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A catalyst-free, temperature controlled gelation system for in-mold fabrication of microgels

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Supplementary Information

Content

1. Materials

Six armed, star-shaped poly(ethylene oxide-stat-propylene oxide) (sPEG) consisting of 80 % ethylene oxide and 20 % propylene oxide (3000 g/mol) is provided by CHT R. Beitlich GmbH. Tetrahydrofurane (anhydrous, ≥ 99.9 %), dichloromethane (anhydrous, ≥ 99.9 %), methanol (analytical grade), triethylamine (≥ 99.5 %), mesyl chloride (> 99 %), sodium hydride (dry, 99 %), sodium azide (≥ 99.5 %), Pd/C (5 wt%), polyvinylpyrrolidone (PVP) and rhodamine B (> 95 % dye) are purchased from Sigma Aldrich and used as received. Methacryloxyethyl thiocarbamoyl rhodamine B is purchased from ThermoFisher Scientific and used as received. Epichlorohydrin (≥ 99 %) is purchased from Sigma Aldrich and dried over calcium hydride. Polydimethylsiloxane (PDMS, Sylgard® 184) is purchased from Dow Corning. Polyethylene terephthalate (PET) sheets are purchased from Goodfellow GmbH.

Precipitations are performed in *n*-pentane and diethyl ether (technical grade). If not mentioned differently, all experiments are performed under nitrogen atmosphere with common Schlenk technique. Before every synthesis, the sPEGs are dried overnight at 75 °C in vacuum. Nitrogen gas is purchased from Linde and dried with a Drierite™ column purchased from Sigma Aldrich.

2. Instrumentation

^{1}H-NMR and ^{13}C-NMR spectra are recorded on a Bruker Ultrashield 400 FTNMR spectrometer at 400 MHz at RT. Deuterated chloroform ($CDCl_3$, Sigma Aldrich) is used as solvent.

Inductively coupled plasma mass spectroscopy (ICP-MS) is carried out with an Agilent 8800 ICP-MS Triple Quad (G3663A) device in a water matrix. Quantitative analysis is performed with a lower detection limit of <1 ppb with a QC1 reference standard.

Size exclusion chromatography (SEC) analysis is carried out with dimethylformamide (DMF) as eluent. Results are evaluated using the PSS WinGPC UniChrom software (Version 8.1.1). SEC is performed using an Agilent 1100 system, equipped with a dual RI-/Visco detector (ETA-2020, WGE). The eluent contains 1 g/L lithium bromide (≥ 99 %, Sigma-Aldrich). The sample solvent contains traces of distilled water as internal standard. One pre-column (8x50 mm) and four GRAM gel columns (8x300 mm, Polymer Standards Service) are applied at a flow rate of 1.0 mL/min at 40 °C. The diameter of the gel particles measure 10 µm, the nominal pore widths are 30, 100, 1000 and 3000 Å. Calibration is achieved using narrowly distributed PEG standards (Polymer Standards Service).

Rheological characterization is performed with a DHR 3 Rheometer with a 20 mm conical geometry from TA Instruments. All measurements are performed at the truncation gap of 51 µM, a strain of 0.5 %, and a frequency of 1 Hz. For time sweep or temperature ramp measurements, 74 µL sample solution is applied between the geometry and the bottom heating plate. After lowering the geometry to the geometry gap (51 µm), the humidity purge is filled with water and the geometry covered with a solvent trap to minimize water evaporation.

Confocal laser scanning microscopy is performed with a Leica SP8 Tandem Confocal system, equipped with a white light laser (WLL). Samples are excited with the dye specific wavelength of 560 nm using the WLL and emission is detected with a HyD detector. Super-resolution microscopy images are acquired with stimulated emission depletion (STED) by exciting the samples as described before and depleting through a pulsed laser with a wavelength of 775 nm. Apart from the planar depletion, a z-depletion of 15 – 25 % is performed. Images are sequentially deconvolved with Huygens Professional (Scientific Volume Imaging B.V).

Atomic force microscopy (AFM) (Bruker, Dimension Icon FastScan System) with nano-indentation is used. An s-Qube® colloidal probe (Nano and more, CP-PNPS-SiO-A-5) with a 2 µm (+/- 5%) diameter silicon dioxide sphere on a triangular gold coated cantilever is calibrated in air using the thermal resonance method built into the AFM software (spring k = 0.1969 N/m). An indentation is applied at 1 µm/s with a maximum force of 5 nN over an area of 100 x 100 nm with four measuring pints. The E moduli are measured by fitting the Hertz model with a non-linear fit.

3. sPEG functionalization

Synthesis of sPEG-epoxy

The procedure is adapted from Laine *et. al*.[1] and optimized for the liquid sPEG system.

5.8 g sPEG (12 mmol OH-groups, 1 eq) is dissolved in THF (50 mL) and added to a stirred dispersion of 0.58 g NaH (24 mmol, 2 eq) in THF (8 mL). After stirring at ambient temperature for 1.5 h, 11 g epichlorohydrin (0.12 mol, 10 eq) is slowly added to the reaction mixture. The solution is then further stirred for 24 h, after which the brown mixture is concentrated to 40 mL under reduced pressure and centrifuged for 10 min at 6000 rpm. The solution is decanted, filtered, and dried in vacuum. The product is purified by precipitation in pentane (500 mL) at –80°C. The sPEG-epoxy is recovered in a yield of 4.1 g (71 %). An endgroup functionalization of 98 % is achieved (according to ^{1}H-NMR spectroscopy).

NMR analysis (400 MHz, $CDCl_3$):

^{1}H-NMR: δ = 3.90-3.30 (m, polymer backbone), 3.07 (1H, m, C*H*-O), 2.70 (1H, m, CH_2-O), 2.53 (1H, m, CH_2-O), 1.05 (3H, d, CH_3-CH-O) ppm.

^{13}C-NMR: δ = 75.14, 71.97, 70.55, 68.50 (polymer backbone), 50.81 (CH_2), 44.25 (CH_2), 17.24 (CH_3) ppm.

SEC analysis (DMF): M_n = 2700 g/mol, M_w = 3000 g/mol, $Đ$ = 1.14.

Synthesis of sPEG-mesylate

The procedure is adapted from Mahou *et. al*.[2] and optimized for the liquid sPEG system.

6.4 g sPEG (12.6 mmol OH-groups, 1 eq) is dissolved in THF (60 mL) and 2.4 g (24 mmol, 1.85 eq) of triethylamine is added to the stirred solution. The solution is cooled to 0 °C and 2.7 g mesyl chloride (24 mmol, 1.85 eq) is slowly added within one hour. The yellowish suspension is stirred for additional 48 h at ambient temperature. The suspension is cooled in an ice bath and filtered over a plug of silica to remove the hydrochloride salt. After drying in vacuum, the polymer is redissolved in DCM and purified by precipitation in pentane/diethyl ether (1:1, v/v) at 0 °C to be left at -80 °C overnight. The sPEG-mesylate is isolated by decantation of the solvent and recovered in a yield of 5.8 g (74 %). An endgroup functionalization of 98 % is achieved (according to ^{1}H-NMR spectroscopy).

