30 mg, 21.70 μmol) were dissolved in 0.3 mL of anhydrous DMF, and this solution was added to a solution of Alkyne-PEO_{45}-*b*-PCL_{100}-*b*-$PMOXA_4$. The reaction was carried out at 100 °C for 15 min under microwave irradiation. To remove salts and unreacted N_3-biotin, the reaction mixture was placed into a regenerated cellulose dialysis membrane (MWCO 3.5-5 kDa, SpectraPor) and dialyzed against THF/H_2O (8/2) mixture for 2 days (solution exchanged 6 times) and against pure THF for 1 day (solution exchanged 3 times). ^{1}H NMR biotin-PEO_{45}-*b*-PCL_{100}-*b*-$PMOXA_4$ (400.13 MHz, δ (ppm), DMF-d_7): 1.30−2.10 (-X-CH_2-CH_2-CH_2-CH_2-CH_2-Y-, where X / Y = C(O) / O / biotin), 2.14−2.41 (-N(C(O)CH_3)-, -NH-C(O)-CH_2-), 2.53 (t, *J* = 7.3 Hz, -(O)C-CH_2-CH_2-), 3.25−3.29 (-C(CH_2)*H*-S-, -CH_2-S-), 3.34−3.42 (-CH_2-NH-C(O)-), 3.59 (m, -N(C(O)CH_3)-CH_2-CH_2-), 3.77 (s, -O-CH_2-CH_2-O-), 4.17−4.32 (-(O)C-CH_2-CH_2-CH_2-CH_2-CH_2-O-, -O-CH_2-triazole-), 4.47−4.58 (-CH_2-C(CH-)*H*-N(H)-C(O)-).

Self-assembly. Self-assembly was performed using film rehydration method. In all experiments 1 mg·mL^{-1} polymer dissolved in CH_2Cl_2 was used as a stock solution, and final polymer concentration in aqueous solution was 2 mg·mL^{-1}. 2 mL of a stock solution was placed in the 5 mL glass round-bottom flask, and CH_2Cl_2 was removed by rotary evaporation. In case of mixtures containing biotinylated polymers, stock solutions of modified and non-modified polymers were mixed accordingly to yield 2 mg of a final mixture. Then 1 mL of Milli-Q water

was added, and the solution was stirred at 350 rpm for 24 h at 62 °C. High temperature, i.e., 62 °C, was required for self-assembly due to the semicrystalline nature of PCL block. To visualize polymersomes via LSM, 50 µL of solution was stained with aqueous solution of 0.5 µL of 0.72 µM Bodipy 630/650 dye.

For investigation of the binding of Cy5-streptavidin to polymersomes, 50 µL of 0.1 mg·mL^{-1} aqueous solution of streptavidin was mixed with 50 µL of 2 mg·mL^{-1} aqueous solution of polymersomes at 20 °C, and then 10 µL of a sample was measured using FCS. To ensure reproducibility of the binding, all experiments were performed at least 3 times with different batches of polymersomes.

Table S1. Characterization of PEO-*b*-PCL-*b*-PMOXA polymers used in this study.

Abbreviation	Polymer	MW of PEO/PCL/PMOXA blocks, kDa[a]	$Đ_M$[b]
ABC	PEO_{45}-*b*-PCL_{110}-*b*-$PMOXA_4$	2.0/12.5/0.3	1.12
biotin-ABC	biotin-PEO_{45}-*b*-PCL_{100}-*b*-$PMOXA_4$	2.0/11.4/0.3	1.15
ABC-biotin	PEO_{45}-*b*-PCL_{103}-*b*-$PMOXA_4$-biotin	2.0/11.7/0.3	1.14

[a]Number-average molecular weight was determined from ^{1}H NMR spectroscopy.
[b]Dispersity was determined from RI data of samples analyzed by DMF GPC using PMMA calibration.

