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Supporting Information

The Evolution of Bicontinuous Polymeric Nanospheres in Aqueous Solution

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Materials and Methods

Poly(ethylene glycol) methyl ether (M_n ca. 2000 g mol^{-1}), 2-bromoisobutyryl bromide (98%), sodium bicarbonate (99%), dimethylamino pyridine (99%), copper(I) bromide (99.99%) 2,2'-bipyridine (>99%) and octadecyl methacrylate were all used as received from Sigma-Aldrich without further purification. Triethylamine (>99.5%) was used as received from Fluka. Aluminium oxide (activated, neutral, for column chromatography 50-200 μm) was used as received from Acros Organics and magnesium sulphate (≥98%, anhydrous) was used as received from Merck. Tetrahydrofuran (analytical reagent grade), methanol (analytical reagent grade), dichloromethane (analytical reagent grade) and acetone (analytical reagent grade) were used as received from Biosolve Nederland without further purification. o-Xylene (analytical reagent grade), isopropanol (analytical reagent grade) were used as received from Merck without further purification. Hydrochloric acid (30%) and sodium hydroxide (> 97%) were purchased from Merck. N-(n-octyl)-2-pyridyl(methanime) was synthesised following a literature procedure.[1,2] Deuterated chloroform (99.8%) was used as received from Cambridge Isotope Laboratories Incorporated without further purification.

Molecular weight parameters were measured via size exclusion chromatography (SEC) on a Waters Alliance system equipped with a Waters 2695 separation module, a Waters 2414 refractive index detector (35ºC), a Waters 2487 dual absorbance detector, a PSS SDV 5 μm guard column followed by 2 PSS SDV linear XL columns in series of 5 μm (8*300) at 40°C. All samples were dissolved in tetrahydrofuran (THF) and filtered through a 0.2 μm PTFE filter (13mm, PP housing, Alltech). THF with 1 v/v-% acetic acid was used as eluent at a flow rate of 1.0 mL min^{-1}. The molecular weights were calculated with respect to polystyrene standards (Polymer Laboratories, M_p = 580 Da up to M_p = $7.1*10^6$ Da).

CryoTEM. Aggregates were visualized using the FEI CryoTitan, equipped with a field emission gun (FEG) operating at 300 kV. A Gatan cryo-holder was used, operating at ca. -170°C. cryoTEM Cu 200 mesh R2/2 Quantifoil Jena grids were purchased from Quantifoil Micro tools GmbH. Prior to the vitrification procedure the grids were surface plasma treated using a Cressington 208 carbon coater. Subsequently, the sample vitrification procedure was carried out using an automated vitrification robot (FEI Vitrobot™ Mark III). For vitrification, 3 μl of the aggregation solution was applied manually to the cryoTEM grids inside the vitrobot chamber, which was conditioned at 100% humidity and at appropriate temperatures between 5 and 35 °C. The excess of liquid on the grid was blotted away and the grid plunged into liquid ethane for vitrification. For 2D imaging, the images were recorded using a 2k x 2k Gatan CCD camera (CryoTitan). Electron tomography was employed using a tilt range of -65° to +65° at 2° increments at a magnification of 19k and -20 μm defocus and a total electron dose of $40e^-.Å^{-2}$.

Small-Angle X-ray Scattering (SAXS) measurements were performed at the Dutch-Belgian BM26B beamline at the ESRF in Grenoble (France). An X-ray photon energy of 10 keV (X-ray wavelength of 1.24 Å) and two different sample-to-detector distances (7 m and 5 m) were used, in order to explore a wide q range ($0.35\ nm^{-1} < q$

< 2.9 nm^{-1}, where q is the modulus of the scattering vector = $q = 4\pi\sin(\theta/2)/\lambda$, with l the X-rays wavelength and θ the scattering angle). The SAXS images were recorded using a 2D Pilatus1M photon counting detector. The q axis has been calibrated using the q positions of known diffraction peaks from a Silver Behenate and rat tail tendon collagen standard samples. Absolute intensity scale in cm^{-1} has been obtained using water and Eltex as secondary standard samples. The samples were contained in 2 mm borosilicate capillaries and left for 2 mins to equilibrate at the appropriate temperature before the measurement was conducted. Measurements of pure THF and water and the solvent mixtures without polymer were measured and were used for background subtraction of the polymer-containing samples. Standard data reduction procedures, i.e. subtraction of the empty capillary contribution, correction for the sample absorption etc. were also applied. The SAXS curves from THF-water mixtures have been analyzed using the Ornstein-Zernike equation in order to quantify the composition fluctuations:

$$I(q) = I(0) / (1 + q^2\xi^2) + bkg$$

where q is the module of the scattering vector, I(0) is the scattering level at zero angles and ξ is the correlation length of the composition fluctuation.