NMR analysis (400 MHz, $CDCl_3$):

^{1}H-NMR: δ = 4.81 (1H, m, C*H*-mesylate), 4.33-4.28 (2H, m, CH_2-mesylate), 3.75-3.31 (m, polymer backbone), 3.09 (1H, s, O*H*), 3.02 (3H, s, CH_3-SO_2), 1.31 (3H, d, CH_3-CH-mesylate), 1.08 (3H, d, CH_3-CH-O) ppm.

^{13}C-NMR: δ = 78.82, 74.96, 73.08, 70.54, 69.32, 69.01, 68.49 (polymer backbone), 38.47 (CH_3), 37.74 (CH_3), 17.96 (CH_3), 17.23 (CH_3) ppm.

SEC analysis (DMF): M_n = 3900 g/mol, M_w = 4100 g/mol, $Đ$ = 1.05.

Synthesis of sPEG-azide

The procedure is adapted from Mahou *et. al*.[2] and optimized for the liquid sPEG system.

The reaction was performed under non-inert conditions.

6.2 g sPEG-mesylate (13 mmol functions, 1 eq) is dissolved in an ethanol-water mixture (95:5 %, v/v, 60 mL) and 1.2 g sodium azide (19 mmol, 1.5 eq) is added to the solution in one portion. The mixture is stirred at 100 °C for 48 h and the solvent is removed under reduced pressure. The polymer is redissolved in DCM and filtered over a plug of silica. After evaporation of the solvent, the sPEG-azide is obtained in a yield of 4.3 g (70 %). An endgroup functionalization of 98 % is achieved (according to ^{1}H-NMR spectroscopy).

NMR analysis (400 MHz, $CDCl_3$):

^{1}H-NMR: δ = 3.80-3.30 (m, polymer backbone), 1.17 (3H, d, C*H*$_3$-CH-mesylate), 1.12 (3H, d, C*H*$_3$-CH-O) ppm.

^{13}C-NMR: δ = 75.03, 70.53, 70.00, 68.48 (polymer backbone), 56.80 (CH), 50.63 (CH_2), 17.22 (CH_3), 16.14 (CH_3). ppm.

SEC analysis (DMF): M_n = 2300 g/mol, M_w = 2400 g/mol, *Đ* = 1.04.

Synthesis of sPEG-amine

The reaction is performed in a 50 mL stainless steel autoclave, equipped with a manometer and a high pressure valve. 0.58 g sPEG-azide (0.96 mmol functions, 1 eq) is dissolved in methanol (5 mL) and the setup is closed after adding 54 mg Pd/C (5 wt%, 0.020 eq Pd) as catalyst. The autoclave is flushed with hydrogen (85 bar) three times. The closed system is left stirring for 24 h at ambient temperature. The hydrogen is released and the catalyst filtered through a syringe filter (PTFE 45/25, 0.45 µm). After solvent evaporation under reduced pressure, the sPEG-amine is recovered in a yield of 0.37 g (64 %). An endgroup functionalization of 91 % is achieved (according to ^{1}H-NMR spectroscopy).

NMR analysis (400 MHz, $CDCl_3$):

^{1}H-NMR: δ = 3.80-3.30 (m, polymer backbone), 2.94-2.85 (2H, m, CH_2-C*H*$_2$-NH_2), 2.61 (1H, t, CH_3-C*H*-NH_2), 1.58 (2H, s, N*H*$_2$), 0.90 (3H, d, C*H*$_3$-CH-O), 0.79 (3H, d, C*H*$_3$-CH-NH_2) ppm.

^{13}C-NMR: δ = 74.89, 74.67, 73.01, 70.20, 68.28 (polymer backbone), 46.11 (CH), 41.51 (CH_2), 19.52 (CH_3), 17.05 (CH_3) ppm.

SEC analysis (DMF): M_n = 2600 g/mol, M_w = 2900 g/mol, *Đ* = 1.12.

ICP-MS analysis: leaching of 0.008 mol% Pd (0.8 µmol).

4. NMR spectra and determination of endgroup functionalization

^{1}H-NMR spectra are analyzed in reference to the PO protons. The non-functionalized sPEG is measured, the backbone signal (δ = 3.80-3.30 ppm) is set to a proton integral of 42H (theoretical amount of protons in a 3000 g/mol sPEG), resulting in a PO integral of 5.02H. All analyzed spectra are normalized to this PO signal. Functionalization degrees are calculated in reference to significant endgroup signals, changing for every compound (details are shown under the following spectra).

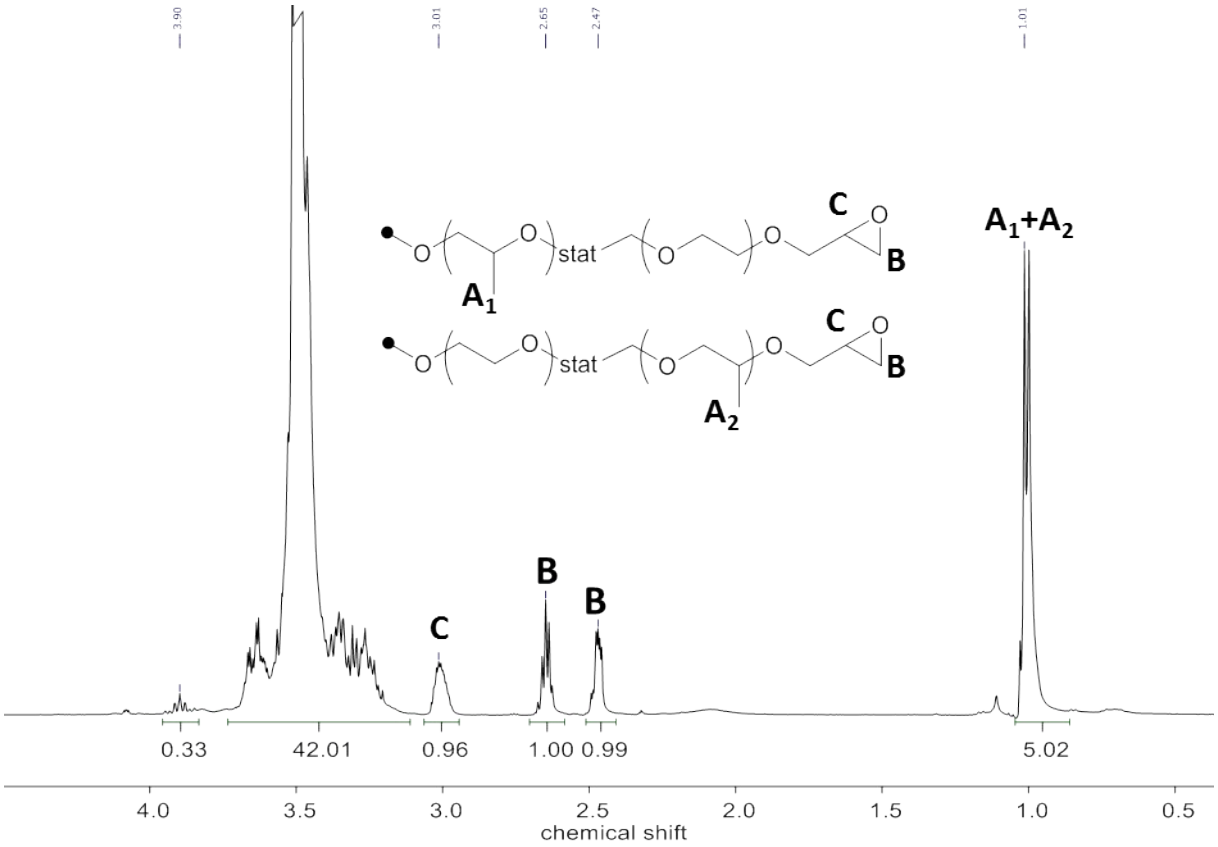

Figure 1: sPEG-epoxy: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$), endgroup functionalization calculated with respect to the average of signals B and C.

