Supporting Information:

Temperature sensitive water-in-water emulsions

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EXPERIMENTAL SECTION

Materials

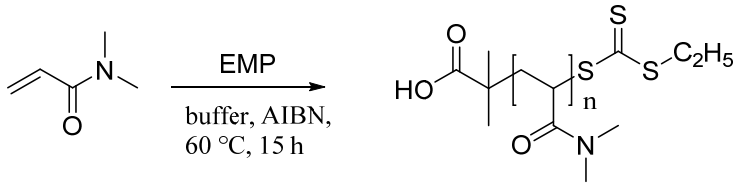
Azobis(isobutyronitrile) (AIBN, 99%, Sigma Aldrich, recrystallized from methanol), acetone (Fisher Scientific), 2-bromisobutyric acid (98%, Sigma Aldrich), carbon disulfide (CS_2, 99%, Sigma Aldrich), *N*,*N*-dimethylacrylamide (DMA, 99%, Sigma-Aldrich, passed over a column of basic aluminum oxide), *N*,*N*-diethylacrylamide (DEA, 99%, TCI, passed over a column of basic aluminum oxide), ethanethiol (97%, Sigma-Aldrich), Rhodamine-labeled Dex (Sigma Aldrich) and potassium phosphate (Sigma-Aldrich). Horseradish-peroxidase (HRP, type VI, EC number 1.11.1.7) was purchased in a form of lyophilized powder from Sigma-Aldrich and used without any further purification. Dextran polymer of 40k was purchased from TCI Deutschland GmbH, PEG 35k was obtained from Sigma-Aldrich. 2-(((Ethylthio)carbonothioyl)thio)-2-methylpropanoic (EMP)[1] and FITC-labeled PEG were synthesized according to a literature procedure.[2]

Aqueous two-phase system

We chose W/W emulsion based on 7 w/v% of PEG 35k and 10 w/v% of Dex 40k. Utilized ATPS was prepared by mixing previously prepared 20 w/v% stock solutions of both polymers in MilliQ water. If needed, appropriate amount of 10 w/v% synthetic copolymer batch solution was added to the mixture and the final concentration adjusted with MilliQ water. Obtained samples were used for all further investigations that included ATPS. The behavior at elevated temperatures was investigated by placing samples in a 60 °C water bath.

Synthesis of PDMA-Macro-RAFT agent.

Reversible addition fragmentation chain transfer (RAFT) polymerization is an easy avenue to synthesize block copolymers like PDMA-*b*-PDEA. 2-(((Ethylthio)carbonothioyl)thio)-2-methylpropanoic acid and AIBN were used to form a macro-RAFT agent with DMA (***Scheme S1***). The macro-RAFT agent was analyzed via ^{1}H-NMR and SEC (***Figure S1-S2*** and ***Table S1***). The ^{1}H-NMR spectra show a characteristic peak around 3.0 ppm originating from the methyl substituents. The PDMA-macro RAFT-agent was obtained with a molar mass of M_n= 62000 g · mol^{-1} and $Đ$ =1.05.



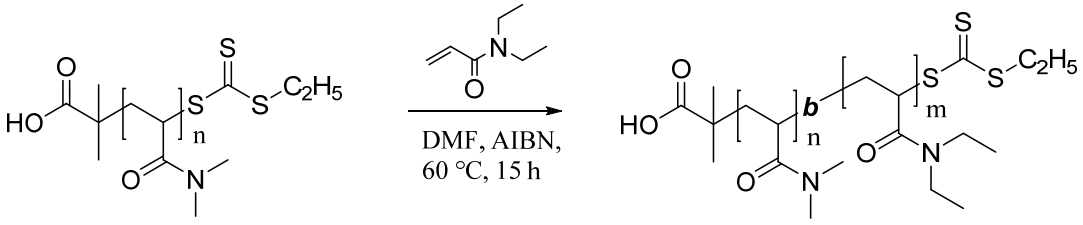
Scheme S1. *Reaction scheme for the synthesis of the PDMA-macro-RAFT agent.*

In a dry argon purged 100 mL round bottom Schlenk flask, EMP (17.3 mg, 0.077 mmol, 1.0 eq.) and AIBN (2.5 mg, 0.015 mmol, 0.2 eq.) were dissolved in DMA (5.0 g, 50 mmol, 650.0 eq.). Acetate-buffer (30 mL, 0.1 M, pH = 5) was added and the tube was sealed. The solution was degassed by three cycles of freeze-pump-thaw, backfilled with argon and placed into a preheated oil bath at 60 °C. Subsequently, the reaction mixture was stirred for 15 hours and stopped by cooling down with liquid nitrogen and exposure to air. After that, the polymer was dialyzed against deionized water (Spectra/Por 3500 Da) for three days, freeze-dried and a colorless solid (2.5 g, M_n = 62000 g · mol^{-1} measured in NMP against PS standards) was obtained.