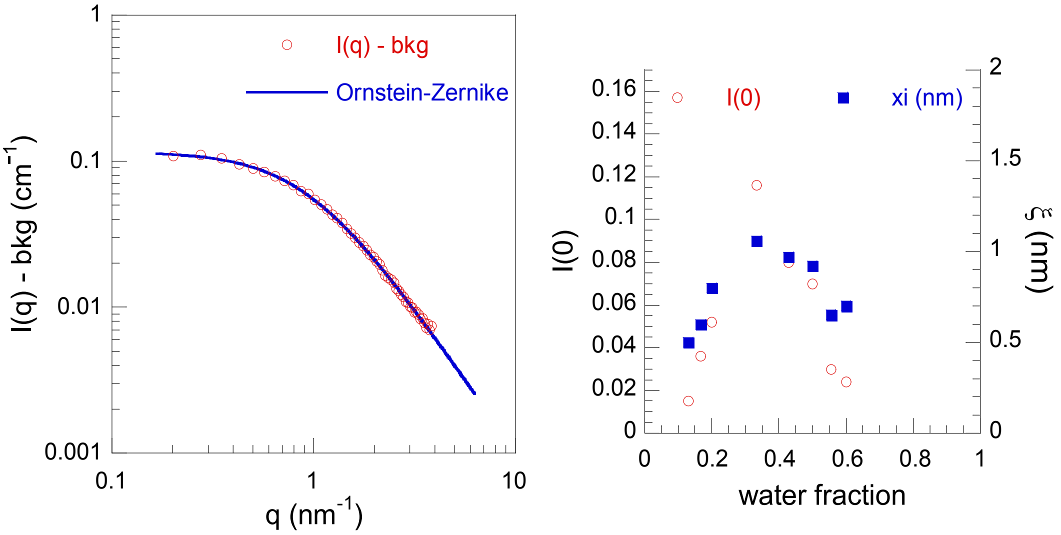
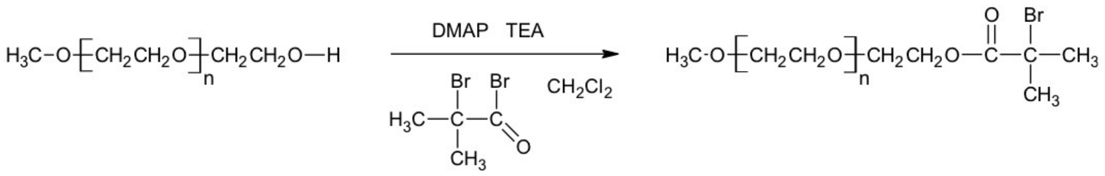


Figure S1. (*Left*) SAXS curve and best fit using the Ornstein-Zernike equation for the water/THF mixture 36 wt% of water. (*Right*) Zero angle intensity I(0) and correlation length as a function of the water content as obtained from the OZ analysis.

The correlation length of the THF/water composition fluctuations obtained via the OZ from SAXS are in agreement with SANS data reported in literature.[3]

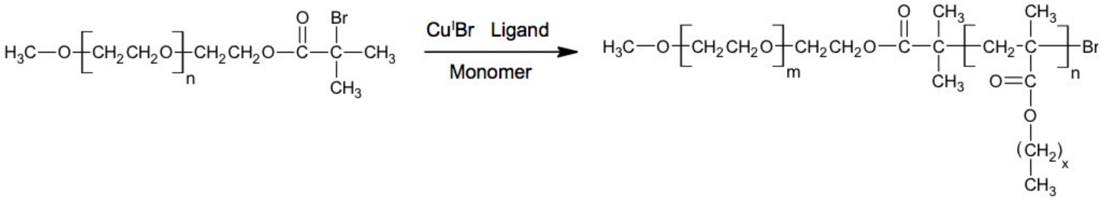
Synthesis of PEO macroinitiator



A modified literature procedure was followed to synthesize a poly(ethylene oxide) (PEO) macro initiator.[4] 2-Bromoisobutyryl bromide (1 ml, 8 mmol) was added dropwise to a stirred solution of triethylamine (1.12 ml, 8 mmol) and dimethylamino pyridine (0.99 g, 8 mmol) in dichloromethane at 0°C. Subsequently, a solution of poly(ethylene glycol) methyl ether (PEGME) (M_n ca. 2600 $gmol^{-1}$) (10 g, 4 mmol) in dichloromethane was added dropwise for a period of at least 1 hour under argon. After this, the solution was stirred once more for 18 hours at ambient temperature. A third of the reaction solvent was removed by rotary evaporation whereupon the resultant yellow precipitate was filtered off. The remaining solution was further diluted with 100 ml dichloromethane and transferred to a separating funnel. The solution was washed three times with a 10% hydrochloric acid solution and subsequently with a saturated sodium bicarbonate solution. The organic layer was collected and dried over anhydrous magnesium sulphate for several hours. The solution was filtered and the dichloromethane evaporated off. Finally, the resultant yellow precipitate was left under vacuum at 50°C for at least four hours. ^1H NMR was used to validate the structure, and the molecular weight parameters were determined by using size exclusion chromatography (SEC).