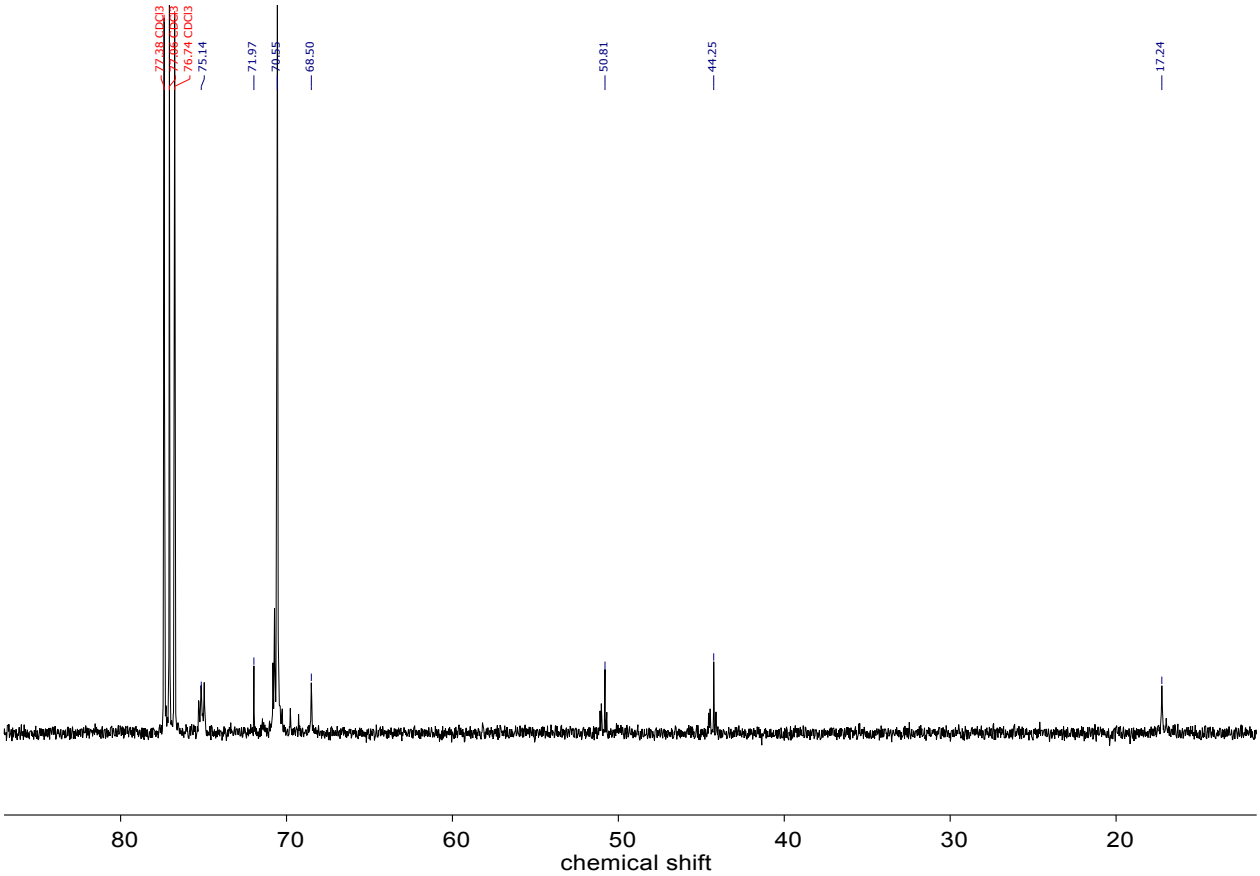

Figure 2: sPEG-epoxy: 13C-NMR spectrum (400 MHz, CDCl3).

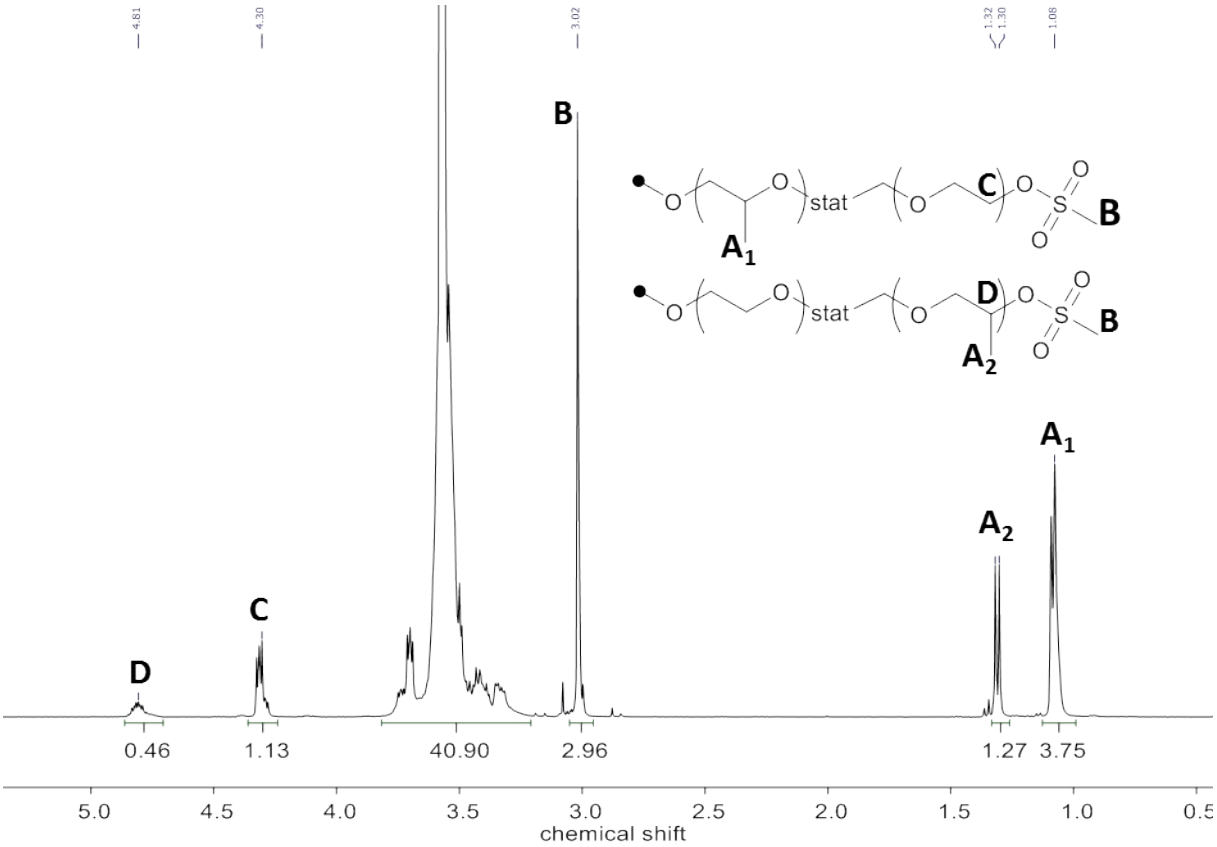

Figure 3: sPEG-mesylate: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$), endgroup functionalization calculated with respect to signal B.

