Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supporting Information –

Nanoscale detection of metal-labeled copolymers in patchy polymersomes

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Materials

2-(Methacryloyloxy)ethyl phosphorylcholine monomer (MPC, 99.9% purity) was donated by Biocompatibles U.K. Ltd. 2-Hydroxypropyl methacrylate (HPMA), 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (CADB), 4,4'-azobis(4-cyanopentanoic acid) (ACVA; V-501, 99 %), bis(2-hydroxyethyl)disulphide (98%), 2-bromoisobutyryl bromide (98%), basic alumina (Brockmann I, standard grade, ~150 mesh, 58 Å, anhydrous ethanol (99 %), anhydrous methanol (≥99.8 %), copper(I) bromide (Cu(I)Br, 99.999%), 2,2′-bipyridine (bpy, 99%), tris(2-carboxyethyl)phosphine hydrochloride (TCEP, ≥ 98.0 %) and indium chloride ($InCl_3$, 99.999 %) were purchased from Sigma Aldrich UK.. Azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) was purchased from Wako Pure Products. The silica gel 60 (0.063-0.200 μm) used to remove the spent ATRP catalyst was purchased from E. Merck (Darmstadt, Germany).HPLC grade dichloromethane, methanol and toluene was purchased from Fisher Scientific (Loughborough, UK). Magnesium sulfate ($MgSO_4$), sodium chloride (NaCl), triethylamine (Et_3N), sodium sulfate (Na_2SO_4) were laboratory reagent grade from Fisher Scientific (Loughborough, UK).. . Maleimido-mono-amide-DOTA (>94 %) was purchased from Macrocyclics™ (Dallas, U.S.A.). All the above were used as received.

2-(diisopropylamino)ethyl methacrylate (DPA) was purchased from Scientific Polymer Products Inc. (Ontario, U.S.A.) and passed through a DHR-4 purchased from the same vendor.

Phosphate-buffered saline (PBS) was prepared from tablets obtained from Oxoid (Basingstoke, UK). Semi-permeable cellulose dialysis tubing (Spectra/Por 6 MWCO 1,000) was purchased from Fisher Scientific (Loughborough, UK).Bis[2-(2-bromoisobutyryloxy)ethyl] disulfide ($BiBOE_2S_2$) was synthesized according to a previously published protocol.[i]Synthesis of PEO_{23}-$PDPA_{15}$ and PEO_{113}-$PDPA_{56}$ was prepared based on our previously.[ii] The $POEGMA_{20}$-$PDPA_{200}$ control polymer was synthesized according to a literature protocol.[iii] The PEO macroCTA used for the RAFT polymerizations was prepared based on our previous work.[*]

Synthesis of $PDPA_{70}$-$PMPC_{25}$-S-S-$PMPC_{25}$-$PDPA_{70}$ by ATRP

A solution of $BiBOE_2S_2$ (0.1850 g, 0.0004 mol, 1 eq., 2 eq. Bromine) in 4 mL anhydrous methanol was transferred to a flask containing MPC (6.045 g, 0.020 mol, 50 eq.) under nitrogen via cannula. The flask was washed with further 4 mL anhydrous methanol. The resulting solution was purged with nitrogen for 35 minutes and kept at room temperature (~ 20 °C). Then, a mixture of bpy (0.2543 g, 0.00163 mol, 4 eq.) and Cu(I)Br (0.1169 g, 0.00081 mol, 2 eq.) was added. A solution of DPA (12.130 g, 0.0569 mol, 142 eq.) in 14 mL anhydrous methanol was prepared and purged with nitrogen for 40 minutes in a separate flask. After 1h, a sample of the polymerization mixture was taken out for assessment of conversion. Then, the DPA solution was added to the polymerization mixture during 5 minutes and the reaction mixture was left overnight at room temperature. After 22 h, ^{1}H NMR analysis confirmed that the conversion was > 99 % and the reaction was opened to the atmosphere and diluted with methanol and dichloromethane (2:3 v/v). The solution gradually turned green, indicating oxidation of the catalyst system. The green solution was passed through silica using a 3:2 dichloromethane:methanol mixture and evaporated partially to give an opaque solution. The solution was dialyzed (MWCO 1,000 Da) against methanol (1 time), dichloromethane:methanol 1:1 (1 time), dichloromethane (1 time). The solvent was removed at reduced pressure and the polymer was dried under vacuum at 30 °C overnight (16.3 g, 92 % yield).