^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 1.91 (singlet, 6H, $(CH_3)_2C$-); 3.37 (singlet, 3H, -OCH_3); 3.62 (broad peak, 4H –OCH_2CH_2–); 3.81 (triplet, 2H, –OCH_2–); 4.31 (triplet, 2H, O=$COCH_2$–).

Synthesis of PEO_{52}-*b*-$PODMA_{26}$



The block copolymer was synthesised via atom transfer radical polymerization (ATRP) using a modified literature method.[1] The PEO macroinitiator (0.45g, 0.21 mmol) was placed in a schlenk tube along with a magnetic stirrer, and dissolved in 2ml of a solution of xylene with 10% isopropanol. To this solution were added octadecyl methacrylate (also dissolved in 2 ml of the above solution) (2.5g, 7.4 mmol), N-(n-octyl)-2-pyridyl(methanime) (0.19g, 0.88 mmol) and Cu(I)Br (22.5mg, 0.16 mmol). The schlenk tube was then sealed and the solution degassed with argon for 30mins, followed by three freeze-thaw cycles. The sealed tube was immersed in an oil bath maintained at 95 °C and the reaction mixture was stirred under argon for 12 h. After this time the seal was removed to expose the reaction mixture to air in order to stop the reaction. The mixture was diluted in THF and passed through an alumina column to remove the catalytic system. Excess THF was evaporated and the polymer was purified by precipitation from cold methanol. The precipitated polymer was subsequently dried in the vacuum oven. The structure was confirmed using 1H NMR and molecular weight parameters calculated using SEC.

^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ: 0.88 (triplet, 3H, -$(CH_2)_{17}$-CH_3); 1.10-1.42 (broad peak, 30H, CH_2-$(CH_2)_{15}$-CH_3); 1.48-1.70 (broad peak, 2H, OC-CH_2-C_{16}); 1.79 (singlet, 2H, C-CH_2-C), 1.82-2.10 (broad peak, singlet, 3H, O=C-C-CH_3), 3.38 (singlet, 3H, -OCH_3 PEO); 3.64 (broad peak, 4H, –(OCH_2CH_2)– PEO); 3.91 (broad peak, 2H, O=$COCH_2$-C)

Aggregate preparation

The following procedure was used to assess the development of the bicontinuous nanospheres in the nanoprecipitation method: in nine separate vials 50 mg of PEO_{52}-*b*-$PODMA_{26}$ (22 wt% PEO) was dissolved in 4 ml THF and a predetermined volume of water (0.2 to 6 ml) was added to each. Typically, the THF-polymer solution was stirred and immersed in an oil bath at 35 °C. After 10 minutes of equilibration, ultrapure water was slowly added at a rate of 0.067 $ml \cdot min^{-1}$. The newly-formed dispersion was removed from the oil bath and kept at 35 °C, taking care to seal the vial to prevent evaporation of THF. Subsequently, the dispersion was analysed by cryoTEM.

The dispersion containing 4 ml THF and 6 ml ultrapure water was then placed in a dialysis membrane and immersed in 3 L of ultrapure water, which was stirred and maintained at 35 °C. Aliquots of the dispersion were removed from the dialysis bag at different time points, kept at 35 °C and analysed by cryoTEM.

The remaining dispersion was removed from the water after 24 h of dialysis and an aliquot was again vitrified at 35 °C. The vitrobot temperature was decreased stepwise and vitrifications were conducted at predetermined temperatures, leaving the dispersion to equilibrate for 15 mins each time. The same procedure was followed for increasing the temperature again.