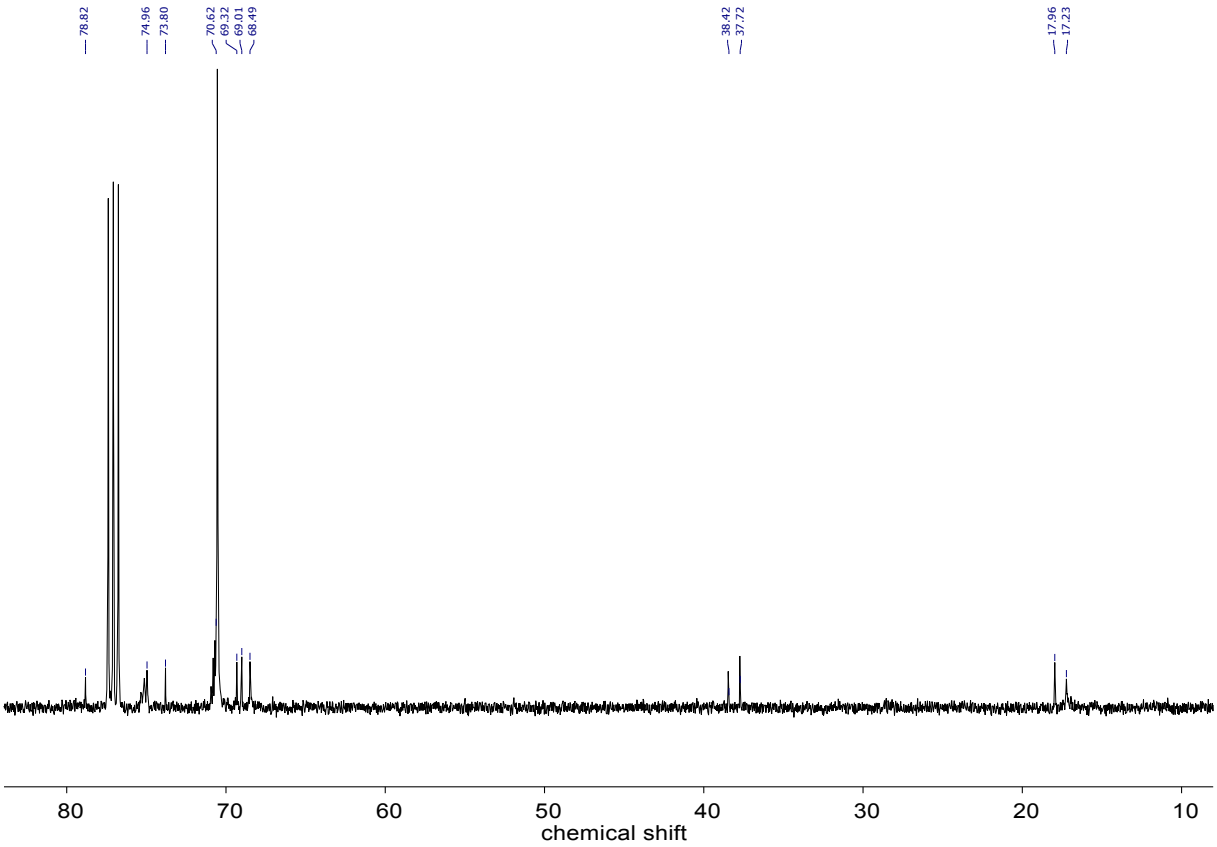

Figure 4: sPEG-mesylate: ^{13}C-NMR spectrum (400 MHz, $CDCl_3$).

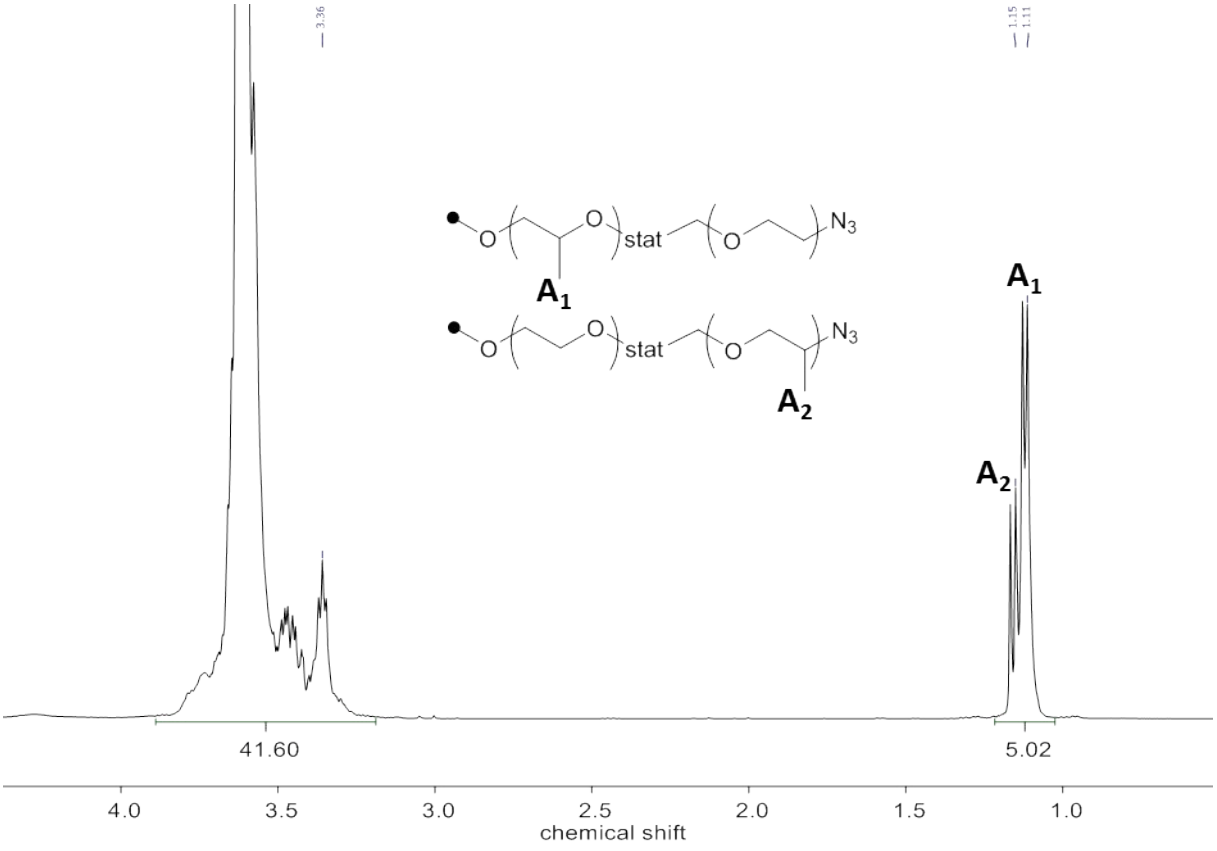

Figure 5: sPEG-azide: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$), endgroup functionalization determined in reference to the conversion of sPEG-mesylate, signal B from Figure 3, here no mesylate residues are detected, thus quantitative conversion is determined.