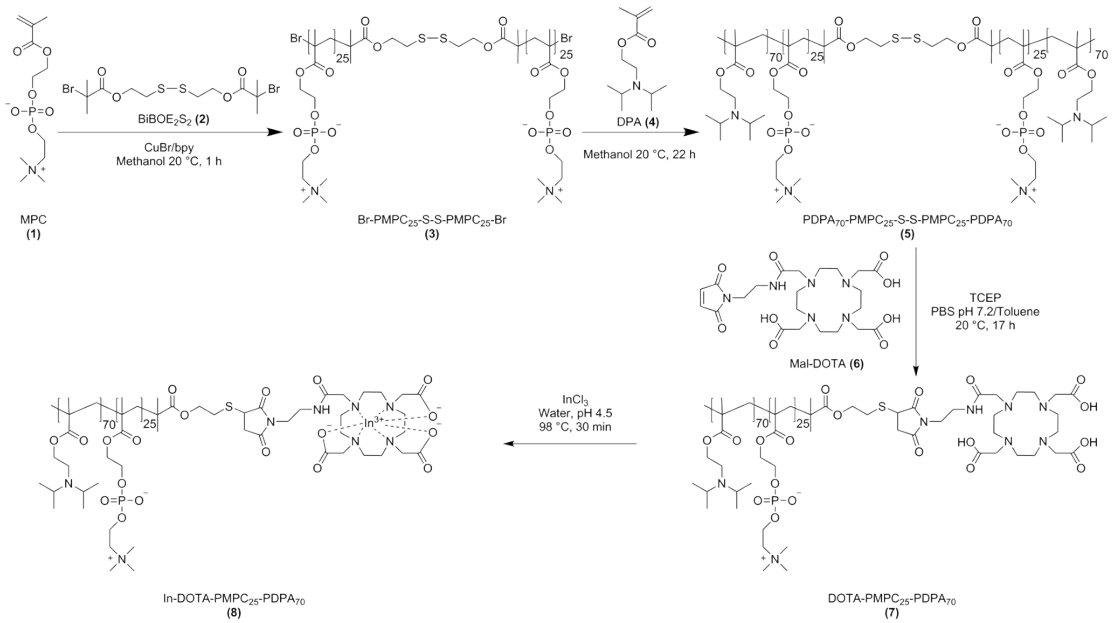
^{1}H NMR ($CDCl_3$/MeOD - 3:1) composition: $PDPA_{67}$-$PMPC_{25}$-S-S-$PMPC_{25}$-$PDPA_{67}$

Preparation of DOTA-S-$PMPC_{25}$-$PDPA_{67}$

$PDPA_{67}$-$PMPC_{25}$-S-S-$PMPC_{25}$-$PDPA_{67}$ (0.5026 g, approximately 10 μmol disulfide) was dispersed in a mixture of PBS buffer (5 mL) and toluene (1 mL). The resulting suspension was purged with nitrogen for 20 minutes. Then, TCEP (20.5 mg, 0.072 mol) and maleimido-mono-amide-DOTA (18.2 mg, 23.2 μmol) were added. After 17 h, the solution was opened in the air, and diluted with water (~10 mL). After that it was dialyzed (MWCO 1,000 Da) against water (4 times) and freeze-dried (0.4366 g, 87 % yield).

The same procedure was used for the Indium-labeled maleimido-mono-amide-DOTA assuming a molecular weight of 871 g/mol.

Scheme SI-1: Synthesis of Indium-labeled diblock PMPC25-PDPA70 copolymers using Atom Transfer Radical Polymerization (ATRP)



Indium labelling of $PMPC_{25}$-$PDPA_{67}$

The Indium-labelling was performed according to a literature protocol.[iv]

A 4.6 mM stock solution of $InCl_3$ was prepared by dissolving InCl3 (0.0173 g, 78.2 µmol) in water (17 mL).DOTA-S-$PMPC_{25}$-$PDPA_{67}$ (0.1164 g, ~5 µmol polymer) was dissolved in 1 M HCl (5 mL). pH was adjusted to 4.5 with 5 M NaOH. $InCl_3$ stock solution (2 mL, 9.2 µmol) was added, which led to a decrease in pH to 3.5. The pH was then adjusted to 4.5 with 1 M NaOH and the solution was left at 98 °C for 30 min. Then, it was dialyzed (MWCO 1,000 Da) against water (5 times) and freeze-dried (99.6 mg, 86 % yield)

A similar protocol was followed for PMPC-PDPA (~5 µmol polymer) without the DOTA and for PEO_{113}-$PDPA_{56}$ (6 µmol polymer) and $POEG_{10}MA_{25}$-$PDPA_{200}$ (~2 µmol polymer).