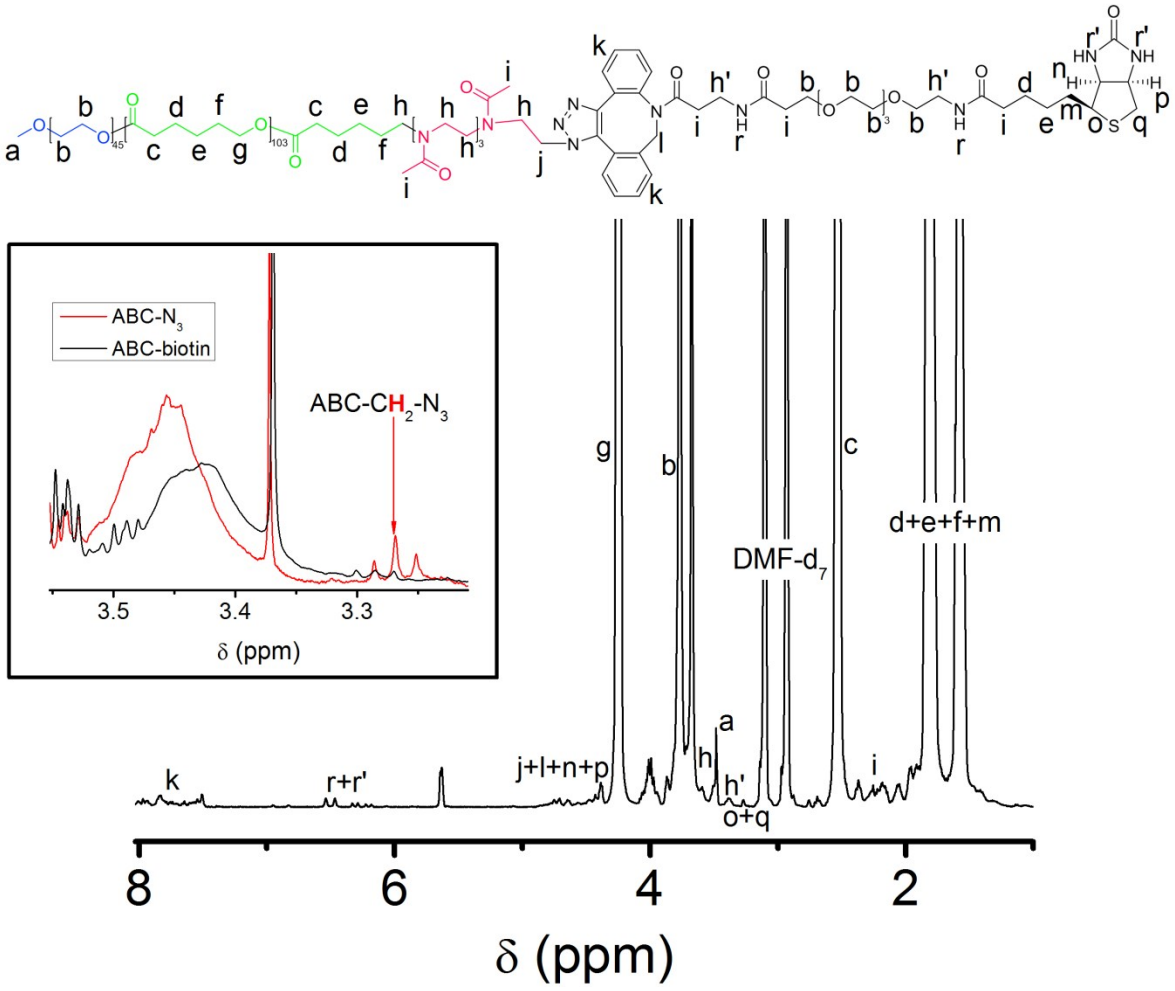

Figure S1. ^{1}H NMR (DMF-d_7) spectrum of ABC-biotin. Inset: Comparison of ^{1}H NMR ($CDCl_3$) spectra of ABC-N_3 and ABC-biotin. The disappearance of a triplet at 3.28 ppm in ABC-biotin spectrum indicates efficient reaction between ABC-N_3 and DBCO-biotin.

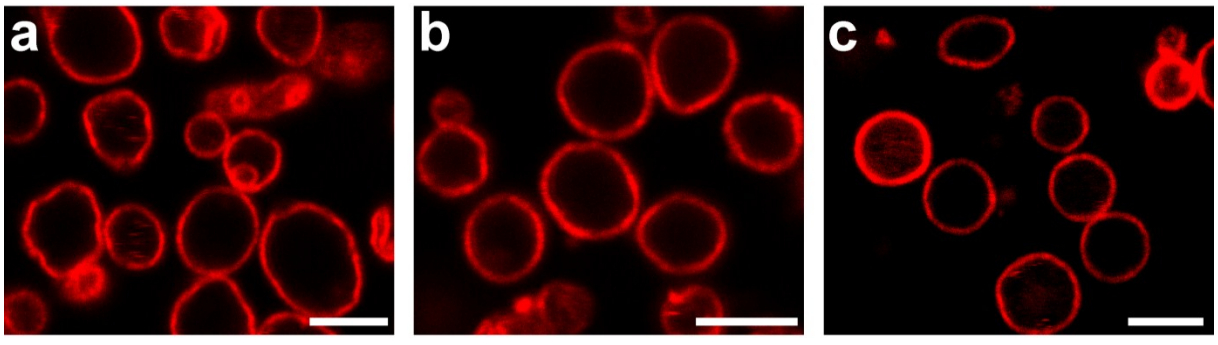

Figure S2. LSM images of polymersomes formed by polymers (a) ABC, (b) ABC containing 10% of biotin-ABC, (c) ABC containing 10% of ABC-biotin. Polymersomes were stained with Bodipy 630/650. Scale bars are 5 μm.