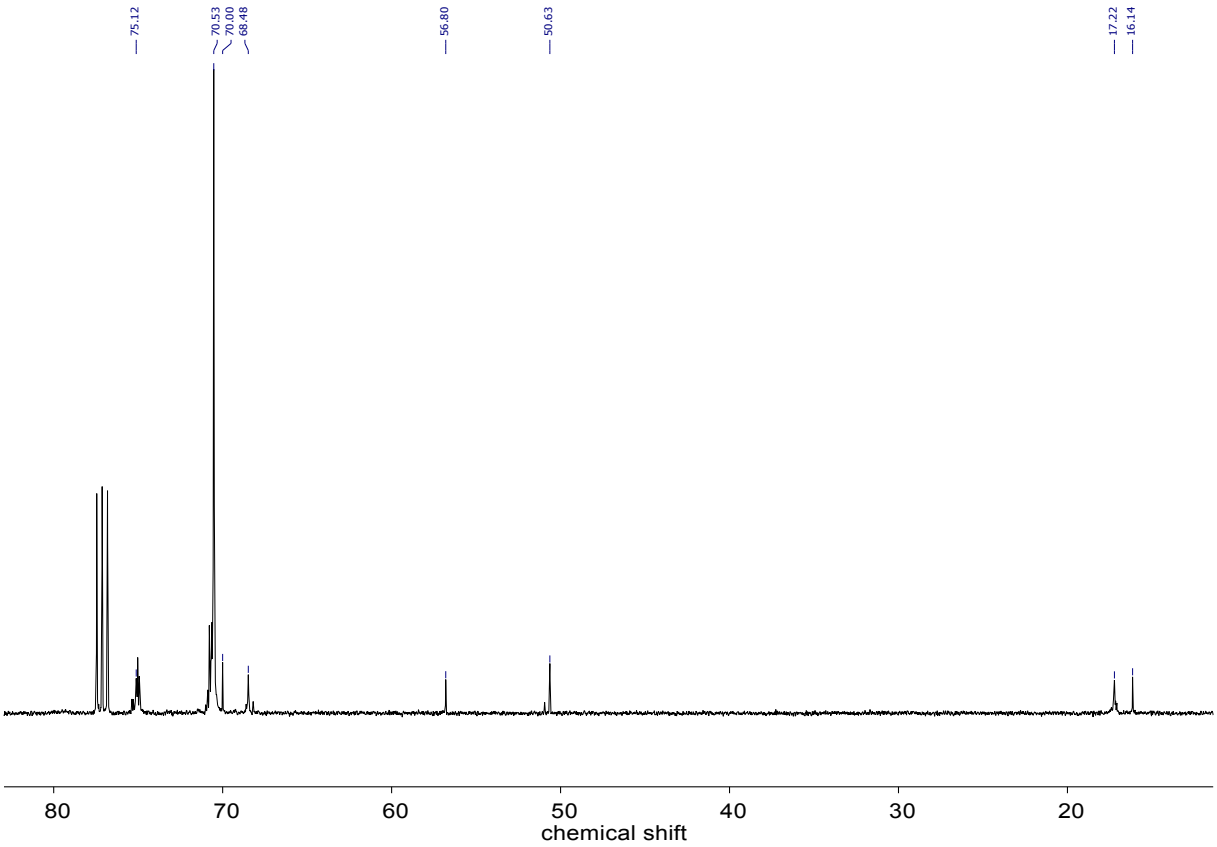

Figure 6: sPEG-azide: ^{13}C-NMR spectrum (400 MHz, $CDCl_3$).

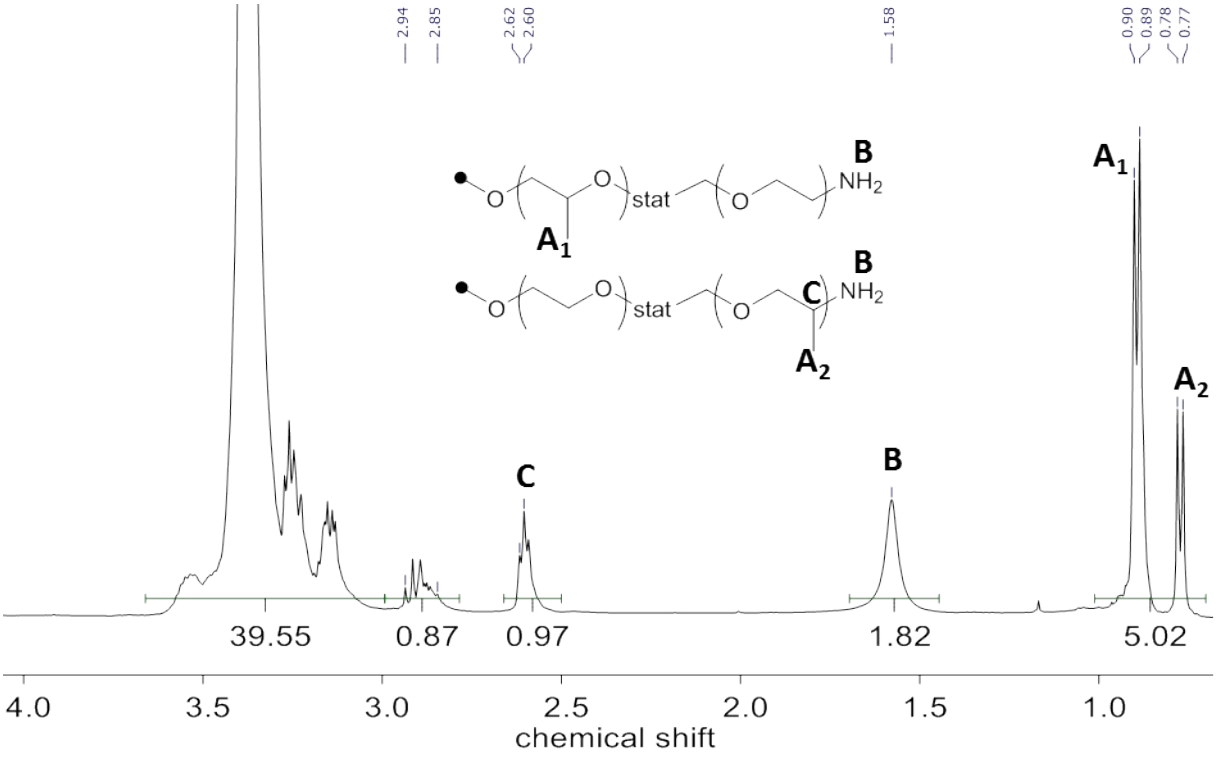

Figure 7: sPEG-amine: ^{1}H-NMR spectrum (400 MHz, $CDCl_3$), endgroup functionalization determined with respect to signal B.