Indium labelling of DOTA-maleimide

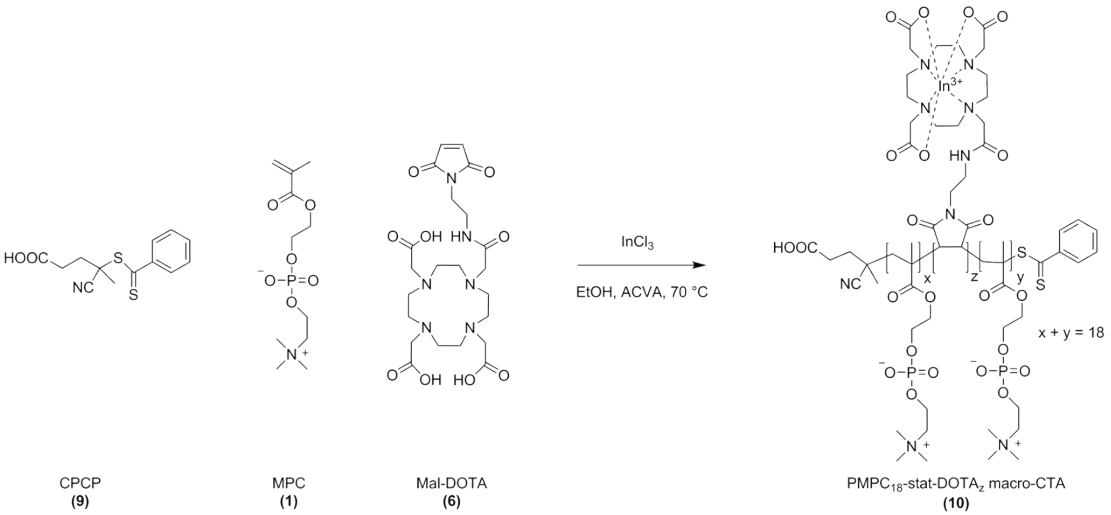
The Indium-labelling was performed according to a literature protocol:[v]A mixture of maleimido-mono-amide-DOTA (50.3 mg, 60 µmol), InCl3 (14.7 mg, 66 µmol) and 0.5 M ammonium acetate buffer pH 6 (0.5 mL) was heated to 80 °C for 1 h. The solution was dialyzed (MWCO 1,000 Da) against deionized water (3 times). andwas then freeze-dried.

For selected polymer samples (see main text and Figure S1), the polymer solution was first dialysed against 0.1 M HCl (2 times) and then against deionized water.

Synthesis of Indium-labelled $PMPC_{18}$ macro-CTA agent by RAFT polymerization in one step

A mixture of MPC monomer (0.23 g; 0.76 mmol), maleimido-mono-amide-DOTA (30.0 mg; 0.04 mmol), CADB RAFT agent (10.7 mg; 0.04 mmol) and 0.51mL anhydrous ethanol was placed in a tube equipped with a magnetic stirrer bar (target degree of polymerization = 20). The sealed reaction vessel was purged with nitrogen for approximately 20 min and ACVA initiator (2.7 mg; 0.01 mmol, CTA/ACVA molar ratio = 4) was added. The resulting solution was purged with nitrogen gas for a further 10 min and was placed in an oil bath at 78 °C. The reaction was left to proceed for 95 min and quenched by cooling down to 25 °C and exposure to air. The final polymer solution was dialyzed (MWCO 1,000 Da) against deionized water (5 times) followed by lyophilization (MPC conversion = 86 %, D.P. = 18 and M_n= 6500 g mol^{-1} as judged by ^{1}H NMR in d_4-methanol). This In-DOTA-$PMPC_{18}$ macro-CTA was used in the RAFT polymerization for the formation of patchy polymersomes by polymer-induced self-assembly (PISA).

Scheme SI-2. Synthesis of DOTA-functionalized PMPC macro-chain transfer agent using reversible addition fragmentation chain transfer (RAFT) polymerization



CPCP (**9**) MPC (**1**) Mal-DOTA (**6**) $PMPC_{18}$-stat-$DOTA_z$ macro-CTA (**10**)

Inductively coupled plasma spectrometry

Indium loaded polymer samples were dissolved in 0.1 M HCl or in water to a concentration of around 0.5 to 3 mg/mL, determined to 3 significant digits. A Spectro Ciros Vision ICP-ES Spectrometer was used for the ICP measurements.

