Cosolvent fractionation of PMOXA-*b*-PDMS-*b*-PMOXA: Bulk separation of triblocks from multiblocks[☆]

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Abstract

Challenges regarding the synthesis, purification, and characterization of the amphiphilic triblock copolymer poly(2-methyl-2-oxazoline)-*block*-polydimethylsiloxane-*block*-poly(2-methyl-2-oxazoline) (PMOXA-*b*-PDMS-*b*-PMOXA) are herein illustrated and discussed. A block copolymer (BCP) library was synthesised by cationic ring-opening polymerisation of 2-methyl-2-oxazoline on broadly and narrowly dispersed PDMS macroinitiators. The crude BCPs were fractionated by a newly developed cosolvent extraction technique. With this it is possible to remove the side products of chain-transfer reactions, intrinsic to the polymerisation of 2-oxazolines. The obtained fractions were characterized by nuclear magnetic resonance, gel permeation chromatography and light scattering, revealing that the cosolvent fractionation separates multiblock copolymers from triblock copolymers. Self-assembly of the individual polymer fractions showed that the cosolvent fractionation is essential to yield polymersomes forming triblock copolymers.

Keywords: cosolvent fractionation, PMOXA-*b*-PDMS-*b*-PMOXA, polymersome self-assembly, triblock and pentablock copolymer, 2-methyl-2-oxazoline polymerisation, multiblock copolymer

1. Introduction

Self-organization and self-assembly are key concepts of nature, enabling the construction of molecular objects with all kinds of different architectures. Cell membranes, for example, are assembled from amphiphilic phospholipids to facilitate compartmentalization, a necessity for spacial separation of chemical reactions within a cell. The spacial confinement alone would only allow chemical reactions to proceed side-by-side until reaching equilibrium within their respective compartment. To

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interconnect these reactions, a controlled exchange of molecules across the membrane is required. In nature, such molecular gate keeping is realized by decorating the membranes with specific proteins, controlling the direction and type of molecules or ions allowed to pass through the membrane. The concept of phospholipid assembly inspired the liposome field, where these molecules are applied for a variety of purposes ranging from basic research on membrane proteins to the development of FDA approved drugs such as Doxil. Large scale chemical synthesis of phospholipids and their work-up requires multiple steps, [1] while the chemical modification of lipids (one method to introduce additional functionalities or targeting moieties) is not straightforward.

In contrast to the naturally occurring lipids, amphiphilic block copolymers (BCP), a special type of copolymers, where blocks of homopolymer with distinctly different physical properties are covalently linked into a single macromolecule, represent a widely explored alternative to lipidic amphiphiles for self-assembly. The BCP's synthetic origin renders them abundant, enables the combination of a vast amount of chemical groups, facilitates the introduction of reactive groups, and allows for linear [2], random [3], dendrimeric [4], branched [5], grafted [6] molecular geometries or every combinations thereof. Furthermore, BCPs can be rationally designed [7] to fine-tune the properties of the macromolecule and thereby give rise to a sheer limitless diversity in self-assembled structures [8] or emergent functions [9]. The amphiphilic triblock copolymer poly(2-methyl-2-oxazoline)-*block*-polydimethylsiloxane-*block*-poly(2-methyl-2-oxazoline) (PMOXA-*b*-PDMS-*b*-PMOXA) represents the first one hosting a functional bacterial membrane protein [10] and still is the flagship for the engineering of polymer based nanoreactors exploiting natural membrane proteins. Over the last 16 years many different membrane proteins have been successfully inserted into PMOXA-*b*-PDMS-*b*-PMOXA membranes despite the hydrophobic mismatch [11] to yield nanoreactors [12], artificial organelles [13] or into PMOXA-*b*-PDMS diblock copolymer based planar membranes to support functional membrane proteins on surfaces [14]. Assemblies based on PMOXA-*b*-PDMS-*b*-PMOXA blended with PDMS-heparin conjugates enabled Najer *et al.* to engineer nanomimics, inhibiting fresh invasion of erythrocytes with Merozoites *in vitro* [15]. Phospholipids self-assemble into membranes which are 3 to 5 nm thick, whereas BCP based membranes are in general much thicker. To compare the lateral diffusion of individual phospholipid chains within a lipid membrane to the lateral diffusion of BCP chains within PMOXA-*b*-PDMS-*b*-PMOXA membranes, polymeric membranes with a thickness similar to the ones of lipids were required. The self-assembly of 6-10 nm thick polymeric membranes is possible from triblock copolymers with well-defined and short block ratios [16] but required the development of a novel purification strategy.

The preparation of BCPs for membrane self-assembly is challenging in several ways: (I) the polymerisation of 2-oxazolines is generally affected by chain-transfer reactions [17], (II) the formation of parasitic PMOXA homopolymer species during the polymerisation of 2-methyl-2-oxazoline, and (III)

the work-up of amphiphilic BCPs requires special attention, since they might co-precipitate with the homopolymers if the respective blocks are short. Therefore, precipitation in hexane was found not to be successful and yields BCPs which are not self-assembling into polymersomes. As alternative methods for the work-up of BCPs either preparative gel permeation chromatography (prepGPC) or selective adsorption based on different hydrophilicity/hydrophobicity might be used. However, polymer fractionation of 5 g crude polymer on a prepGPC system is time-consuming and requires dedicated expensive equipment. Furthermore, PMOXA is known to interact with cross-linked polystyrene, the common stationary phase in GPC columns [18]. Selective adsorption based on the hydrophobic/hydrophilic properties might be applicable to remove homopolymers, but is not expected to remove the side-products formed via chain-transfer reactions. The parasitic PMOXA homopolymer chains are completely water soluble and most likely become encapsulated but are not expected to affect the self-assembly process negatively, supported by many experiments where hydrophilic cargo is encapsulated into polymersomes. In contrast, the presence of unreacted PDMS macroinitiator or interlinked chains, formed by the covalent linkage of two active PDMS chains, shifts the self-assembly from polymersomes towards PDMS precipitates. Here, we report the development and demonstrate the robustness of a novel cosolvent extraction method. To the best of our knowledge the concept of using two organic solvents to fraction an amphiphilic BCP is not yet reported in literature and represents a powerful new technique for BCP work-up. In a series of cosolvent extractions, where in each iteration an extraction solvent with higher polarity is used, it was possible to fraction commercial PDMS to yield narrowly dispersed macroinitiators. The cosolvent fractionation of crude PMOXA-*b*-PDMS-*b*-PMOXA enables the separation of multiblock copolymers, formed by chain-transfer reactions, from triblock copolymers and is essential to yield BCPs for the self-assembly of polymersomes.