Figure S2: ^1H NMR spectrum of $PEO_{52}Br$ macroinitiator

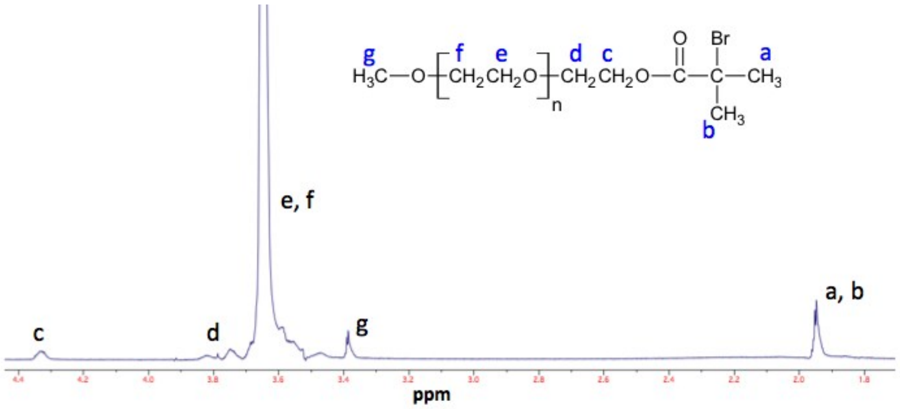


Figure S3: ^{1}H NMR spectrum of PEO_{52}-*b*-$PODMA_{26}$

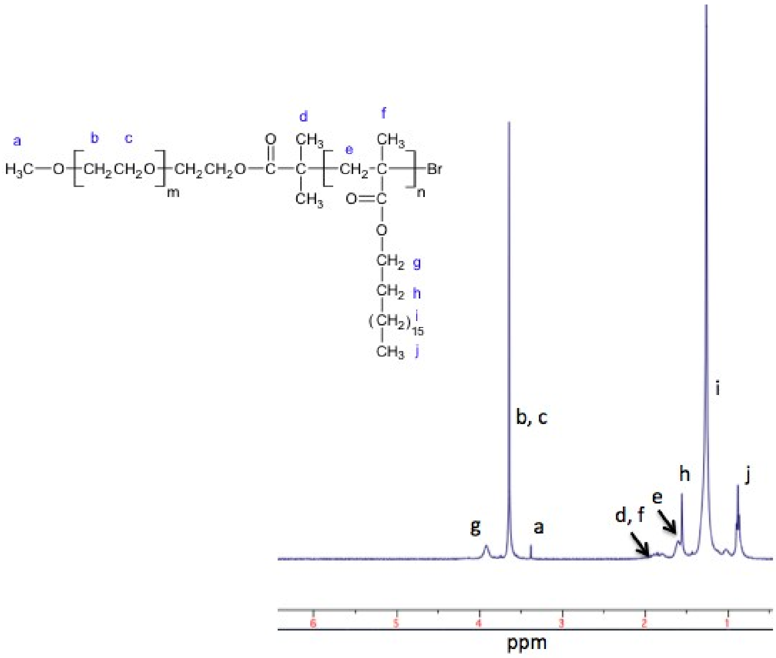


Figure S4: SEC chromatogram of $PEO_{52}Br$ macroinitiator and PEO_{52}-*b*-$PODMA_{26}$