NMR spectroscopy

^{1}H NMR spectra were recorded using a Bruker Avance 400 spectrometer and analyzed using MestRe-C vs. 2.3a and ACD/NMR Processor Academic Edition vs. 12.01

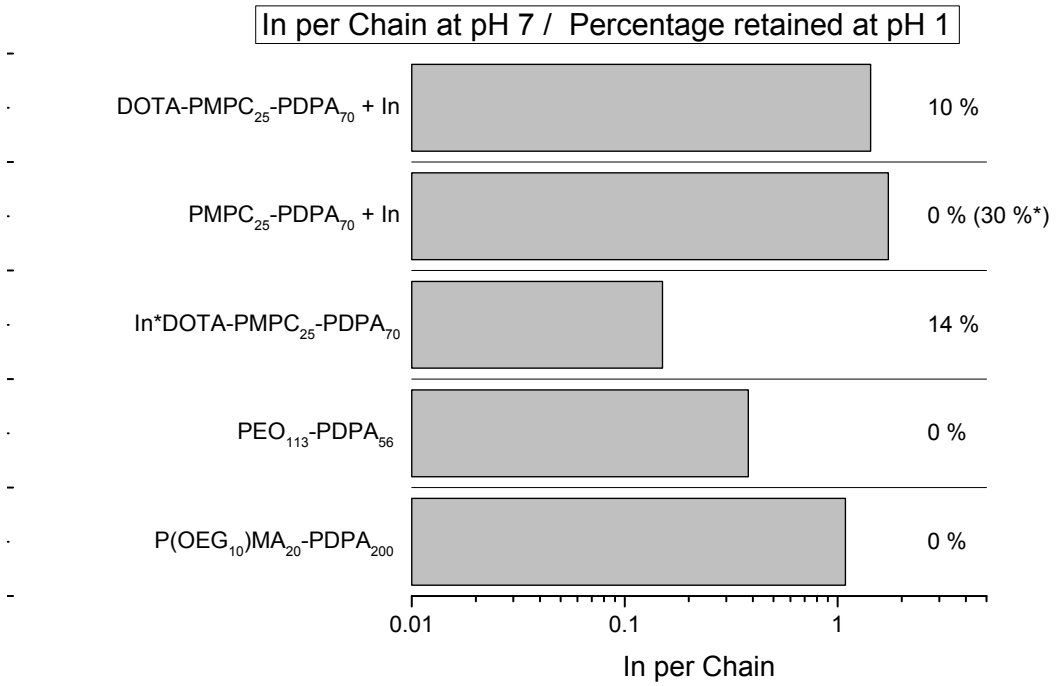


Figure S1. Chart showing the Indium content per copolymer chain as determined by ICP-AES. The values for the PMPC-based copolymers are obtained using the ratio between Indium and Phosphorous. The values for PEO and POEGMA-based copolymers are obtained using the molecular weight calculated from the target composition. The percentage given represents the corresponding amount of Indium left after an acidic workup in relation to its neutral counterpart. Percentage with a (*) represents the In retained after attempted removal by the means of EDTA after polymersome formation.

Figure S1 shows that the addition of EDTA to the formed polymersomes does not remove Indium bound to the polymer (30% In retained for $PMPC_{25}$-$PDPA_{70}$ + In). A method to completely remove non-DOTA bound polymer was eventually found by removing it by dialysis against acidic water. Here, only DOTA-labelled polymers were able to retain Indium during the procedure, showing Indium being bound to the ligand.

We analyzed whether the PMPC-PDPA gets decomposed during the labeling process and conducted a GPC before and after the Indium treatment. As both GPC traces show a complete overlap, decomposing can be ruled out. NMR analysis showed a change in PDPA peaks of the polymer, as 2 of them vanished completely and 2 other ones shifted slightly. This might be due to partial protonation and partial Indium binding, but cannot be taken as a conclusive indication of where the Indium is bound in the PMPC-PDPA.