Synthesis of PDMA-*b*-PDEA

The block copolymer was synthesized via RAFT polymerization of DEA and PDMA-macro-RAFT agent in DMF (***Scheme S2***). The block copolymers were analyzed via ^{1}H-NMR and SEC (***Figure S1, S3, S4*** and ***Table S1***). Block copolymers of either 4:1 or 2:1 monomer ratio were obtained with molar masses of 79500 g · mol^{-1} and 93300 g · mol^{-1} of and $Đ$ =1.08 and 1.07, respectively. In ^{1}H-NMR both peaks for PDMA around 3.0 ppm and PDEA around 3.2 ppm

are present. The integral ratio of these peaks, was for one block copolymer around 4:1 (***Figure S4***) and 2:1 (***Figure S5***). In this way, we indicate successful synthesis of the desired block copolymers and performed basic characterization (***Table S1***).



Scheme S2. *Reaction scheme for the synthesis of PDMA-b-PDEA.*

In a dry argon purged 50 mL Schlenk tube, PDMA-macro-RAFT agent (710 mg, 0.11 mmol, and 1.0 eq.) was dissolved in DMF (3.0 mL). DEA (400 mg, 3 mmol, 275.0 eq.) and AIBN (38 µL, from a DMF stock 10 mg mL^{-1} DMF, 0.2 eq.), was added and the tube was sealed. The solution was degassed by three cycles of freeze-pump-thaw, backfilled with argon and placed into a preheated oil bath at 60 °C. Subsequently, the reaction mixture was stirred for 15 hours and stopped by cooling down with liquid nitrogen and expose to air. After that, the polymer was dialyzed against deionized water (Spectra/Por 3500 Da) for three days, freeze-dried and a colorless solid (0.99 g, M_n = 79500 g · mol^{-1} measured in NMP against PS standards) was obtained. The second PDMA-*b*-PDEA was synthesized in a similar way (0.95 g, M_n = 93300 g · mol^{-1}).

Methods

Size exclusion chromatography (SEC) was conducted in 1-methyl-2-pyrrolidone (NMP) (Fluka, GC grade) with 0.05 mol L^{-1} LiBr and BSME as internal standard at 70 °C using a column system with a PSS GRAM 100/1000 column (8 × 300 mm, 7 µm particle size), a PSS GRAM precolumn (8 × 50 mm), a Shodex RI-71 detector and a PS calibration with standards from PSS.

Cloud point (T_{cp}) measurements were performed with a T70+ UV/Vis Spectrometer (PG Instruments Ltd). Sample in glass cuvette was placed in a sample holder and equilibrated and held at 20 °C for 5 min. Afterwards, samples were heated with 1 °C/min rate up to 60 °C. Over the entire time, transmittance at 660 nm was recorded and plotted as a function of temperature. T_{cp} was determined as a temperature at which samples exhibits half of the initial transmittance.

Dynamic light scattering (DLS) was performed on a ZetaSizer by Malvern. Due to high scattering of samples at elevated temperatures, block copolymers were dissolved at 0.25 wt% in 1 mL of MilliQ water. Scattering was recorded at ambient temperature and consequently that procedure was repeated after the samples were equilibrated at 60 °C. Particle size distributions are displayed as a number weighted function. All experiments were performed three times and average size distribution was calculated.

Optical microscopy with temperature-control stage and confocal microscopy were employed to characterize emulsions at various temperatures. Prepared emulsions were imaged by optical microscopy (OM, DM1000 LED, Leica, Germany). In order to screen emulsion stability at different temperatures, sample was prepared, heated to 60 °C in a water bath and placed on a pre-heated temperature stage by Linkam Scientific (THMS600). Afterwards sample was cooled down with a 5 °C/min rate and optical micrographs were taken at every 10 °C.

Confocal laser scanning microscopy (CLSM) was performed on an TCS SP5, Leica, Germany. In order to get an insight into which water phase is continuous and which is dispersed, the emulsion was stained with fluorescently labeled polymers and confocal images acquired. Staining was performed by addition of either FITC-labeled PEG or Rhodamine-labeled Dex (<1 × 10^{-5} mol/L). Samples were prepared, thermostated at 40 °C water bath and transferred to pre-heated stage. The emulsions were imaged at 40 °C, right above T_{cp} of both DHBCs.