2. Experimental procedures & Materials

2.1. Materials

Chemicals. Bifunctional, 2-propoxyethyl-1-ol terminated polydimethylsiloxane (PDMS) macroinitiators were purchased from ABCR (AB116675), Shinetsu (6003 and 6002 carbinol fluids) and Dow-Corning (Corbinol Fluid 5562), further referred to as MI0, MI1, MI2 and MI3, respectively. Solvents for the fractionation and dialysis were distilled from technical-grade supplies and anhydrous solvents for the polymer synthesis were obtained from Sigma-Aldrich. Formvar-coated copper grids for transmission electron microscopy (TEM) were received from the Centre for Microscopy at the University of Basel (ZMB). GPC eluents were obtained from Scharlau in HPLC-grade quality. All other chemicals and standards were obtained from Sigma-Aldrich in the highest available purity and used as received if not stated otherwise. Argon was dried by flowing it through phosphorous pentoxide prior to use for either polymer synthesis or storage.

76 *Glassware.* Glassware for polymerisation, fractionation or film formation was cleaned over night in
77 KOH saturated 2-propanol solution followed by at least 4 hours of glass surface regeneration in
78 aqueous HCl (ca. 5 %). All glassware used for polymerisation and distillation was kept at 120 °C for
79 at least 48 hours prior to use. For all polymerisations, one-neck round-bottom flasks with a Young
80 valve were used. During PDMS-bi-triflate filtration a custom made one-neck round-bottom flask
81 with two Young valves was used for collection of the activated PDMS to allow for convenient and
82 simultaneous argon and vacuum connection. All glassware was assembled directly out of the oven,
83 connected to the vacuum line and evacuated (10^{-2} mbar) while being heated with a heat gun set to
84 600 °C, flushed with dry argon and reevacuated while cooling to room temperature. This procedure
85 was repeated at least three times.

86 2.2. Synthesis

87 *Distillation.* Triethylamine (TEA) and 2-methyl-2-oxazoline (MOXA) were stirred over CaH_2 under
88 argon in a two-neck round-bottom flask for 12 hours prior to distillation. TEA was heated in an
89 oil bath set to 121 °C. The fraction condensing at 87-89 °C was collected and used for not more
90 than two weeks after distillation (storage of TEA over molecular sieves caused the solution to turn
91 yellow over night and was avoided). MOXA was heated in an oil bath set to 133 °C. The fraction
92 condensing at 108-110 °C was collected and stored over dried 4 Å molecular sieves in a glove box ($<$
93 0.5 ppm O_2 , $<$ 0.5 ppm H_2O) and not used for more than one year after distillation. No difference
94 between freshly distilled and stored monomer was observed.

95 *PDMS activation.* The desired amount of bi-functional PDMS, typically 16 g, was weighed under
96 a normal atmosphere into a 250 mL argon flushed, dry round-bottom flask, containing a large oval
97 magnetic stirrer, closed with a septum and stirred at 630 rpm, 91 °C under reduced pressure (10^{-2}
98 mbar). After 12 hours, the PDMS was degassed by three cycles of argon/vacuum and left to cool at
99 room temperature in an argon atmosphere. Approximately 125 mL of dry hexane was added under
100 argon stream. The septum was fitted with an argon balloon reassuring an oxygen and water free
101 atmosphere while cooling the mixture to ca. -18 °C (measured bath temperature) in a stirred and
102 cold-finger cooled 2-propanol bath (cooling aggregate set at -20 °C, Julabo FT901). After ca. 1 hour
103 of cooling, 2.075 molar equivalents of dry TEA were added in one shot and the reaction mixture was
104 left stirring to distribute the TEA homogeneously. After ca. 15 minutes 2.05 molar equivalents of
105 trifluoromethanesulfonic anhydride in dry hexane were added drop-wise over ca. 2 minutes to avoid
106 high local concentration effects. The reaction was left stirring 3 to 4 hours to form PDMS-bi-triflate.
107 After activation, the mixture becomes cloudy-white due the formation of TEA-triflate salts which are
108 only white and solid at temperatures below -5 °C. The solids were filtered off under argon pressure
109 through a 2-propanol-dry-ice mixture cooled G4-frit into a two Young valve round-bottom flask. After

110 filtration, the final PDMS-bi-triflate macroinitiator was obtained through evaporation of hexane and
111 residual TEA at room temperature until a pressure less than $5 \cdot 10^{-2}$ mbar was reached. Depending
112 on the type of reaction to be performed, the PDMS-bi-triflate macroinitiator was transferred into a
113 glove box for splitting.

114 *2-Methyl-2-oxazoline polymerisation.* The 1st generation polymers were polymerised solely in ethyl-
115 acetate (EtOAc) at random monomer concentrations because EtOAc was added via stainless steel
116 needle under an argon stream directly into the septum-sealed round-bottom flask used to collect
117 the filtered PDMS-bi-triflate, rendering any reasonable volume estimations impossible. The amount
118 of monomer was calculated to be two times the desired repeating units per side plus 20 %. As an
119 example, the targeted number of repeating units for BCP00 was 11 on both sides and consequently
120 26.4 molar equivalents of monomer were added. The mixture was then left polymerising at 42 °C,
121 630 rpm for 66 hours and quenched as described below.