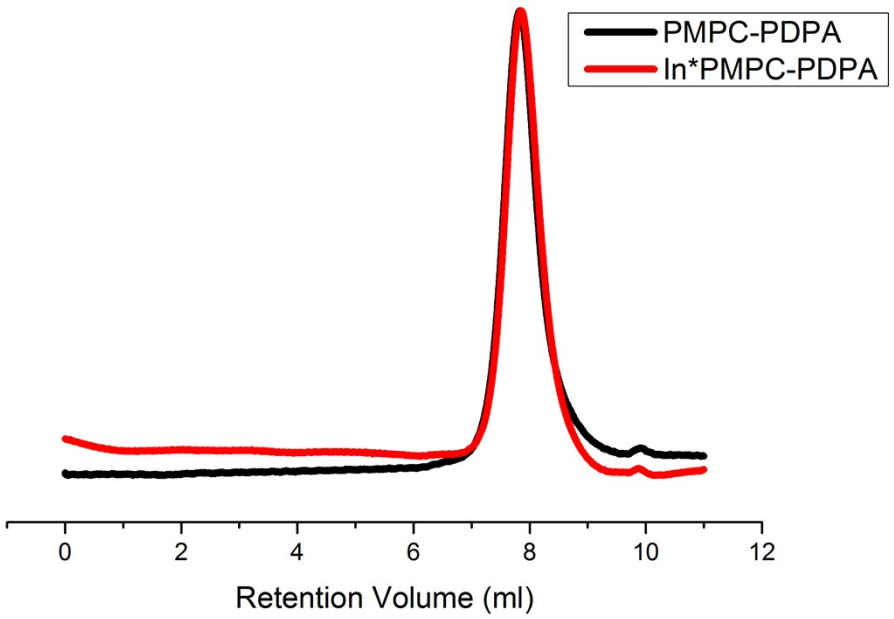


Figure S2. GPC trace of pure PMPC-PDPA and after it has been labelled with Indium. There are no visible signs of decomposing or partial hydrolysis, since both traces overlap completely.

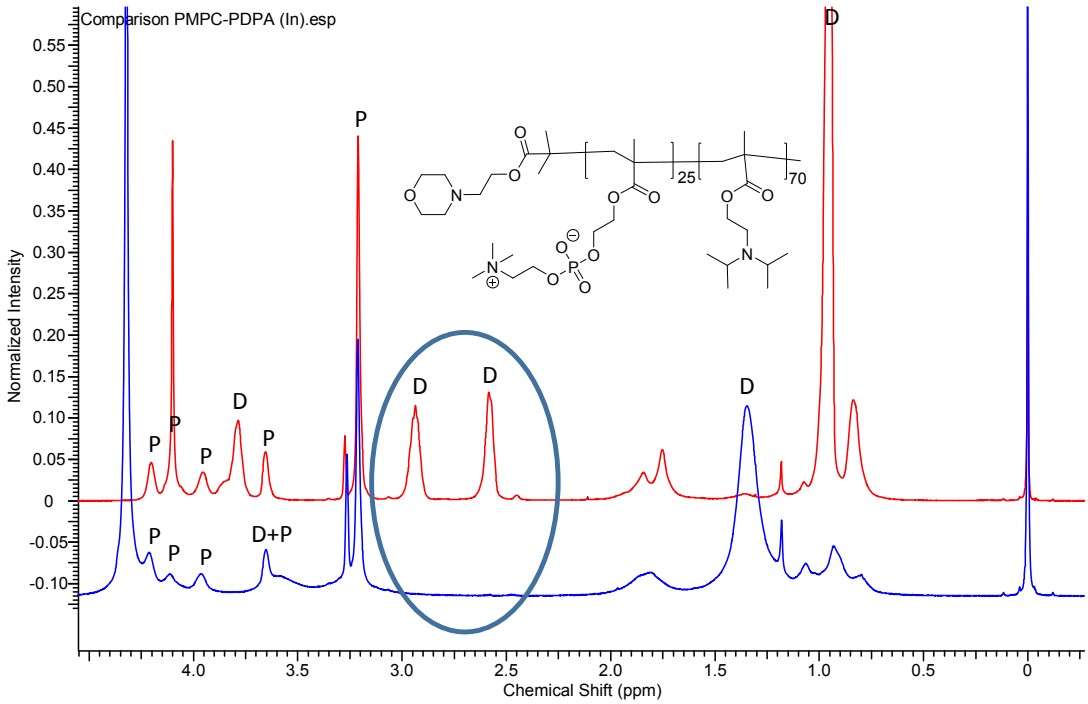


Figure S3. 1H NMR spectra of pure PMPC-PDPA (red) and In labelled PMPC-PDPA (blue). Peaks belonging to the PMPC part have been labelled as “P” and all peaks belonging to PDPA marked as “D”. Differing CDCl3/MeOD compositions explain the shift in the methanol-peak. Apparently, both peaks relating to positions directly neighboring the Nitrogen vanish completely, while the other ones stay. Since no change in the GPC trace was observed, this must be due to protonation and/or In labelling. Final clarification could not be reached.

