

Supporting Information

Dual pH- and temperature-responsive RAFT-based polymers for transiently soluble block copolymer micelles and polymer-protein conjugates

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Materials and Instrumentation

All chemicals and solvents were commercially available and use as received unless otherwise stated. Dichloromethane (DCM), Toluene, dimethylacetamide (DMA), THF, methanol, CDCl₃, hexane are from Sigma Aldrich. DCM was distilled before use. Azobisisobutyronitrile (AIBN, 98%, Aldrich) was recrystallized from MeOH (twice) and stored at in freezer. 2-(butylthiocarbonothioylthio)propanoic acid, methyl-2-(n-pentyltrithiocarbonyl)propanoate (MPTTCP) were prepared according to the established procedures¹. pH buffers (pH4, pH5, pH6, pH7.4) were prepared according to literature. Specifically, pH4 was prepared by mixing 100 ml 0.1 M potassium hydrogen phthalate and 0.2 ml of 0.1 M HCl; pH 5 was prepared by mixing 100 ml 0.1 M potassium hydrogen phthalate and 45.2 ml of 0.1 M NaOH; pH6/pH7.4 was prepared by mixing 100 ml 0.1 M KH₂PO₄ and 11.2 ml/78.2 ml of 0.1 M NaOH, all the solutions were then made up to 200ml with distilled water. Poly(ethylene glycol) monomethyl ether (average Mn ~2,000, Sigma Aldrich) was characterized by size exclusion chromatography with THF as eluent (Mn=2400, PDI=1.06) and ¹H NMR spectroscopy using CDCl₃ as solvent (DP=48). All polymerization were performed under an argon atmosphere.

¹H NMR spectra were recorded on a Bruker 300 MHz FT-NMR spectrometer using CDCl₃ as solvent. Chemical shifts (δ) are given in ppm relative to TMS.

Size exclusion chromatography (SEC) was carried out in THF at 45 °C at a flow rate of 1 mL/min with a SFD S5200 auto sampler liquid chromatogram pH equipped with a SFD refractometer index detector 2000. The PL gel 5 lm (105 Å, 104 Å, 103 Å, and 100 Å) columns were calibrated with polystyrene standards.

Gas chromatography was performed on 7890A from Agilent Technologies with an Agilent J&W Advanced Capillary GC column (30 m, 0.320 mm, and 0.25 μm). Injections were performed with an Agilent Technologies 7693 auto sampler. Detection was done with a FID detector. Injector and detector temperatures were kept constant at 250 and 280 °C, respectively. The column was initially set at 50 °C, followed by two heating stages:

from 50 °C to 100 °C with a rate of 20 °C /min and from 100 °C to 300 °C with a rate of 40 °C /min, and then held at this temperature for 0.5 minutes. Conversion was determined based on the integration of monomer peaks using DMA as internal standard.

Turbidity measurements were performed on a Cary 300 Bio UV-Visible spectrophotometer at a wavelength of 600 nm. The samples were first cooled to a suitable temperature to fully dissolve the copolymer (5 mg ml⁻¹), after which the sample was placed in the instrument and cooled to 5 °C. The transmittance was measured during at least two controlled cooling/heating cycles with a cooling/heating rate of 1 °C min⁻¹ while stirring. Cloud point (CP) is given as the temperature when the transmittance goes through 50% during heating.

Dynamic light scattering (DLS) was performed on a Zetasizer Nano-ZS Malvern apparatus (Malvern Instruments Ltd) using disposable cuvettes. The excitation light source was a He-Ne laser at 633 nm, and the intensity of the scattered light was measured at 173°. This method measures the rate of the intensity fluctuation and the size of the particles is determined through the Stokes-Einstein equation

$$d(H) = kT/3\pi\eta D \quad (1)$$

where $d(H)$ is the mean hydrodynamic diameter, k is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the dispersing medium, and D is the apparent diffusion coefficient. All samples were filtered through Millipore membranes with pore sizes of 0.2 μm prior to measurement.

Fluorescence measurements were carried out on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) equipped with a Varian Cary Temperature Controller. The emission spectra excited by a 200 nm laser were monitored from 520 - 700 nm, and the slit width was kept at 5 nm during the measurements.