122 The 2nd generation polymers were polymerised in EtOAc, chloroform:acetonitrile (7:3 v/v) mix-
123 ture (TCM:ACN) and in N,N-dimethylacetamide (DMAC). The activated macroinitiator was trans-
124 ferred into a glove box for safe handling since a reliable comparison of the reaction kinetic in different
125 solvents requires the same degree of activation and molecular composition of the macroinitiator.
126 The polymerisation was carried out in a 250 mL round-bottom flask equipped with a Young valve.
127 0.4 mmol of the respective PDMS-bi-triflate was dissolved in 80 mL of the respective solvent. The
128 final monomer concentration was 0.5 M. After the addition of the monomers, the round-bottom flask
129 was sealed with a septum, discharged from the glove box, left polymerising at 42 °C, 630 rpm and
130 sampled regularly for analysis. All reactions were heated conventionally in a stirred oil bath on a
131 IKA RTC basic heating plate equipped with an external temperature sensor.

132 *Kinetic analysis.* At each time point 100 µL of reaction mixture was aspirated, transferred into an
133 NMR tube and diluted with K₂CO₃ saturated deuterated chloroform without tetramethylsilane. The
134 multiplet at 0.36-0.56 ppm served as reference signal (normalized to 4.0 protons) and the integral of
135 the multiplet at 3.27-3.60 ppm was plotted against the polymerisation time.

136 *Quenching.* If not stated otherwise, polymerisation reactions were quenched by adding a mixture
137 of H₂O:TEA (4:1 v/v) in one shot, where the amount of TEA was 2.075 molar equivalents of the
138 macroinitiator. Alternatively, a methanolic KOH solution could be used for quenching [19], but
139 was here avoided due to possible alkaline degradation of the PDMS block. After the desired MOXA
140 conversion was observed in ¹H-NMR spectra, the reaction mixture was either quenched or transferred
141 into a glove box and split into different dry round-bottom flasks for individual quenching. To yield
142 NH₂ functionality, a 50 molar excess of ethylenediamine was added in one shot, for N₃ functionality, a
143 four-fold molar excess of sodium azide was dissolved in dry DMF or DMAC and added to the reaction

144 mixture in one shot. Thiol functionality was achieved in a two step reaction, where first a four-fold
145 molar excess of potassium thioacetate in dry DMAC was added to the reaction to yield thioacetate
146 terminated polymer chains which were then deprotected on demand after purification/fractionation
147 by a molar excess of tris(2-carboxyethyl)phosphine in methanol at room temperature over night. After
148 addition of respective quenching agents the mixtures were stirred over night at room temperature.
149 The mixtures were transferred into one-neck round-bottom flasks and the solvent was evaporated to
150 yield $-OH$ or $-NH_2$ terminated crude block copolymers (BCP). The reactions quenched with either
151 NaN_3 or thioacetate were re-suspended in pure TCM, filtered through ca. 4 cm of Celite S, supported
152 by a G4-glass frit, to remove the excess of quenching agents, followed by evaporation of TCM to yield
153 the respective crude BCPs as white or slightly yellow crystalline crude products.

154 2.3. Purification and fractionation

155 *PDMS fractionation.* To yield narrowly dispersed PDMS macroinitiators, commercial PDMS prod-
156 ucts with a broad molar mass dispersion were fractionated in a series of cosolvent extractions. Typically
157 100 g of PDMS was dissolved in a one litre round-bottom flask in 200 mL hexane. 600 mL of EtOH
158 was added and the mixture was stirred over night at room temperature at 430 rpm. The milky so-
159 lution was poured into a freshly cleaned separation funnel until two clear phases were formed, which
160 required up to two days. The top fraction contained mostly hexane and higher molecular weight
161 PDMS species, whereas the bottom fraction contained the lower molecular weight PDMS chains. In
162 a next step, the PDMS from the bottom fraction was redissolved in hexane and extracted with MeOH
163 in the same way. The procedure was repeated with ACN and four distinct fractions of PDMS were
164 obtained.

165 *Purification.* The crude BCP was purified from unreacted monomers or small molecular weight
166 species by ultrafiltration or dialysis. Ultrafiltration was carried out in an EtOH:H₂O (1:1 v/v) mix-
167 ture. The molecular weight cut-off for ultrafiltration or dialysis was chosen according to the target
168 molecular weight between 1000 and 3000 Da (RC dry dialysis tubs, Spectrapor and RC ultrafiltration
169 membranes, Millipore). For dialysis, the dry BCPs were dissolved in 30 mL of pure EtOH, filled into
170 the dialysis tube and dialysed against TCM for 2-4 rounds. Then a TCM:EtOH mixture (1:1 v/v)
171 was used for 2 rounds before using pure EtOH. The dialysis was carried out at room temperature for
172 ca. a week and 6 to 8 cycles before the purified polymer was recovered as a white, slightly transparent
173 solid.

174 *Block copolymer fractionation.* 5 to 10 g of dialysed or ultrafiltrated BCP was dissolved in a one litre
175 round-bottom flask in 400 mL MeOH. The solution was stirred and 400 mL of hexane was added. The
176 mixture was stirred for at least one hour at room temperature before cooling to $-60\text{ }^{\circ}\text{C}$. It was further
177 stirred at low temperature for at least another hour before pouring it directly into a separation funnel

where it was left separating until two clear phases were formed. The higher the molar mass dispersity of the BCP, the more time the phase separation required. Narrowly dispersed BCP fractions would separate within a few hours, whereas their broadly dispersed analogues would require up to three days to separate. The bottom fraction was evaporated at 40 °C until no further solvent evaporated at a pressure of 300 mbar. Then 30 mL of H₂O were added to the mixture and the evaporation continued to a pressure of 91 mbar. The solution was then filtered through a 0.45 µm syringe filter, portioned into 20 mL scintillation vials, frozen in liquid nitrogen, freeze-dried, flushed with argon and stored at -20 °C until further use. This procedure was not suitable for the top fractions, and consequently they were concentrated to a small volume, transferred into scintillation vials and left evaporating at room temperature in the fume-hood for several days.