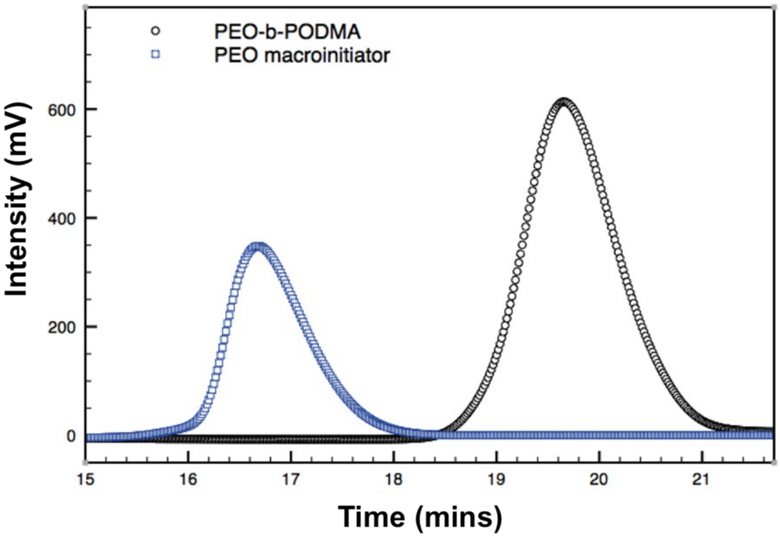
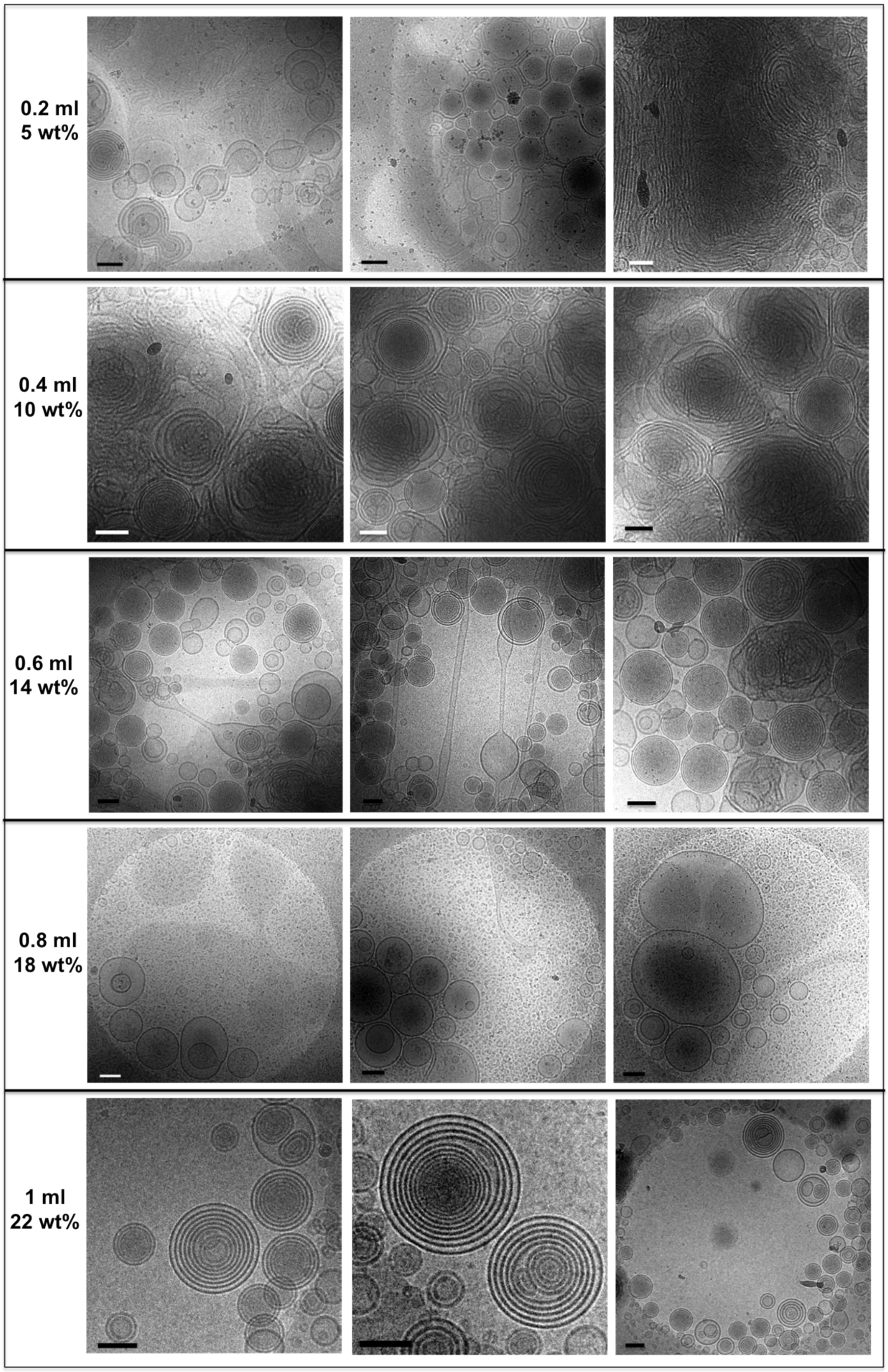
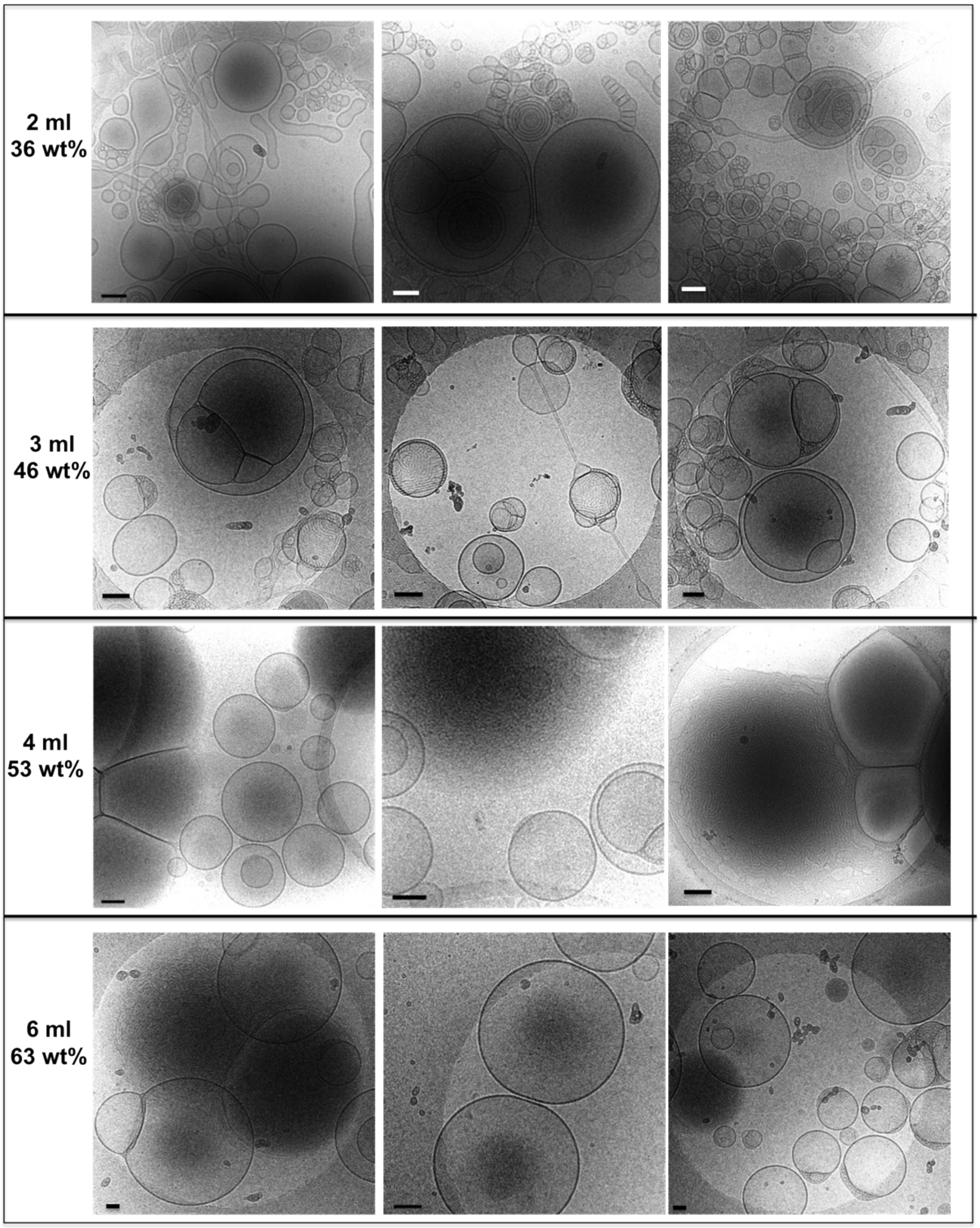