Synthesis and characterization

Synthesis of (2,2-dimethyl-1,3-dioxolane-4-yl)methyl acrylate (DMDMA) monomer

The synthesis of this monomer was performed in two steps based on reported methods². For the first step, glycerol (92 g, 1 mol), acetone (52g, 0.9 mol) p-toluenesulfonic acid monohydrate (0.1 g) and hexane (300 ml) were placed in a 500 ml flask and refluxed for 48h. 20ml of acetone were added after 12, 24 and 36 hours during reaction. Then the reaction solution was neutralized by adding potassium bicarbonate (0.22 g, 2.6 mmol) followed by filtration. The filtrate was dried under reduced pressure. Then 85.2 g (64%) of acetone glycerol was collected by distillation under reduced pressure. ¹H NMR (300 MHz, CDCl₃), δ (ppm): 4.19 (m, 1H, CH₂CHCH₂), 3.49–4.04 (m, 4H, HOCH₂CHCH₂), 2.52–2.75 (br, 1H, HO), 1.25–1.45 (d, 6H, C(CH₃)₂).

For the second step, a solution of acetone glycerol (22.36 ml, 180 mmol) and triethylamine (25.1 ml, 180.0 mmol) in anhydrous dichloromethane (DCM, 100 mL) was cooled to 0 °C in an ice water bath. Acryloyl chloride (16.2 ml, 200 mmol) in DCM (30 mL) was added dropwise with vigorous stirring. After 12h of reaction, the solution was filtered. The filtrate was washed twice with distilled water after neutralization by adding sodium hydroxide solution (0.1 M). Then the residual water and DCM was evaporated under reduced pressure. 26.0 ml (ca. 78%) DMDMA was purified by reduced-pressure distillation in the presence of hydroquinone as inhibitor. ¹H NMR (300 MHz, CDCl₃), δ (ppm): 5.70–6.40 (m, 3H, CH₂CH), 3.65–4.32 (m, 5H, CH₂CHCH₂), 1.25–1.42 (d, 6H, C(CH₃)₂).

Synthesis of methoxy tri(ethylene glycol) acrylate (mTEGA) monomer

The preparation procedure of mTEGA was similar as reported literature.³ A solution of triethylene glycol monomethyl ether (32 ml, 0.2 mol), triethylamine (33.47 ml, 2.4 mol) in DCM (240 ml) was cooled to 0 °C in an ice water bath. Acryloyl chloride (19.4 ml, 0.24 mol) in DCM (60 mL) was added dropwisely with vigorous stirring. After 12h of reaction, ca. 20g of silica gel was added to the solution. The mixture was purified by column chromatography using silica gel as stationary phase and DCM as eluent. Then the solvent was evaporated in *vacuo*. The product was finally purified by reduced-pressure distillation in the presence of hydroquinone as inhibitor. Yield: 33g (76%). ¹H NMR (300 MHz, CDCl₃), δ (ppm): 5.70–6.42 (m, 3H, CH₂CH), 4.20–4.30 (m, 2H, CH₂CHCOOCH₂), 3.42–3.72 (m, 10H, CH₂(OCH₂CH₂)₂OCH₃), 3.3 (s, 3H, CH₃). WARNING: THIS MONOMER MIGHT CAUSE CHEMICAL BURNS WHEN IN DIRECT CONTACT WITH SKIN!

Synthesis of poly(ethylene glycol) macro chain transfer agent PEG-CTA

A solution of 2-(butylthiocarbonothioylthio)propanoic acid (1.43g, 6 mmol), poly(ethylene glycol) monomethyl ether (10g) in DCM (100 ml) was cooled to 0 °C in an ice water bath. N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (1.15g, 6 mmol) and 4-dimethylaminopyridine (61.08 mg, 0.5 mmol) in DCM (40 ml) was added dropwise with vigorous stirring. Then the solution was allowed to react for 16 h in room temperature. After that, the solution was washed with water for two times and dried under reduced pressure. The solid residue was dissolved in DCM and precipitated for three times in hexane/diethyl ether mixture (80/20 *vol*). PEG-CTA was collected by drying under reduced pressure at room temperature for 48h. Mn (THF SEC)= 2600, Đ=1.06. ¹H NMR spectra of PEG and PEG-CTA are shown in Fig. S1.