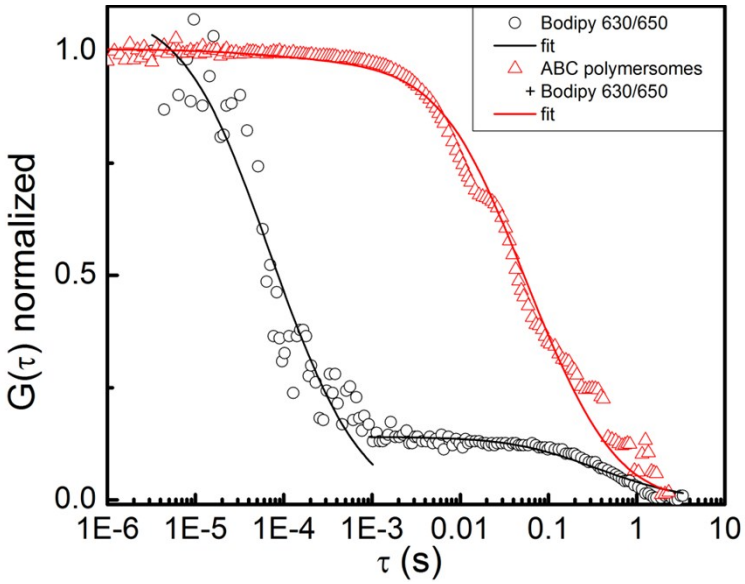

Figure S3. Normalized autocorrelation curves from FCS data in water: 7.2 nM Bodipy 630/650 (black), 2 mg·mL^{-1} ABC polymersomes stained with aqueous solution of 7.2 nM Bodipy 630/650 (red). The lower step in the black curve corresponds to the aggregation of hydrophobic Bodipy 630/650 dye in water. The two steps in the latter curve were fitted separately with one-component functions, because two-component function failed to fit the data.

Calculation of average polymersome volume V_{pol}:

$$V_{pol} = \frac{4}{3}\pi r^3 = \frac{4}{3}\pi(2\ \mu m)^3 \approx 3 \cdot 10^{-17} m^3 = 30\ fL$$

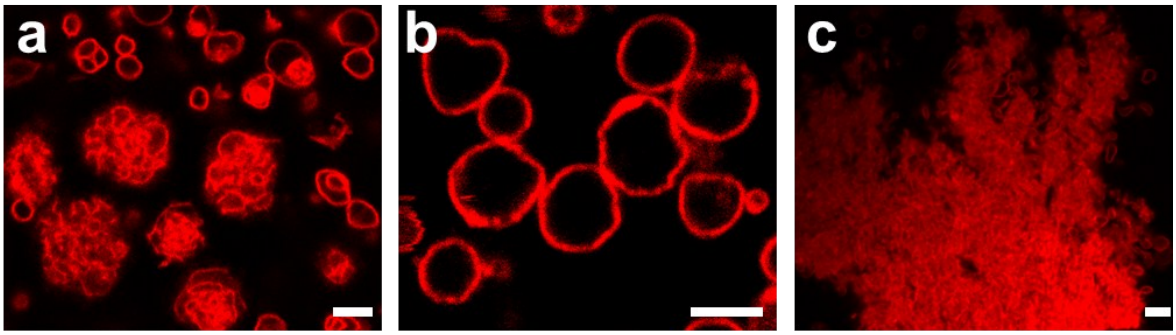

Figure S4. LSM images of structures formed by polymers (a) ABC-biotin (PEO_{45}-*b*-PCL_{103}-*b*-$PMOXA_4$-biotin), (b) ABC (PEO_{45}-*b*-PCL_{110}-*b*-$PMOXA_4$), (c) PEO_{45}-*b*-PCL_{135}-*b*-$PMOXA_{20}$. ABC-biotin forms a mixture of polymersomes and cloud-like aggregates indicating that the packing geometry of ABC-biotin molecules is somewhat intermediate between cylinder

corresponding to polymersomes and double-cone shape corresponding to cloud-like aggregates. Structures were stained with Bodipy 630/650. Scale bars are 5 μm.

References:

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2. Konishcheva, E. V.; Zhumaev, U. E.; Meier, W. P., PEO-b-PCL-b-PMOXA Triblock Copolymers: From Synthesis to Microscale Polymersomes with Asymmetric Membrane. *Macromolecules* **2017,** *50* (4), 1512-1520.