Patchy polymersome formation

(a) Film rehydration

Nanometer-sized In-DOTA-PMPC-PDPA/PEO-PDPA polymersomes were formed by Film rehydration method. In-$PMPC_{25}$-$PDPA_{70}$ and PEO_{23}-$PDPA_{15}$ were premixed at 1:9 and 1:1 molar ratios and dissolved in 2:1 v/v chloroform/methanol at 10 mg/ml total copolymer concentration in the organic solvent. The solution was placed in a vacuum oven at 40°C and left overnight in order to evaporate the organic solvent. Acopolymer dried thin film was formed on the sample vial surface. Rehydration of the In-$PMPC_{25}$-$PDPA_{70}$/PEO_{23}-$PDPA_{15}$ film was performed using 0.1 M PBS (pH 7.4) at a copolymer concentration of 5 mg/mL. The aqueous dispersions were stirred with a magnetic stirrer at 2000 rpm for two weeks at room temperature. The polymersome solution was then centrifuged 15 minutes at 500 relative centrifugal force (rcf) followed by 5 minutes at 2000 rcf using an Eppendorf Microcentrifuge. Centrifugation was performed in order to purify the solution and narrow down polymersome sizes as large and slighter particles remained in the pellet and supernatant respectively.

For Film Rehydration including EDTA, the EDTA (1mg per 2 mg polymer) was added 2 weeks after the film rehydration and the solution then dialysed against pH = 9 water for 4 times. The sample was then first analysed by TEM , liophilised and then subjected to ICP analysis as described above.

(b) by PISA

In-DOTA-PMPC-PHPMA/PEO-PHPMA polymersomes were formed *in-situ* during polymerization by a RAFT PISA approach using an equimolar mixture of two macro-CTAs. In-DOTA-$PMPC_{18}$ macro-CTA (45.2 mg, 0.007 mmol), PEO_{113} macro-CTA* (36.9 mg, 0.007 mmol), HPMA (0.522 g. 3.62 mmol, 260 equiv. vs macro-CTA mixture) and 2.4 mL water (total solids content = 20% w/v) were added in a 5 mL round-bottom flask equipped with a magnetic stir bar. After purging the mixture with nitrogen for 20 min, VA-044 (1.5 mg, 0.005 mmol, CTA mixture / VA-044 molar ratio = 3.0) was added in the reaction flask. The solution was purged with nitrogen for a further 10 min and was immersed in an oil bath set at 40 °C. The reaction was left to proceed under

stirring for 19 h to ensure complete monomer conversion (HPMA conversion ~ 100% as judged by ^{1}H NMR in d_4-methanol) and quenched by cooling at 20 °C followed by exposure to air. After the RAFT polymerization, a dilute polymer solution (0.1 w/v) of the formed polymersomes was prepared for DLS measurement (sphere-equivalent intensity-average diameter of polymersomes = 484 nm) and TEM grid preparation.

Patchy polymersome characterization

Dynamic light scattering (DLS)

A Zetasizer Nano-ZS (Malvern Instruments, UK) was used for the DLS experiments. Aqueous copolymer solutions (0.1% w/v) were analyzed using disposable cuvettes. The DLS data were averaged over three consecutive runs.

Transmission Electron Microscopy (TEM) imaging

Conventional TEM imaging was performed using a FEI Tecnai G2 Spirit TEM microscope at 80 kV equipped with an Orius SC1000 camera. Although the polymersomes contained Indium for enhancing the contrast seen by TEM they were also stained using a phosphotungstic acid (PTA) solution at 0.75% (w/v). Sigma Aldrich supplied PTA at 10% (w/v) was used. The solution was prepared by dissolving 37.5 mg of PTA in boiling distilled water (5 mL). The pH was adjusted to 7.0 by adding a few drops of 5 M NaOH under continuous stirring. The PTA solution was then filtered through a 0.2 μm filter.