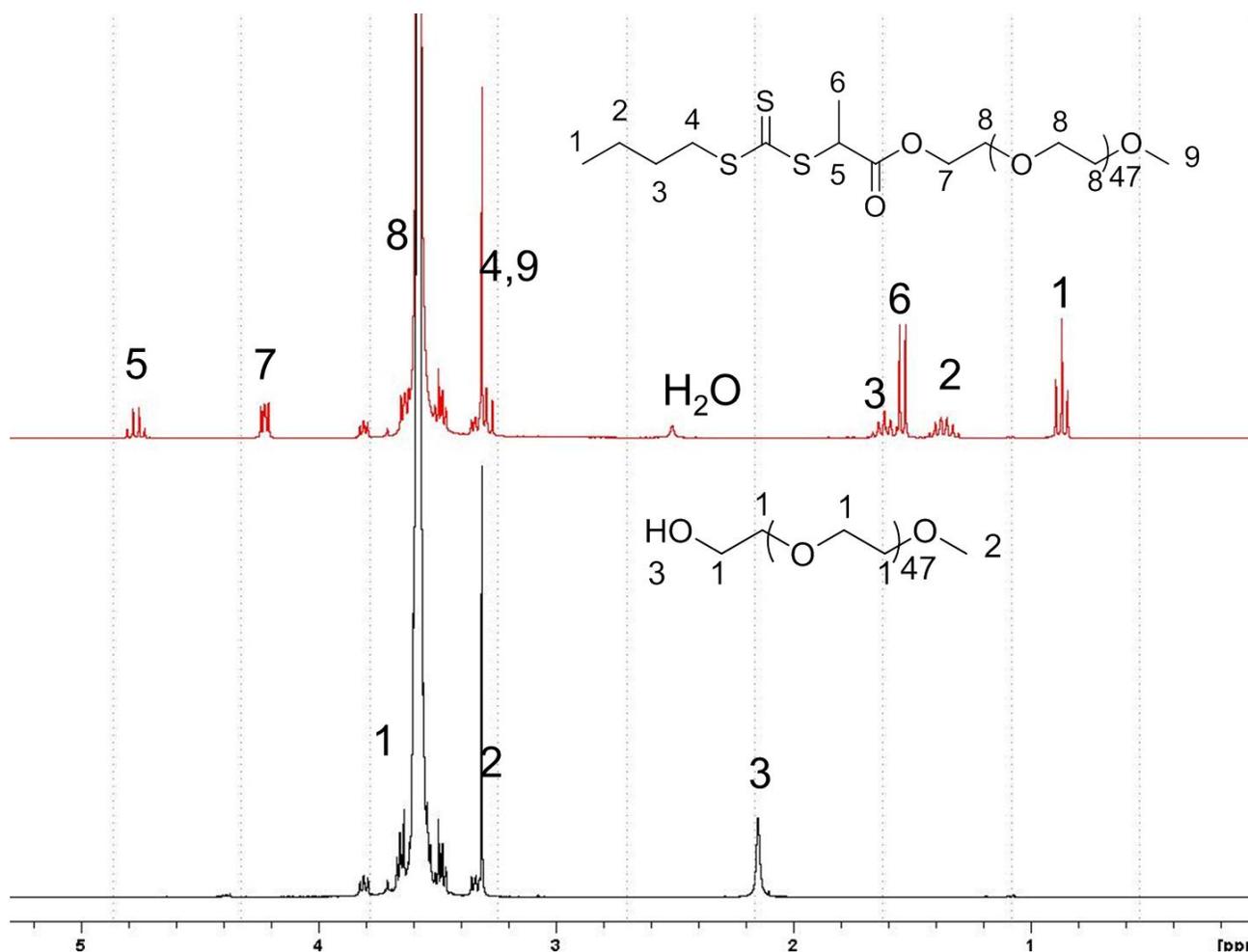


Fig. S1 ^1H NMR of PEG and PEG-CTA in CDCl_3

Synthesis of poly(DMDMA-co-mTEGA)