2.4. Self-assembly of triblock copolymers

The self-assembly of the synthesised triblock copolymers into polymersomes was performed in aqueous buffered saline (PBS, pH 7.4). Typically, 100 µL of a triblock copolymer stock solution (100 mg mL⁻¹) was diluted in a 10 mL round bottom flask to 400 µL with absolute EtOH. The solution was evaporated slowly at about 50 rpm, 42 °C and 165 mbar for 1 hour on a rotary evaporator to form a polymer film on the glass wall of the round-bottom flask. The film was further dried for 15 minutes at 4 mbar on the rotary evaporator. The film was rehydrated by adding an oval 7.5 mm magnetic stirrer, 2 mL of PBS, followed by stirring at 630 rpm at room temperature for at least 12 hours.

2.5. Analytical methods

Gel permeation chromatography (GPC). GPC traces were recorded and analysed in WinGPC UniCrom (V 8.20 - build 4815, Polymer Standard Service (PSS), Germany). If not stated otherwise 100 µL sample of was injected. TCM and THF systems were calibrated against narrowly dispersed polystyrene standards and the DMF system was calibrated against poly(methyl-metacrylate) standards. Traces of pure PDMS were recorded using an Agilent-based system composed of a 1200 series pump and autosampler followed by a 1100 series RI detector, equipped with a series of PLgel mixed-C columns (pre-column 3 µm particles (5 cm), three 5 µm particles (30 cm), all 0.75 cm diameter, Agilent). Detector and columns were kept at 40 °C, using 250 ppm 2,6-di-tert-butyl-4-methylphenol (BHT) stabilized THF as the eluent at a flow rate of 1 mL min⁻¹. Traces of the crude triblock copolymers or of the reaction mixtures were recorded using an Agilent-based system composed of a 1200 series pump and autosampler followed by a VWD (1100 series), RI (1100 series) and a viscometer (PSS DVD1260), equipped with a series of linear-S SDV columns (pre-column (5 cm), three analytical columns (30 cm) all 5 µm particles and 0.8 cm in diameter, PSS, Germany) or a pair of SDV columns (pre-column (5 cm), a 10³ Å and a 10⁵ Å both 30 cm, all 5 µm particles and 0.8 cm

in diameter, PSS, Germany). The column-set used is indicated by the abbreviation SDV-lin and SDV respectively. Detector and columns were kept at 35 °C using TCM, stabilized with EtOH, as the eluent at a flow rate of 1 mL min⁻¹. Traces of the final and fractionated triblock were recorded from an Agilent Viscotek/Malvern mixed system, composed of a 1100 series pump and autosampler followed by a TDA 305 (RI, VWD, viscometer and 90° light scatterer, Viscotek/Malvern) equipped with a series of gram columns (pre-column (5 cm), 30 Å (30 cm) and two 10³ Å (30 cm), all 10 µm particles and 0.8 cm in diameter, PSS, Germany). Detector and columns were kept at 60 °C using DMF with 20 mM LiBr as the eluent at 1 mL min⁻¹.

Nuclear magnetic resonance (NMR) spectroscopy. ¹H-NMR spectra were recorded at 128 scans on a Bruker DPX-400 spectrometer in CDCl₃ without tetramethylsilane, saturated with K₂CO₃ to buffer any possible acidity of TCM to achieve best possible resolution. The analysis, phase correction along f1 and Whittaker Smoother to adjust the baseline prior to integration was performed using MestReNova 10.0.1-14719 (Mestrelab Research S.L., Spain). All spectra were aligned to the chemical shift of CDCl₃ at 7.26 ppm.

Transmission electron microscopy (TEM). 5 µL of polymersome solution was left adsorbing on a formvar-coated and glow discharged 200 mesh copper grid and blotted off after 1 minute. Twice a drop of 5 µL of water was placed on the grid and blotted off immediately. This procedure was repeated with 5 µL of 2 % aqueous uranyl acetate, where the sample was stained for 10 seconds in the second step. Prepared grids were left drying in air for at least 10 minutes before imaging them at an acceleration voltage of 80 kV on a Philips CM100 transmission electron microscope.

Light scattering. Static or dynamic light scattering was performed on a setup from LS Instruments, equipped with a 30 mW HeNe laser. Polymers were dissolved in the respective solvent and filtered through a 0.2 µm PTFE syringe filter, evaporated and redissolved to yield an exact concentration of the solution. A series of concentrations were measured by diluting the stock solution after the first measurement and then refiltering it to yield the next lower concentration. All measurements were performed at 100 % laser intensity.

Determination of dn/dc. Light scattering experiments require the dn/dc value of the analyte in the respective solvent to determine the molecular weight. A way to determine the dn/dc value is to inject a series of different amounts from the substance of interest on the GPC, and plot the injected mass against the total area of the RI signal (negative and positive part of the peak) [20]. With this procedure dn/dc · K_D is found, where K_D is the detector constant. From a substance like polystyrene with known dn/dc value, K_D is obtained to calibrate the detector and enable the determination of dn/dc for the respective PMOXA-*b*-PDMS-*b*-PMOXA fractions.