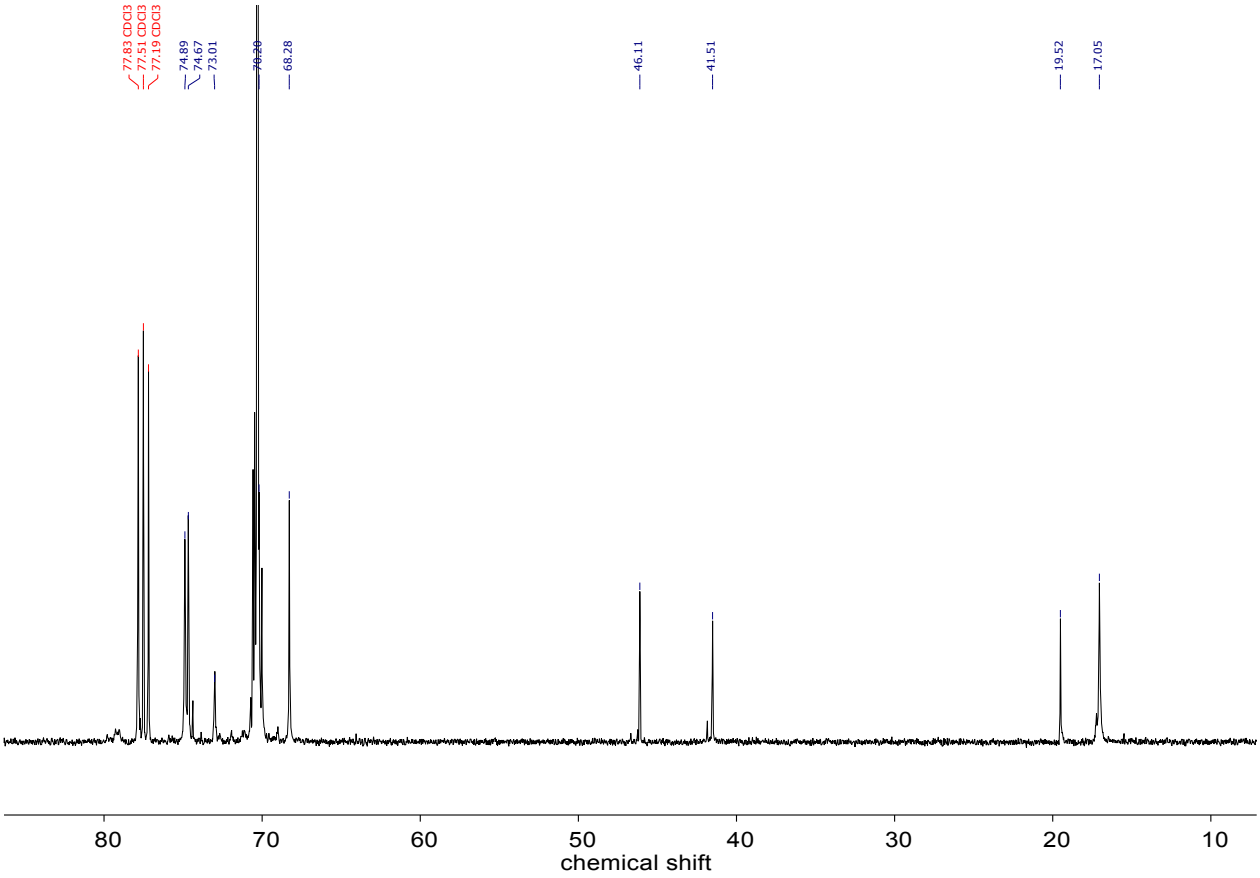

Figure 8: sPEG-amine: ^{13}C-NMR spectrum (400 MHz, $CDCl_3$).

5. SEC results

Table 1: SEC results.

Polymer	M_n [g/mol]	M_w [g/mol]	$Đ$
sPEG	3800	4000	1.04
sPEG-mesylate	3390	4100	1.05
sPEG-azide	2300	2400	1.04
sPEG-amine	2600	2900	1.12
sPEG-epoxy	2700	3000	1.14

6. Rheology experiments: temperature variation

In Figure 9a, the storage modulus G' of aqueous prepolymer solutions and subsequent hydrogels is determined at different gelation temperatures. In Figure 9b, a comparable experiment is shown with linear PEG (200 g/mol) as non-volatile alternative to water. In the rheometer setup, the geometry is equipped with a humidity purge and solvent trap to reduce water evaporation. In general, a maximum storage modulus of around 95 kPa is achieved for all samples, independent of temperature or solvent. As expected, a variation in gelation time is observed for different temperatures, while at high temperatures, G' decreases at longer measuring times as an artifact due to water evaporation from

the gel and solvent trap. In addition, the water based prepolymers crosslink up to ten times faster than in linear PEG. Fortunately, longer measurements of 50000 s are possible with the filler as the problem of evaporation is here not relevant. At 50 °C, the prepolymer solution crosslinks significantly slower but is expected to plateau at a comparable storage modulus.