Table S1: Molecular weight parameters of the polymers

	M_n NMR	M_n SEC	M_w SEC	M_w/M_n SEC	wt% PEO
$PEO_{52}Br$	2500	2550	2700	1.07	100
PEO_{52}-*b*-$PODMA_{26}$	11300	10400	12900	1.25	22

Figure S5: CryoTEM images of the dispersions at different water contents. All scale bars represent 200 nm.




2 ml
36 wt%
3 ml
46 wt%
4 ml
53 wt%
6 ml
63 wt%

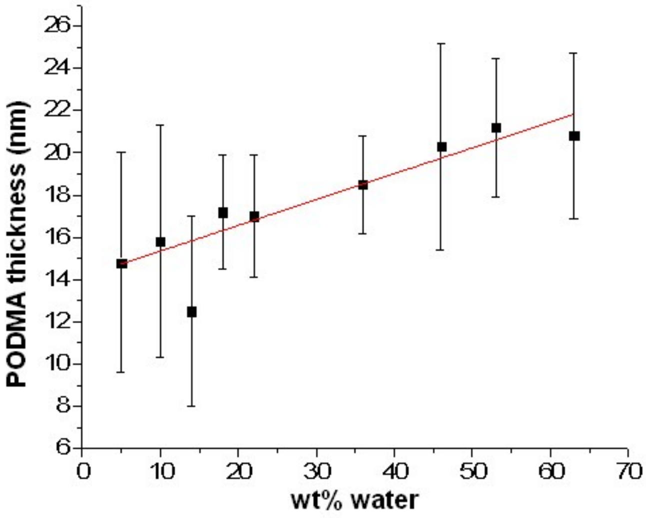


Figure S6: Average thickness of PODMA domain as measured by electron density profiles of calibrated cryoTEM images. Error bars depict an average of 50 measurements from cryoTEM images.

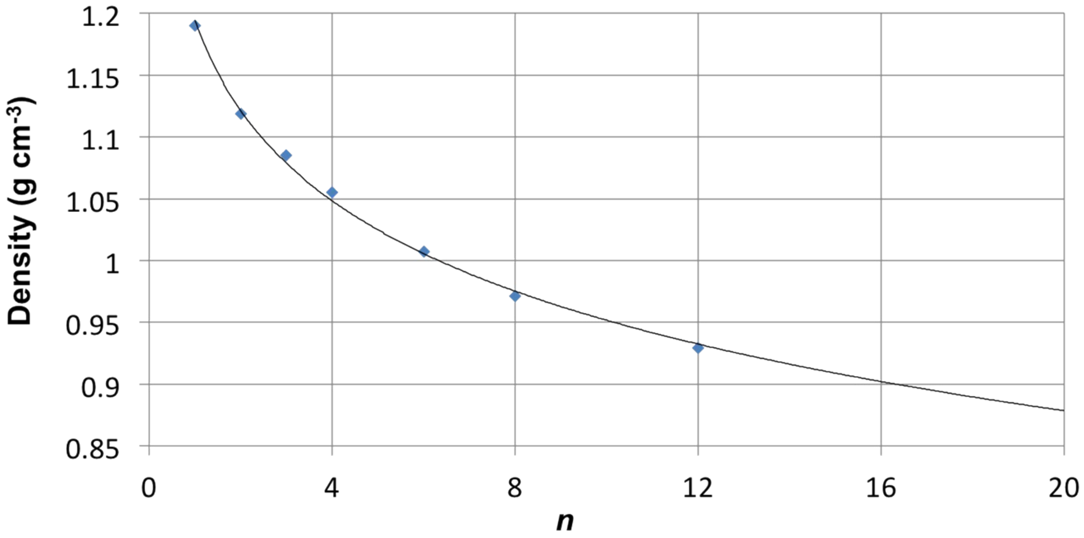


Figure S7: Graph of density vs length of alkyl side chain (*n*). Density of *n* = 18 extrapolated from this graph.