Figure 1: GPC traces of two commercially available PDMS products, fractionated by cosolvent extraction (black: original, blue: top fraction, orange: EtOH fraction, violet: bottom fraction and green: ACN fraction. 100 g of the Shinetsu 6003 carbinol fluid ($\bar{M}_n$: 1.97) were fractionated into the hexane fraction (64.0 g, $M_{n,NMR}$: 7636 g mol⁻¹, $\bar{M}_w$: 1.23, 6003H), an EtOH fraction (26.5 g, $M_{n,NMR}$: 4551 g mol⁻¹, $\bar{M}_w$: 1.18) and a MeOH fraction (4.4 g, $M_{n,NMR}$: 2590 g mol⁻¹, $\bar{M}_w$: 1.31). 100 g of the Shinetsu 6002 carbinol fluid yielded a hexane fraction (40.7 g, $M_{n,NMR}$: 6306 g mol⁻¹, $\bar{M}_w$: 1.20, 6002H), an EtOH fraction (43.4 g, $M_{n,NMR}$: 4280 g mol⁻¹, $\bar{M}_w$: 1.21) and a MeOH fraction (5.9 g, $M_{n,NMR}$: 2134 g mol⁻¹, $\bar{M}_w$: 1.34). ACN fractions were not of interest due to their low molecular weights and small amounts. The low molecular weight shoulders (below 1000 g mol⁻¹) are artefacts from the stabilizers contained in THF used as eluent (see Fig. S2 for illustration)

3. Results & Discussion

A small library (Table S1) of poly(2-methyl-2-oxazoline)-*block*-polydimethylsiloxane-*block*-poly(2-methyl-2-oxazoline) (PMOXA-*b*-PDMS-*b*-PMOXA) block copolymers (BCP), was synthesised via cationic ring opening polymerisation of 2-methyl-2-oxazoline (MOXA) on commercial PDMS macroinitiators (MI) yielding broadly dispersed 1st generation BCPs and on fractioned PDMS MIs (6003H and 6002H) yielding narrowly dispersed 2nd generation BCPs. To assess the optimal solvent for the polymerisation and to record reaction kinetics, MOXA was polymerised on narrowly distributed MIs.

3.1. PDMS macroinitiator fractionation

The received PDMS products were characterized by GPC and ¹H-NMR and found to be pure, but quite disperse (Fig. S1). The solubility of PDMS in polar solvents depends on the molecular mass of the respective polymer chains which allowed the cosolvent fractionation of PDMS homopolymers. In a series of extractions against hexane, EtOH, MeOH and ACN were used one after another as described in the experimental section. The series of cosolvent extractions enabled the fractionation of the carbinol fluids 6003 and 6002, each into three distinct, relatively low dispersed PDMS fractions (Fig. 1). The chromatograms depicted in Fig. 1 exhibit peaks below 1000 g mol⁻¹ for all fractions analysed, which are attributed to interactions with BHT, the stabilizer present in the eluent. This is supported by the absence of these peaks when using TCM as an eluent which is stabilized with EtOH (Fig. S2).

Figure 2: Kinetic analysis of 2-methyl-2-oxazoline polymerisation on different molecular weight PDMS macroinitiators in EtOAc (red squares) and in TCM:ACN (7:3 v/v, green diamonds). Blue and black lines represent linear fits of the ^1H -NMR data (details in Fig. S3): **(A)** Shinetsu 6003 hexane fraction with $M_{\text{NMR}}: 7636 \text{ g mol}^{-1}$ in EtOAc (slope: 2.02, $R^2: 0.952$, blue) and in TCM:ACN (slope: 1.48, $R^2: 0.997$, black) and **(B)** Shinetsu 6002 hexane fraction with $M_{\text{NMR}}: 6306 \text{ g mol}^{-1}$ in EtOAc (slope: 0.88, $R^2: 0.918$, blue) and in TCM:ACN (slope: 1.21, $R^2: 0.996$, black).

3.2. Solvent selection & reaction kinetics of 2-methyl-2-oxazoline polymerisation

The choice of solvent for the synthesis of BCPs is not trivial, since the individual blocks are not necessarily soluble in the same solvent or the common solubilising agent is not optimal for the polymerisation. Historically, PMOXA-*b*-PDMS-*b*-PMOXA was synthesised in either ethylacetate (EtOAc) [19] or in chloroform:acetonitrile (7:3 v/v, TCM:ACN) [21]. Alternatively, *N,N*-dimethylacetamide (DMAC) was successfully used for the polymerisation of 2-ethyl-2-oxazoline [22]. It solubilises the PDMS-bi-triflate macroinitiators, but it was found to cause brownish coloured PMOXA-*b*-PDMS-*b*-PMOXA BCPs and was difficult to remove on the rotary evaporator. Therefore, the reaction kinetics in EtOAc and TCM:ACN were compared and DMAC was not further used for the polymerisation of MOXA on PDMS MIs.

MOXA polymerisation kinetics were assessed on narrowly dispersed MIs to ensure the most homogeneous MI composition. For this, samples of 6002H and 6003H (Fig. 1) were activated with trifluoromethanesulfonic anhydride as described in the experimental section. The kinetic data (Fig. 2, Fig. S4) represents two activated batches and four individual polymerisations (BCP10, BCP11 and BCP09, BCP08 respectively) of which two (BCP08, BCP09) were split before quenching to yield -hydroxyl, -azide, -thioacetate and -amine end-groups (BCP0X A, B, C and D Table S1). The separate quenching has the benefit to yield functionalised BCPs for further modifications and enables individual cosolvent fractionations for the analysis of the technique.

Kinetic data recorded from the polymerisation in EtOAc and TCM:ACN revealed a more linear character for TCM:ACN than for EtOAc, reasoned by the respective R^2 values of the linear regression (Fig. 2). The slope of the linear regression (Fig. 2) suggests that the reaction kinetics of the polymerisation does not depend on the molecular mass of the macroinitiator, but on other factors.

285 This is expected and further supported by the lower molecular mass MI 6002H exhibiting for both
286 solvents slower kinetics than the higher molecular mass MI 6003H. However, the more linear reaction
287 kinetic in TCM:ACN together with the low solubility of PMOXA in EtOAc renders TCM:ACN the
288 preferred solvent mixture for the polymerisation of MOXA on PDMS MIs.