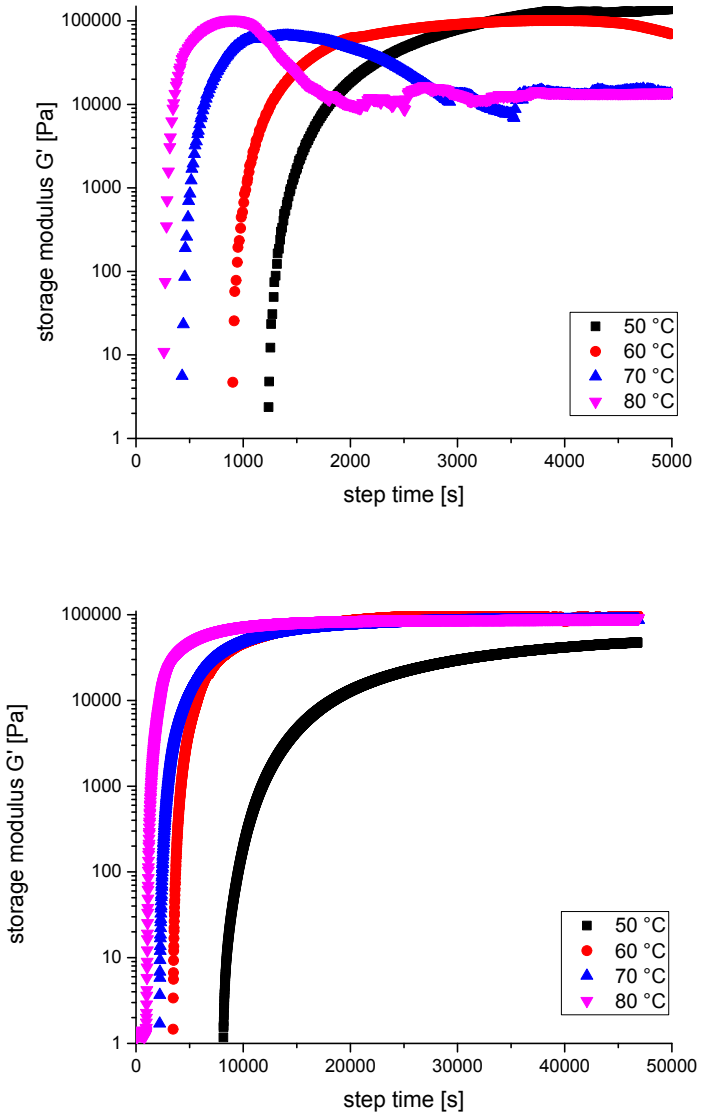

Figure 9: Representative curves of rheology experiments with amine-epoxy prepolymer solutions at different temperatures, 74 µL of 20 wt/V% sPEG-amine and sPEG-epoxy at a 1:1 ratio is added in the geometry, which is covered and contains a humidified purge to avoid fast water evaporation. a) aqueous polymer solution; b) comparable conditions with linear PEG-filler (200 g/mol) as gelation medium.

7. Fabrication of hydrogel discs

Hydrogel discs are fabricated in PDMS molds. The molds are prepared by placing a PDMS layer with a 12 mm punched hole onto a second full PDMS bottom layer. Both are bound together via plasma induced surface activation. The layers create a cylindrical well with a volume of around 200 µL. Prepolymer solutions are prepared in water or with an inert PEG (200 g/mol, Sigma Aldrich) at defined concentrations and stored at 4 °C. The solutions are mixed at equimolar ratio and pipetted into the mold. Gelation is performed in the oven at

elevated temperature. The hydrogel disc is sequentially removed from the mold and swollen overnight in a water filled petri dish. Swelling behavior is measured by comparing the weight of the swollen and dry gel. Measurements are reproduced three times.

$$Swelling\ degree\ [\%] = \frac{m_{swollen} - m_{dry}}{m_{dry}} \cdot 100\ \%$$

$$Water\ content\ [\%] = \frac{m_{water}}{m_{water} + m_{dry}} \cdot 100\ \%$$

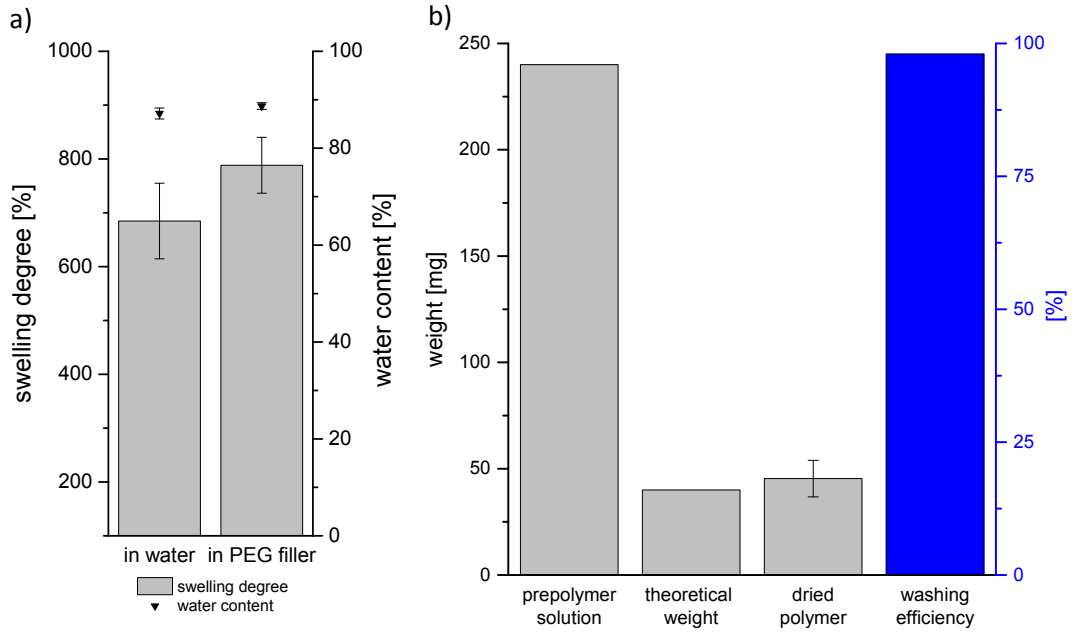

Figure 10: Comparison of hydrogel discs, fabricated in different dispergent agents (20 wt/V% prepolymer at a 1:1 ratio of sPEG-amine and epoxy, 200 µL samples, gelation temperature 60°C, gelation time 1 h). After gelation, the sample are washed to remove the inert filler and left in water overnight to ensure complete swelling. a) A comparable swelling and water content is detected for both water and inert filler as dispergent agents. b) After washing, the samples are dried to calculate the washing efficiency of the PEG filler is examined, revealing a 98 % washing efficiency.

8. Endgroup modification with rhodamine acrylate

Rhodamine B-acrylate is covalently coupled to the free amine functions of amine-epoxy hydrogel discs, post-fabrication. After the disc is prepared and swollen in water overnight, the samples are stored in 1 mL borax buffer (pH = 9) and 200 µL DMSO containing 5.4 mg rhodamine B acrylate for 12 hours. As comparison, non-functionalized rhodamine B is applied to a disc under comparable conditions. In an ultrasound bath, both gels are washed 3 times in 10 mL acetone and DMSO, respectively. The rhodamine B-acrylate treated gels maintain their color, whereas non-functionalized rhodamine B is washed out of the hydrogel network.