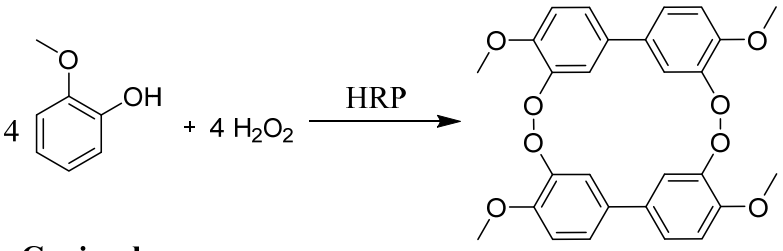
Partitioning experiments

HRP partitioning coefficient (*K*) in PEG/Dex ATPS was performed by Bradford assay protein quantification in both phases.[3, 4] Bradford reagent was prepared by dissolving Coomassie Brilliant Blue G-250 dye in 50 mL of 95% ethanol. Afterwards, 100 mL of 85% phosphoric acid was added and the solution was completed to 1 L by Milli-Q water. HRP calibration curve was prepared in the range from 1 to 20 mg/L. Sample was prepared by diluting 100 μL of the tested phase with 100 μL of Milli-Q water and 800 μL of the Bradford reagent. Absorption spectra was recorded by employment of a T70+ UV/vis spectrometer (PG Instruments Ltd). Difference between two absorption peaks that correspond to the absorption maximum of the free dye (465 nm) and dye-protein complex (595 nm) was determined and compared with a calibration curve to obtain enzyme concentration in both phases. Ration between these two concentrations is determined *K* value.

Partitioning of the colored enzymatic reaction product is displayed in Figure S6. Reaction of guaiacol oxidation (*Scheme S3*) catalyzed by HRP was performed in ATPS ay 50 mM substrate concentration. After 5 min sample was placed in a boiling water bath for 2 min in order to denaturate enzyme and stop the reaction. Afterwards, sample was left to cool down to room temperature and image of the partitioning was taken (*Figure S6*).

Enzymatic activity

Enzymatic activity of HRP in different environments was probed by a modified method based on guaiacol oxidation in presence of hydrogen peroxide.[5] Modification was applied due to high turbulence of the sample that prevented us to follow an increase in the absorbance over time at 470 nm, as it was predicted by the original assay. Initially all stock solutions were equilibrated at 37 °C in a water bath. Sample was prepared by mixing stock solutions of PEG 35k and Dex 40k to the final concentration of 7 wt% and 10 wt% respectively. Consequently, we added 20 μL of hydrogen peroxide to final concentration of 2.7 mM and added desired volume of stock guaiacol solution to obtain contraction in a range from 1 to 50 mM. If we wanted to test emulsion with stabilizer, we added block copolymer 100 μL of 100 mg/mL block copolymer stock solution. Samples were completed to a volume of 970 μL and vigorously stirred. Finally, HRP stock solution to a concentration of 0.05 mg/L was inserted in order to initiate guaiacol oxidation to a colored product. Every minute 300 μL of sample was taken and completed with cold 10 M urea solution to 900 μL. This served well to inhibit any further reaction due to high concentration of denaturation agent (urea), break the emulsion through dilution and cooling sample down below T_{cp} to break copolymer micelles. All of this resulted in a transparent, dyed sample that we could use to measure absorbance due to dye formation. Rates of the dye formation over 3 minutes were calculated and plotted versus substrate concentration as a Michaelis-Menten plot.[6]