Density of PEO_{52}-*b*-$PODMA_{26}$ (22 wt% PEO; 0.936 $g.cm^{-3}$) estimated from density of PEGME 5000 $gmol^{-1}$ (1.10 $g.cm^{-3}$)[5] and density of octadecyl methacrylate extrapolated from graph above with data from references [5,6] .

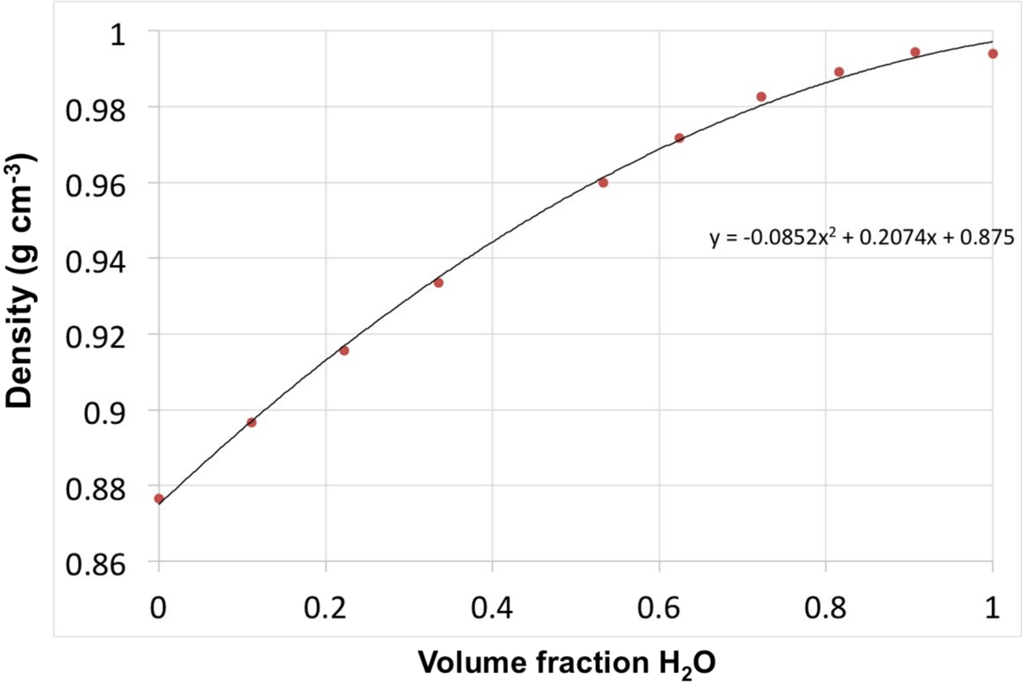


Figure S8: Density of THF-water solutions at 30 °C vs mole fraction as reproduced from reference [7].