Copper grids were glow-discharged for 40 seconds in order to render them hydrophilic. Then 5 μL of polymersome/PBS dispersion (diluted 10-fold, concentration 0.5 mg/ mL) was deposited onto the grids for one minute. After that, the grids were blotted with filter paper and immersed into the PTA staining solution for 5s for negative staining. Then the grids were blotted again and dried under vacuum for 1 min.

In-PMPC$_{25}$-PDPA$_{70}$/PEO$_{23}$-PDPA$_{15}$ polymersomes were imaged in the absence of selective staining to quantify the contrast provided by Indium labelling. Fig. S2 shows a typical polymersome at 1:9 copolymer molar ratio formed after two weeks of stirring with clearly defined dark domains formed in

the surface. A magnified image of a chosen domain is also shown in Fig. S4 where the darker circular areas correspond to electron dense Indium labelled PMPC molecules within the domains. The ability to detect nanoscale morphology inside the polymersome domains in the absence of selective stain demonstrates the efficiency of Indium labelling when used as a tool for enhanced TEM imaging and resolution.

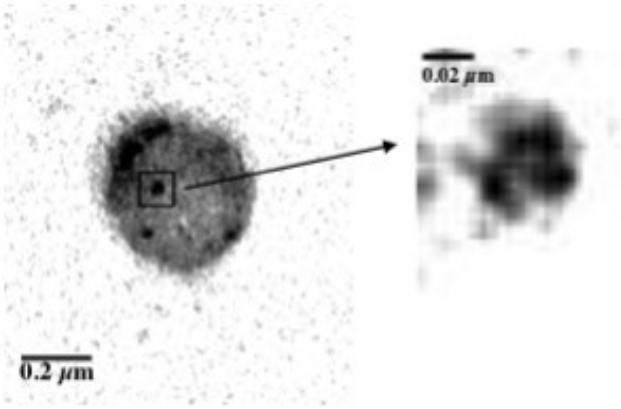


Figure S4 TEM image of unstained In-PMPC-PDPA/PEO-PDPA polymersomes at 1:9 molar ratio after two weeks of stirring. The segregated Indium rich domains in the polymersome surface as well as their nanoscale morphology can be observed even in the absence of selective staining.

Statistical analysis on five different polymersomes after two weeks of stirring at 1:9 copolymer molar ratios has been performed in order to obtain quantitative information from the TEM images.