Guaiacol

Scheme S3. *Reaction scheme for HRP catalyzed guaiacol oxidation.*

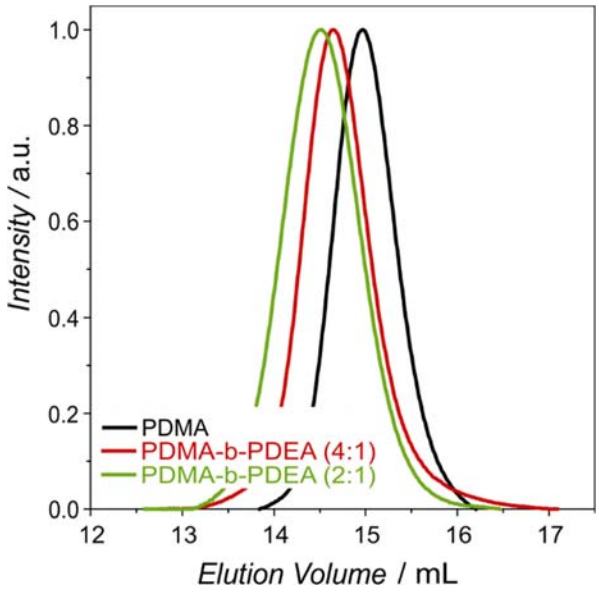


Figure S1. *SEC measurement of PDMA, PDMA-b-PDEA measured in NMP against PS standards.*

Table S1. *Results of SEC measurements of PDMA-macro-RAFT agent and PDMA-b-PDEA copolymers measured in NMP against PS standards.*

Polymer	M_n *(kg·mol^{-1})*	*Đ*
PDMA	62.0	1.05
PDMA-*b*-PDEA (4:1)	79.3	1.08
PDMA-*b*-PDEA (2:1)	93.3	1.07