289 To test for a living character of the 2-oxazoline polymerisation, demonstrated by Hoogenboom
290 *et. al* [23], of MOXA on PDMS MIs, the polymerisation was accelerated by the addition of 10 mL
291 monomer after 95.5 (Fig. S4A) and 47.5 (Fig. S4B) hours. It was found that the polymerisation is
292 living, despite the use of highly reactive triflate catalysts, long polymerisation times (47.5 and 95.5
293 hours) and high molecular weight PDMS MIs. This suggests that it might be possible to sequentially
294 synthesise symmetric multiblock copolymers with different poly(2-oxazoline) blocks on PDMS-bi-
295 triflate.

296 3.3. Cosolvent fractionation

297 After synthesis the crude BCP is soluble in MeOH, and a turbid solution indicates the presence
298 of unreacted PDMS chains. If observed, unreacted chains were removed by centrifugation and the
299 polymer was further purified from any small molecular mass species by dialysis or ultrafiltration to
300 yield a chemically pure BCP. Ultrafiltration was found to require more time (up to two weeks) and
301 the choice of solvents is limited due to pressure and solvent-membrane compatibility. Due to the
302 amphiphilic character of the BCP, the choice of solvent for an effective dialysis or ultrafiltration is
303 limited. It is required to solubilise both blocks (PDMS and PMOXA), unreacted monomers and
304 small molecular weight impurities best possible. The time benefit rendered dialysis the method of
305 choice and TCM, a good solvent for PDMS and PMOXA, the best performing solvent for dialysis.
306 However, after removal of unreacted monomers and small molecular weight species, the obtained
307 BCPs were not self-assembling into polymersomes upon film rehydration in an aqueous environment
308 even though the hydrophilic weight fraction f_{OX} was in the range of 0.20 to 0.35, required for vesicular
309 self-assembly [24].

310 The self-assembly of amphiphilic macromolecules is a fragile process and is easily affected by a
311 tiny amount of hydrophobic species, whereas hydrophilic species are tolerated in a larger amount due
312 their solubility. Mixtures of small organic molecules can be separated by extraction in immiscible
313 solvents due the molecules preference for one or the other solvent. Herein it is reported, that a similar
314 concept is applicable to amphiphilic BCPs. MeOH and hexane are immiscible, PMOXA is soluble in
315 MeOH and PDMS is soluble in hexane. The preference for one or the other phase is determined by
316 BCP chain's f_{OX} . A stirred, methanolic solution of the pure BCP is clear and becomes slightly turbid
317 only when hexane is added. At around 50 % (v/v), the solution turns white, like fresh snow, and the
318 solvents are emulsified by the amphiphilic macromolecules. Decreasing the temperature of such an
319 emulsion results in decreased solubilities of the individual blocks and forces the macromolecules into

Figure 3: Transmission electron micrographs of micelles (A) assembled from a block copolymer with a block ratio of 18:49:18 and $f_{OX}=0.44$ compared to polymersomes assembled from BCP07-bottom (B) and assembled worm-like structures and a few polymersomes from BCP08A-bottom (C). All samples were prepared by film rehydration in 100 mM phosphate buffer pH 7.4 and imaged directly after 24 hours of stirring at room temperature and 630 rpm. All scale bars are 200 nm.

hexane or MeOH. In the separation funnel, the mixture reaches room temperature while the solvents start separating to yield two fractions (Fig. S5). Sometimes a third fraction (inset Fig. S5) of less than 150 mg was observed in a typical separation of 5.0 to 10 g crude BCP. $^1\text{H-NMR}$ analysis of this third fraction revealed a high amount of both, PMOXA and PDMS (23:104:23 as an example). This fraction was discarded if observed and not further analysed since it occurred rarely for 1st generation BCPs and its amount was negligible. The comparison between the separation of room temperature mixtures and mixtures cooled to -60 °C was assessed by GPC and demonstrated the benefit from cooling (Fig. S6) for the fractionation efficiency. The two main fractions are further referred to as 'bottom' and 'top' fraction because both of them contain hexane and MeOH, implied by the volumes of the respective fractions (see Fig S5) and reasoned by solvent molecules being solubilised within the other fraction by the amphiphilic polymer chains. An example for the evolution of the molecular weight distributions upon cosolvent fractionation is illustrated for BCP08 in Fig. S7.

Separated polymer fractions exhibit distinct properties; polymers from the top fractions are not soluble in MeOH, DMF or DMSO as examples of polar solvents and additionally do not self-assemble into polymersomes. BCP08A-top is not soluble in pure hexane, whereas BCP00-top still is, illustrating the effect of the block length on the solubility profile of the BCPs. The polymers from the bottom fractions are soluble in polar solvents such as MeOH, EtOH or DMF and self-assemble in aqueous conditions into micelles (Fig. 3A), polymersomes (Fig. 3B) or worm-like structures (Fig. 3C) depending on the respective f_{OX} . The deflated structures observed in transmission electron microscopy (TEM) images (Fig 3B) are characteristic for PMOXA-*b*-PDMS-*b*-PMOXA polymersomes and their vesicular character was confirmed by cryo-TEM analysis earlier [16].