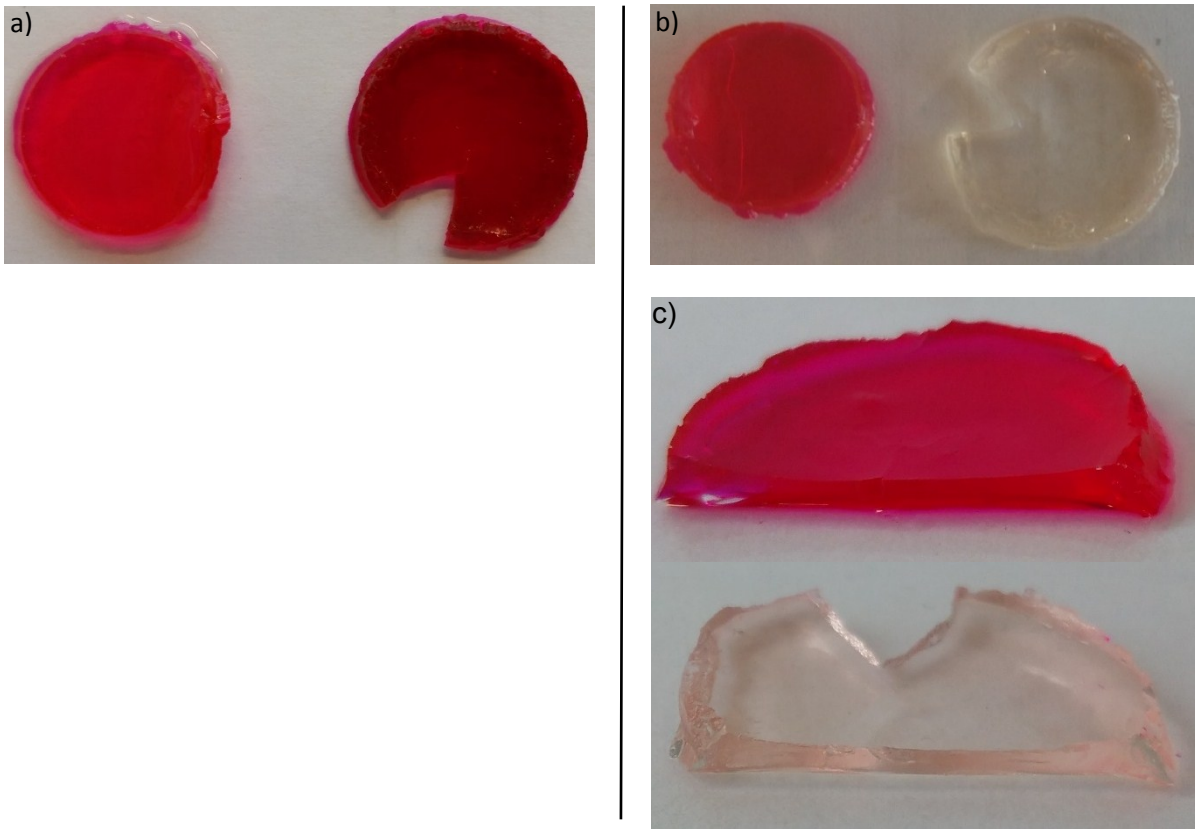

Figure 11: a) Hydrogels after post-gelation with rhodamine B, left: treated with rhodamine B-acrylate, right: treated with non-functionalized rhodamine B; b) Hydrogels after washing, left: covalent binding of rhodamine B-acrylate, right: rhodamine B is washed out completely from the hydrogel; c) Cross-sections of the hydrogels in b, the dye diffuses into the gel and binds throughout the entire gel in the case of rhodamine B-acrylate.

9. Microgel fabrication

Microgel fabrication is performed by an in-mold polymerization technique according to a procedure recently reported by our working group.[3] In short, PDMS molds are produced as described before. sPEG-amine and epoxy are mixed with PEG (200 g/mol) at different concentration, including 1 vol% of a 10 wt% rhodamine acrylate in DMSO, and poured into the mold's cavities. Excess polymer solution is removed using a polyethylene terephthalate (PET) film to avoid film formation in between the cavities. Inside the cavities, the polymers are crosslinked via heating at 60 °C in an oven overnight. A polyvinylpyrrolidone (PVP) layer is added on top of the microgels and hardened at 40 °C for several days to interpenetrate the microgels. The PVP layer is then dissolved in water to extract and collect the microgels. PVP residues are eliminated via repetitive centrifugation in water.

10. Self-assembly experiments

Self-assembly experiments are performed with bright field microscopy at 10x magnification. 10 µL of aqueous microgel solution is monitored in a grid like order, taking 9-15 pictures with a total amount of 200-300 microgels. The entire area is imaged and pictures are combined via Image J with the Grid/collection stitching method[4] at an overlap of 20 %. The total amount of rods is counted manually and the different stacking modes are characterized (single, double, stacked "side-to-side", stacked "random"). Stacks are defined to contain at least 3 microgels.

11. Self-assembly of microgels dispersed in water

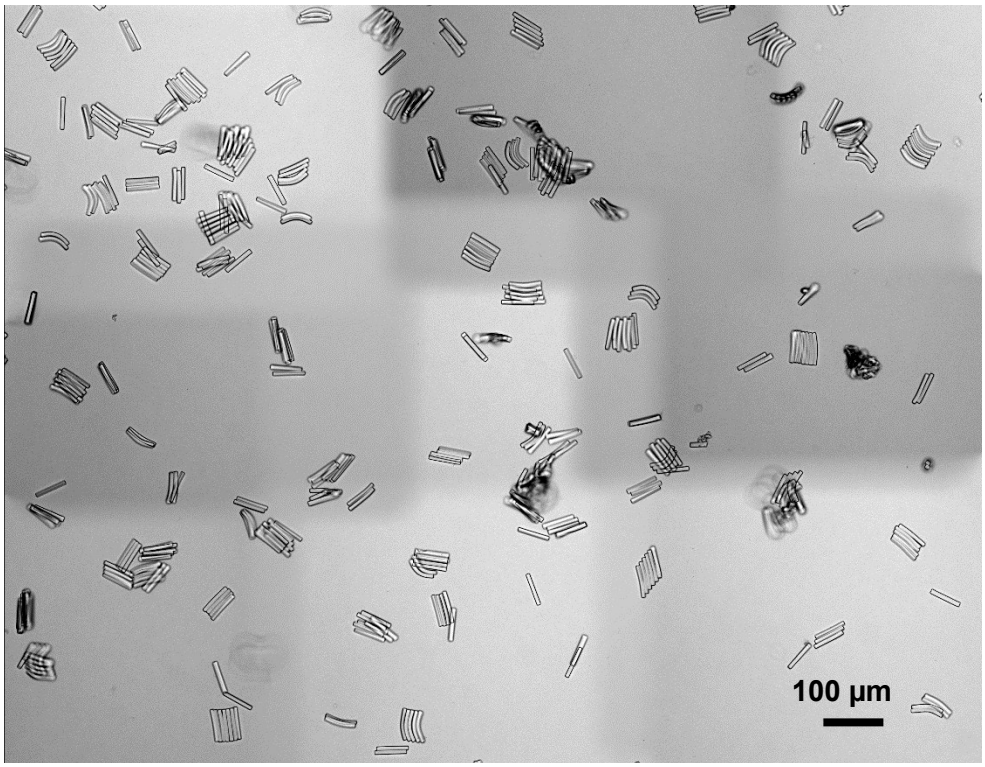

Figure 12: An exemplary image for the quantification of microgel assembly is shown (5x5x50 µm microgels, 100 wt/V% sPEG-amine-epoxy, gelation temperature: 60°C, gelation time: 1 h, purified samples by repetitive centrifugation), bright field microscopy images are taken in a 3x3 grid and stitched together.

12. References

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2. R. Mahou and C. Wandrey, *Polymers*, 2012, **4**, 561.
3. J. C. Rose, M. Cámara-Torres, K. Rahimi, J. Köhler, M. Möller and L. De Laporte, *Nano Letters*, 2017, **17**, 3782-3791.
4. S. Preibisch, S. Saalfeld and P. Tomancak, *Bioinformatics*, 2009, **25**, 1463-1465.