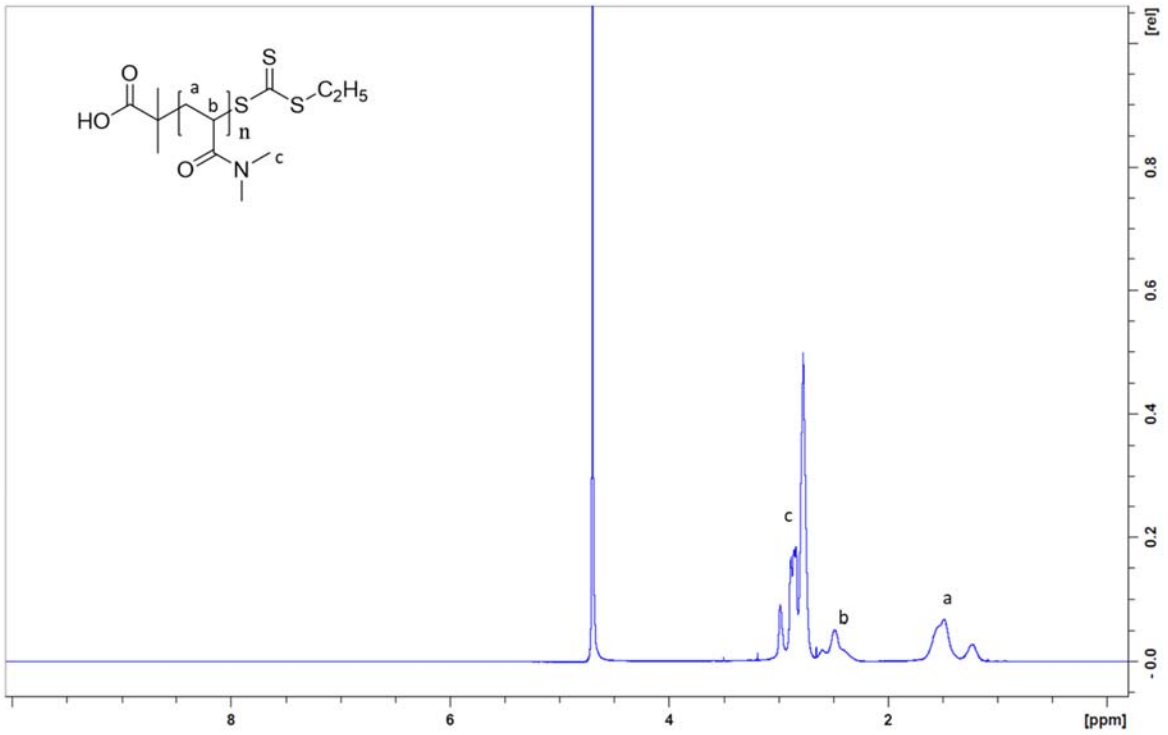


Figure S2. *^{1}H-NMR of the PDMA-macro-RAFT agent, measured in D_2O.*

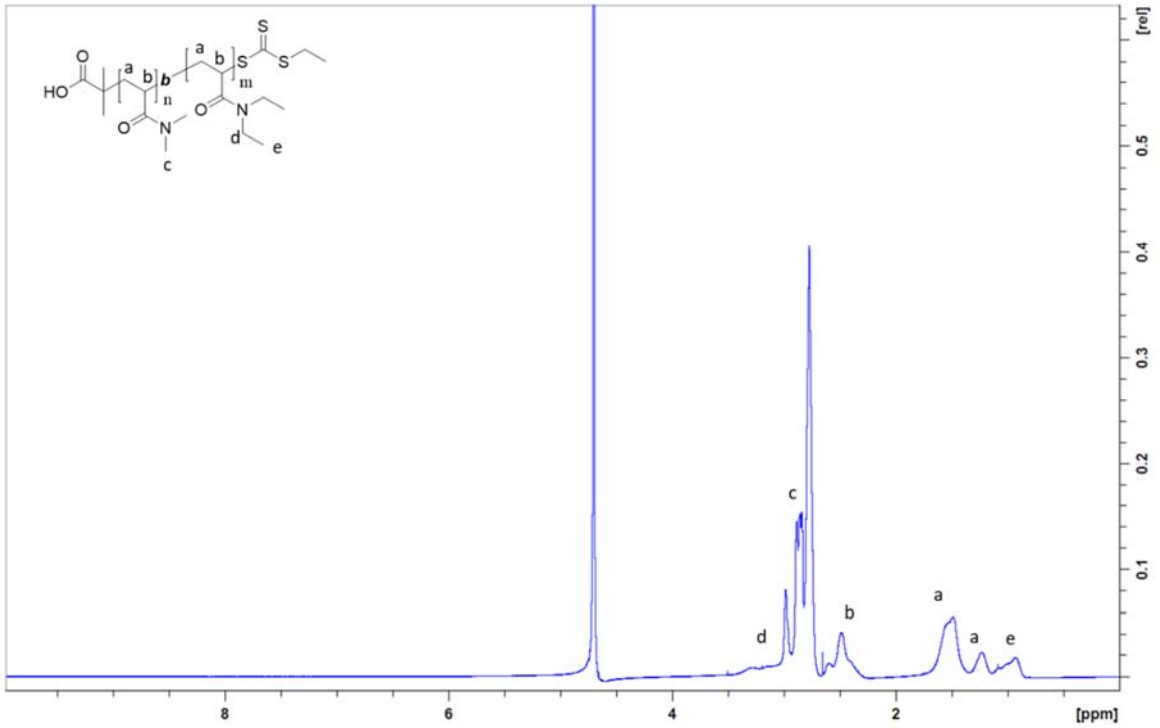


Figure S3. *[1]H-NMR of the PDMA-b-PDEA (4:1) after dialysis, measured in D_2O.*

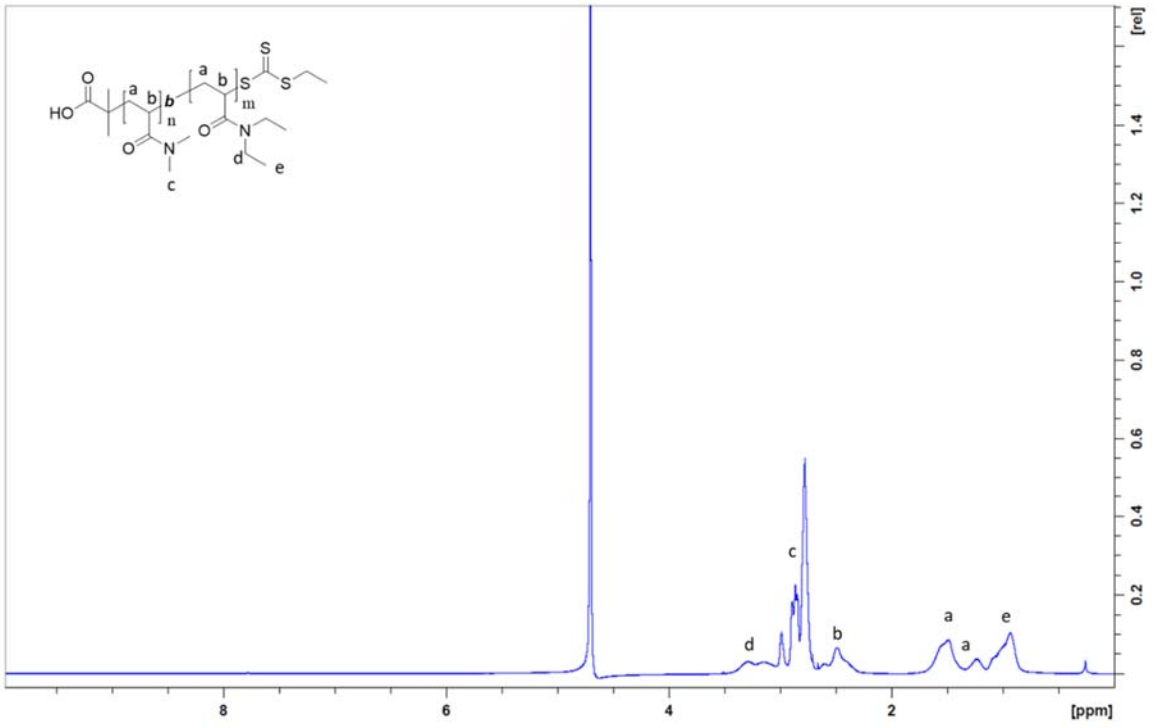


Figure S4. *[1]H-NMR of the PDMA-b-PDEA (2:1) after dialysis, measured in D_2O.*

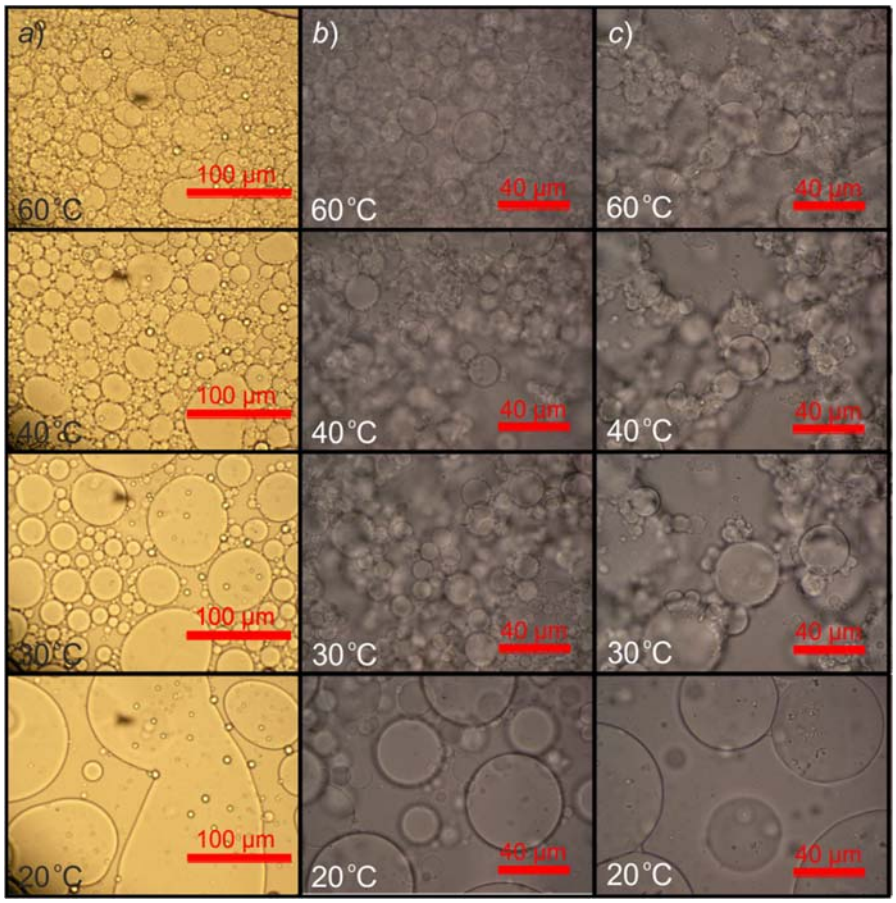


Figure S5. *Optical micrographs of W/W emulsion stabilized by PDMA-b-PDEA 2:1 copolymer at different temperatures at (a) 20x magnification and (b) 50x magnification and by PDMA-b-PDEA 4:1 at 50x magnification (c). Cooling rate was 5 °C/min.*

In **Figure S5c** at 20 °C membrane-like features are observed between several droplets. The presented micrographs capture the droplet coalescence when cooling down the emulsions in a rather narrow time regime. During the coalescence an intermediate membrane-like formation between droplets cannot be fully excluded but also not evidenced from these experiments. As the coalescence of two or more drops resulted in complete phase separation very quickly and in a very narrow temperature range, such a membrane formation would be highly unstable and thus not applicable, which we also expected from the structures of the stabilizing polymers. Most likely, the image shows the moment of commencing droplet coalescence.

Figure S6. *Image of the sample after enzyme catalyzed reaction product partitioned in a PEG/Dex ATPS.*

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