Another interesting observation was made when the polymers from the respective fractions were dissolved in TCM and evaporated steadily at 42 °C, 600 mbar. The polymers from the bottom fractions form an optically opaque, crystalline, non-uniform coverage on the bottom of a glass vial,

Figure 4: Structure (A) of the block copolymer and representative ¹H-NMR spectra of the bottom fraction (from BCP07), recorded in K₂CO₃ saturated CDCl₃ without TMS (B): 400 MHz, ppm: -0.50-0.35 (m, 237.5 H, -O-Si((CH₃)₂)-), **0.38-0.49 (m, 4.00 H (ref), -Si-CH₂-)**, 1.95-2.19 (m, 38.0 H, -N(C(O)-CH₃)-), 3.18-3.62 (m, 55.6 H, -Si-CH₂-CH₂-CH₂-O-CH₂-CH₂-(N-CH₂-CH₂)_{m-1}-N-CH₂-CH₂-), 3.61-3.88 (m, 4.0 H, -CH₂-OH) and top fraction (C): 400 MHz, ppm: -0.50-0.35 (m, 483.8 H, -O-Si((CH₃)₂)-), **0.38-0.49 (m, 4.00 H (ref), -Si-CH₂-)**, 1.95-2.19 (m, 26.5 H, -N(C(O)-CH₃)-), 3.18-3.62 (m, 42.8 H, -Si-CH₂-CH₂-CH₂-O-CH₂-CH₂-(N-CH₂-CH₂)_{m-1}-N-CH₂-CH₂-), 3.61-3.88 (m, 3.5 H, -CH₂-OH). No significant difference is observed between spectra recorded from the bottom (B) and top (C) fractions, confirming identical chemical compositions and suggesting that the respective polymers only vary in their block ratios or block compositions.

whereas the polymers from the top fractions form a homogeneous, non-crystalline, optically transparent and solid layer as depicted in Fig. S8. This was observed for both generations of BCPs and is independent of their dispersity.

The overall yield of the synthesis is not a concern of the present work, whereas the yield of the cosolvent fractionation was always quantitative. The mass ratio between the polymers contained in the top and bottom fractions was quite constant and determined to be $59 \pm 11 \%$ ($n = 19$) of the initial BCP mass in the bottom fraction.

3.4. Composition of Top & bottom fraction

The BCP structure and representative ¹H-NMR spectra from the top and bottom of the BCP07 are presented in Fig. 4, indicating identical chemical compositions of the respective BCPs (Fig. 4B, C). Due to the dispersity of the BCPs, the only reasonable reference peak to determine the block ratio and Mn is the one representing the -CH₂- group at 0.38-0.49 ppm (b in Fig. 4). The number of dimethylsiloxane repeating units is calculated from a, whereas the number of 2-methyl-2-oxazoline (MOXA) repeating units can be calculated ambiguously from the signal of d and e. Therefore, the number of MOXA units obtained from either of these signal must be the same and the most accurate way of determining the PMOXA block length is consequently according to the following equation: $m = (x/6.0 + (y - 12)/8.0)/2.0$ where m is the number of PMOXA repeating units per side, x is

the integral of the signal from **d** and y the integral of the signal from **e**. The correction of y by subtracting 12 is necessary due to the signal overlap (**e**) of the $-\text{CH}_2-$ groups in the spacer, linking the PDMS block to the PMOXA blocks (three $-\text{CH}_2-$ groups on both sides), with the signal from the protons in the PMOXA backbone. Respective f_{OX} are calculated by dividing the total molecular weight by the molecular weight of the PMOXA blocks and are summarized together with the block ratios of the respective bottom and top fractions in Table S1.

The molecular mass distribution of the fractionated BCPs was assessed by GPC. In an ideal system the analyte would not interact with the column material, hence the separation would solely be based on the molecular weight of the analyte. It was reported elsewhere that PMOXA interacts with cross-linked polystyrene, the filling material used in SDV columns and it has been demonstrated that the use of a TCM:TEA:2-propanol 94:4:2 v/v (TCM:TEA:IPA) mixture as an eluent reduces such interactions [18]. For our small molecular weight polymers it was found that the use of TCM:TEA:IPA as an eluent leads to an apparent high molecular weight shoulder in the UV trace (Fig. S9, green trace), which is not observed in the respective delta pressure signal recorded from the viscometer (Fig. S9, red trace). The delta pressure is not affected because the reference and sample channel contain the same amount of TEA and IPA, assuring that the observed signal originates from the analyte alone. Furthermore, the overlaid delta pressure traces (Fig. S9, blue and red trace) indicate no significant difference in the elution pattern between pure TCM and TCM:TEA:IPA as eluents. The UV traces however, (Fig. S9, green and magenta trace) recorded in pure TCM (254 nm) and in TCM:TEA:IPA (310 nm, necessary due to the eluent mixture) are significantly different. The better agreement between delta pressure and UV trace in pure TCM lead to the preference of pure TCM as the eluent over the elution mixture. The refractive index of PMOXA is greater than that of TCM and the one of PDMS lower, rendering the interpretation of the RI signal from the bottom fractions tricky as illustrated in the raw elugrams (Fig. S10C). The RI signal of the top fractions is found to be negative (Fig. S11C) for all analysed fractions, supported by a shift in the physical properties of the respective BCPs towards that of PDMS (compare f_{OX} of top and bottom fractions, Table S1). All M_n values are therefore calculated based on the UV traces recorded at 254 nm. The baseline for the UV traces of the bottom fractions were all set as illustrated in Fig. S10C. The obtained molecular mass distributions and the corresponding dispersities are summarized in Table S1, Fig. 5 and Fig. 6.

3.5. Chain interlinkage during synthesis

The narrowly dispersed MIs enabled the synthesis of equally narrowly dispersed BCPs as listed in Table S1. Quenching the reaction at the time when ^1H -NMR spectra indicate the desired conversion is not resulting in BCPs with the desired block ratios due to the changes of the block ratio upon fractionation. For example BCP08 and BCP09 were quenched when f_{OX} was 0.30 to yield polymer-some forming triblock copolymers. After the cosolvent fractionation, f_{OX} of the respective bottom

Figure 5: Graphical representation of M_n determined by GPC (yellow bars) and $^1\text{H-NMR}$ (brown bars) for the polymers isolated from the bottom (**left**) and the top (**right**) fractions. The left block (BCP00-07) represents the 1st generation, the right block (BCP08-11) the 2nd generation block copolymers. The discrepancy between M_n determined from GPC and $^1\text{H-NMR}$ is negligible for the bottom fractions and becomes more pronounced for the top fractions.