SAXS

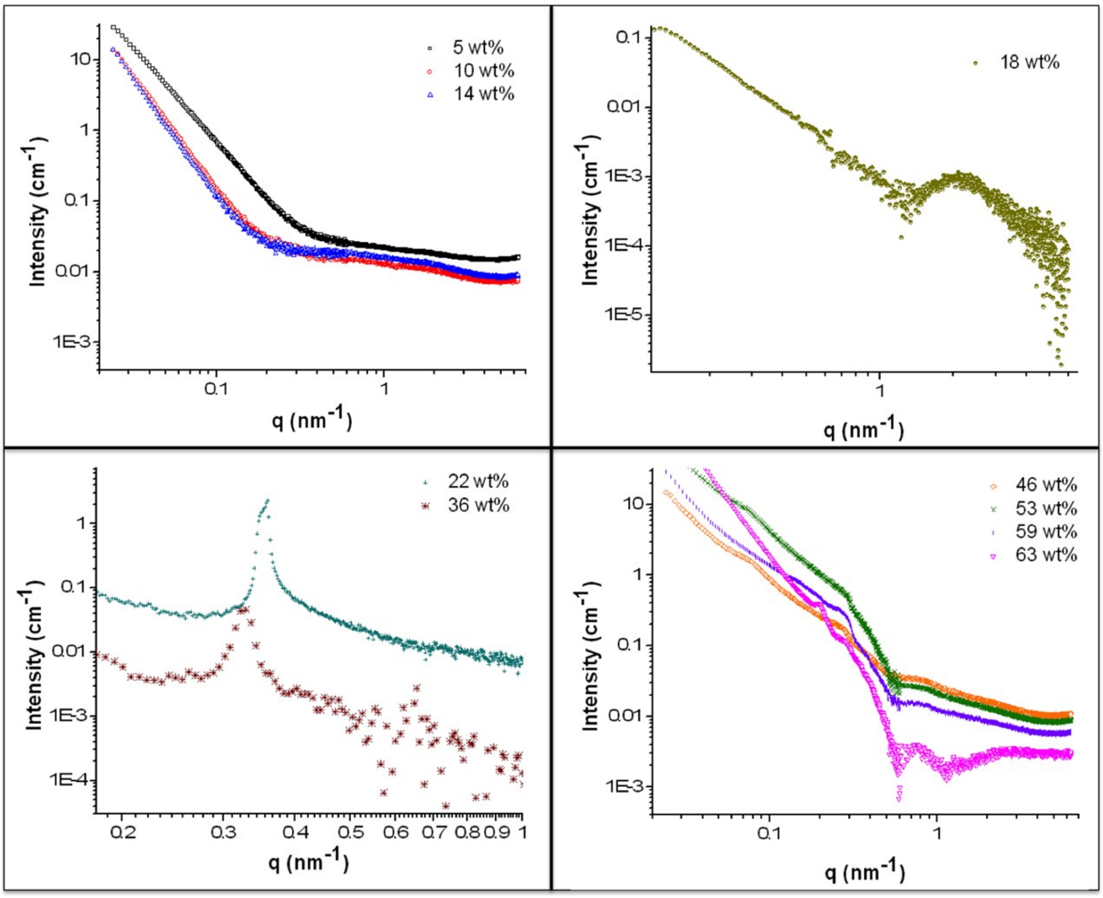


Figure S9: SAXS data recorded for the dispersions at different water contents. All data recorded at 35 °C.

The evolution of structure with the addition of water was ascertained by the evolution of the SAXS intensity. A quantitative analysis of the SAXS curves was impossible due to the complexity of the system (i.e. polydispersity in size and shape of the structures). However, for some samples, data modeling was possible and the main structural parameters could be obtained. The SAXS curve for the dispersion at 18 wt% water yielded a form factor with a low angle slope of q ~ -2, confirming the vesicle morphology observed in cryoTEM. At 22 and 36 wt% (multi lamellar vesicles by cryoTEM) the SAXS curves show a strong correlation peak, yielding a lamellar thicknesses of 17 and 19 nm respectively, in good agreement with that obtained from cryoTEM (21 ±4). The SAXS profiles at 46 - 63 wt%, are in agreement with a vesicle form factor with similar wall thickness (i.e. scattering minima at 0.6 and 1.25 nm^{-1}).

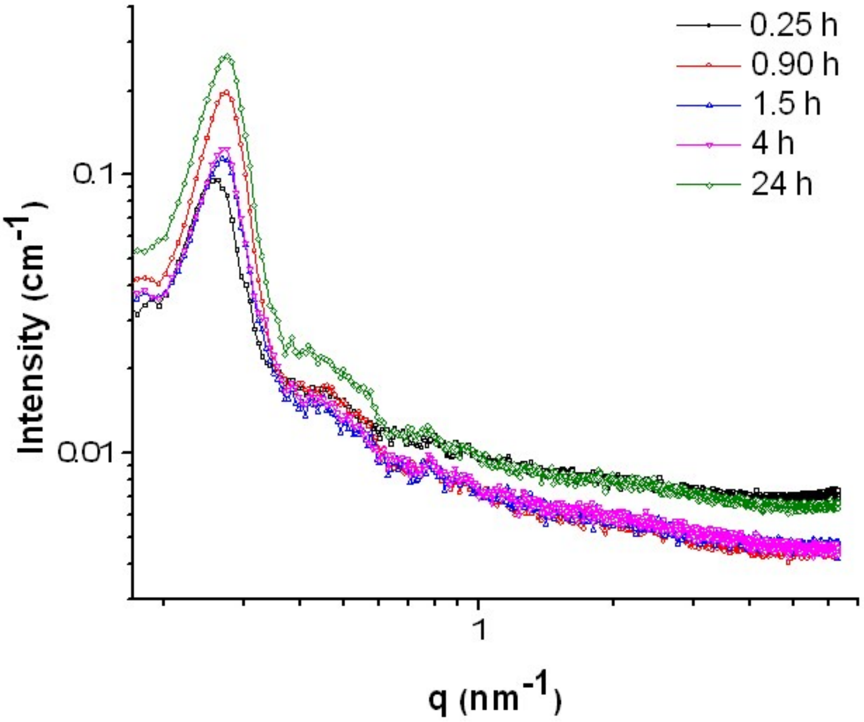


Figure S10: SAXS data recorded for the dispersion throughout the dialysis. All data recorded at 35 °C.

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