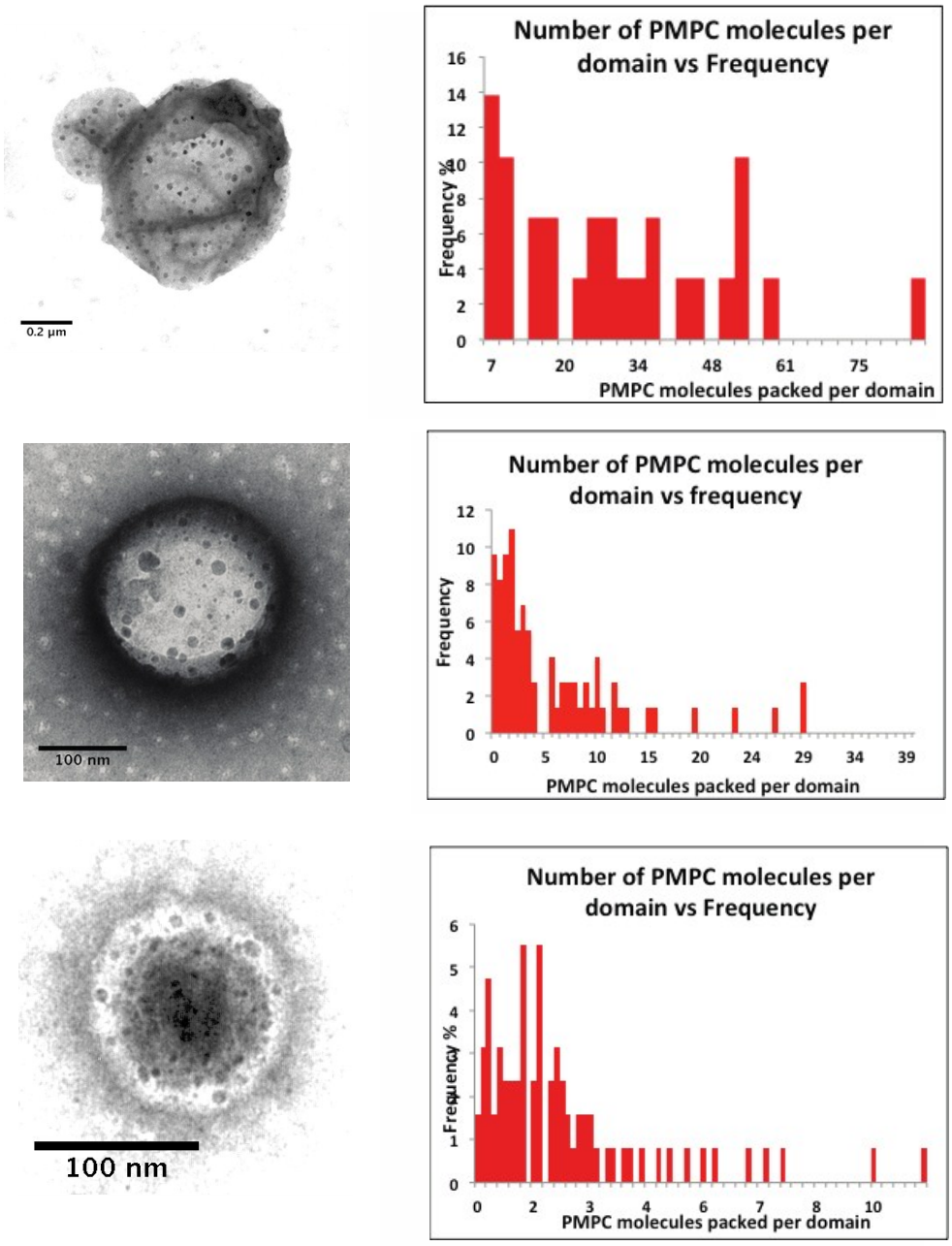


Figure S5 TEM image of different In-PMPC-PDPA/PEO-PDPA polymersomes at 1:9 molar ratio after two weeks of stirring with their respective statistical distribution of the PMPC domains.

The clearly defined domain areas were measured using Image J software. Data binning was performed in the measured areas dividing them into intervals and computing the frequency for each interval. This process served to reduce errors and as a way of quantization.

Parameters such as domain area, number of PMPC molecules per domain and fractional area vs. frequencies have been calculated (Fig. S3).

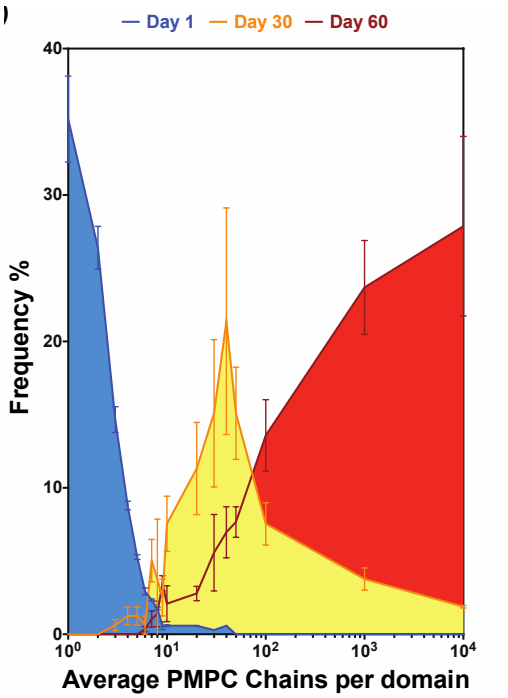


Figure S6 Distribution of the average number of PMPC chains per domain for the vesicles shown in the main paper (Figure 2) as a function of the polymersome ageing. This was calculated by averaging over 20 vesicles per sample.

Scanning Transmission Electron Microscopy (STEM) imaging, and Energy Dispersive X-ray Spectroscopy (EDX)

Indium distribution within polymersomes was mapped using a JEOL 2100 microscope operating at 200kV on STEM mode and equipped with an EDX detector X-Max^N 80 T from Oxford Instruments.

For each sample, the following procedure was applied. Indium containing polymersomes were first imaged by conventional TEM in the absence of PTA staining using a CCD Camera Orius SC2001 from Gatan. The microscope was then switched to STEM mode and the EDX detector was inserted. Indium mapping and X-ray spectra were then acquired using Aztec software from Oxford Instruments. In the EDX spectrum, the peak at 3.28 kV corresponds to the Lα X-ray energy level for the Indium element.

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