Figure 6: Graphical representation of the dispersities ($\bar{D}$, left axis, red bars) and the hydrophilic weight fractions (f_{OX} , right axis, blue bars) determined for the bottom (**left**) and top (**right**) fractions. The mean dispersity indicates a clear benefit for the bottom fraction when polymerised from low dispersed macroinitiators. The difference in f_{OX} between top and bottom fractions is similarly significant for 1st and 2nd generation polymers.

fractions was clearly above 0.30 (Fig. 6, Table S1). From the experience of all polymers synthesised and fractioned, the final f_{OX} can be estimated as approximately 0.05 times higher than determined at the time of quenching.

The M_n values obtained from GPC and $^1\text{H-NMR}$ are in good agreement with each other for most bottom fractions from 1st and 2nd generation triblock copolymers (Fig. 5 left, Table S1). In contrast, the values obtained for top fractions exhibit larger differences between the M_n values from GPC and $^1\text{H-NMR}$ (Fig. 5 right, Table S1). Alternatively, the molecular weight of the BCPs might be determined by light scattering, a method depending on (I) complete solubilisation of the macromolecules in the respective solvent and (II) a large enough dn/dc to yield enough scattering contrast. To facilitate light scattering measurements of the BCPs and thereby confirm the M_n from GPC or $^1\text{H-NMR}$, the refractive index increment of four polymers was determined as described in the experimental section and illustrated in Fig. S12. The respective increments in TCM, the only solvent found to solubilise both blocks, were found to be 0.0088, 0.0282, 0.0162 and 0.0154 for BCP01-bottom, BCP01-top, BCP09A-bottom and BCP09A-top, respectively. With such low dn/dc values, it was not possible to get reliable molecular weight from light scattering measurements to confirm the values obtained from either GPC or $^1\text{H-NMR}$.

For the 2nd generation of BCPs, the GPC traces of top and bottom fractions show clear molecular mass differences which can not be reasoned by artefacts. The multiplet at 0.38-0.49 ppm, used as a reference signal to determine the block ratios, originates from the -CH₂- group (Fig. 4A **a**) connected to the last dimethylsiloxane repeating unit and is relative to the amount of connected PDMS chains. Therefore, Mn values from ¹H-NMR can only match the those from GPC when a covalent interlinkage of two chains is assumed to yield pentablocks: PMOXA-*b*-PDMS-*b*-PMOXA-*b*-PDMS-*b*-PMOXA.

The amount of polymer in the respective fractions, averaged over the synthesised BCPs, revealed that 41 ± 11 % (*n* = 19) of the crude polymer is composed of these pentablocks. Furthermore, the differences in Mn values obtained from GPC and ¹H-NMR becomes negligible for polymers quenched with H₂O:TEA (BCP08A and BCP09A) or potassium thioacetate (BCP08C and BCP09C) and differs only slightly when quenching with sodium azide (BCP08B and BCP09B) when the reference signal in the respective ¹H-NMR is corrected to 8.0 protons (0.38-0.49 (m, 8.00 H (ref), -Si-CH₂-)). Quenching the polymerisation with ethylenediamine, a bi-functional molecule, intrinsically favouring chain interlinkage, due to its bi-functionality results in a significant difference between the Mn values (BCP08D and BCP09D). These findings confirm that the performed synthesis yields pentablocks via chain interlinkage together with triblocks, yielding a BCP mixture which is effectively separated by the developed cosolvent fractionation. The fractionation enables the essential isolation of PMOXA-*b*-PDMS-*b*-PMOXA triblock copolymers for polymersome self-assembly.

4. Conclusion & Outlook

The developed cosolvent fractionation method was successfully used to yield PMOXA-*b*-PDMS-*b*-PMOXA triblock copolymers for polymersome assembly. This novel method is unique because two unmixable organic solvents are used to fraction amphiphilic block copolymers (BCP) based on their hydrophilic weight fraction (*f*_{OX}). It was demonstrated that the temperature at which the emulsion is stirred prior to the separation has a significant effect on the efficiency of the cosolvent fractionation. The method was successfully extended to fraction commercial PMDS homopolymers to yield relatively narrowly dispersed fractions of macroinitiators. Based on these results, it is anticipated that the adaptation of the solvent combination would allow the fractionation of other amphiphilic BCPs where, as for PMOXA-*b*-PDMS-*b*-PMOXA, the combination of hexane and methanol was ideal to overcome the drawback of chain interlinkage during the cationic ring-opening polymerisation of MOXA on PDMS macroinitiators.

Polymerization on narrowly dispersed macroinitiators yield narrowly dispersed triblock copolymers which self-assemble into polymersomes upon film rehydration. Such polymersomes offer a membrane platform of more uniform character than the ones assembled from the highly dispersed triblock copolymers. In a theoretical work [25], an increase in perturbation energy upon protein

insertion into a bilayer is predicted. It is further stated that the range of perturbation increases with the polymeric chain length. The polymer model applied is constructed with one length of polymer chains, an equal length of hydrophilic and hydrophobic blocks and zero spontaneous curvature of the membrane. The dispersity of a real polymer and the lateral fluidity [16], originating from the unique properties of the hydrophobic PDMS block, are together responsible for the membrane perturbation energy of the assembled membrane. It is envisioned that the access to narrow dispersed PMOXA-*b*-PDMS-*b*-PMOXA triblock copolymers, enabled by the presented work, will provide experimental means to assess the manifold of properties of polymeric membrane assemblies and inspire more advanced theoretical studies. These triblock copolymers give rise to the possibility of creating blends of narrowly dispersed BCPs and thereby introduce a controlled and tunable dispersity. The self-assembly of such blends might answer important questions regarding membrane protein insertion and more profound understanding of the membrane assembly, curvature and composition with respect to the dispersity and the chain length of the amphiphilic BCP.

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