DMDMA, mTEGA azobisisobutyronitrile (AIBN) and MPTTCP were first dissolved in toluene/DMA solvent mixture (80/20 vol) in a schlenk vial. The concentration of monomer was fixed at 2M. After degassing the solution three times by freeze-vacuum-thaw cycles, the schlenk vial filled with Argon and immersed in a preheated oil bath at 70 °C while stirring. The polymerization was performed for about 2 hours and stopped by immersing the schlenk vial into dry ice/isopropanol bath. The resulting polymer was isolated by precipitation in hexane (three times) followed by drying under reduced pressure at 50 °C. Conversion of the monomers was analysed by GC with DMA as internal standard. Size exclusion chromatography was used to evaluate number average molecular weight (M_n) and polydispersity indices (\mathcal{D}) of the obtained polymers. For kinetics study, samples were withdrawn from the polymerization mixture under a flow of argon at different times. An induction period of about 20 mins has been detected, which is commonly observed for the RAFT polymerization of acrylates⁴.

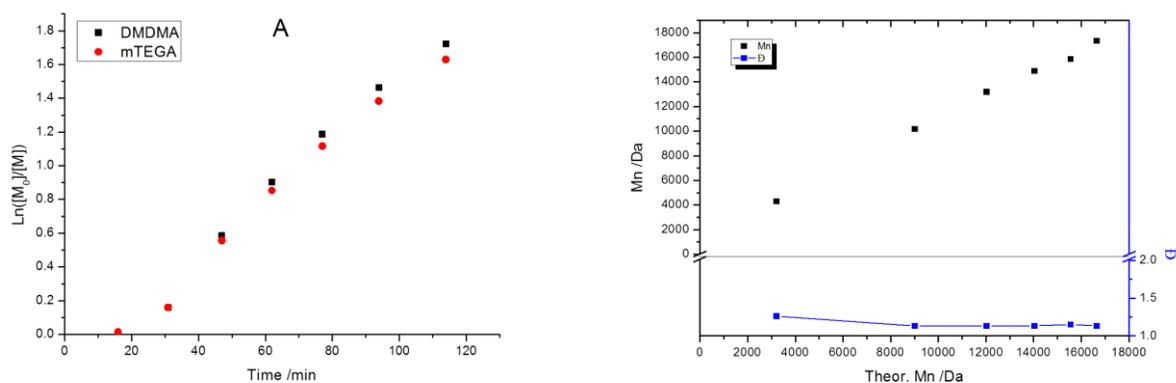


Fig. S2 Left: Pseudo-first-order kinetic plot of DMDMA and mTEGA for RAFT polymerization with DMDMA: mTEGA: CTA: AIBN=50: 50: 1: 0.1. Right: Corresponding M_n and \bar{D} versus theoretical M_n plot. The CTA used in this kinetics study was MPTTCP

Synthesis of PEG-block-poly(DMDMA-co-mTEGA)

DMDMA, mTEGA, AIBN and PEG-CTA were first dissolved in toluene/DMA mixture solvent (80/20 vol) in a schlenk vial. The concentration of monomer was fixed at 1.5 M. After degassing the solution three times by freeze-vacuum-thaw cycles, the schlenk vial filled with Argon and immersed in a preheated oil bath at 70 °C while stirring. The polymerization was performed for 2 hours and stopped by immersing the reaction flask into dry ice isopropanol bath. The resulting polymer was isolated by precipitation in hexane (three times) followed by drying under reduced pressure. Conversion of the monomers was analysed by GC with DMA as internal standard. Size exclusion chromatography was used to evaluate number average molecular weight (M_n) and polydispersity indices (\bar{D}) of the obtained polymers.

Methods

pH triggered hydrolysis of poly(DMDMA-co-mTEGA)

Hydrolysis of poly(DMDMA-co-mTEGA) was performed in pH buffers (pH=4, 5, 6, and 7.4 respectively) at a concentration of 5 mg/ml at 37 °C in test tubes. Samples for cloud point measurement were taken directly from the hydrolysis solution. For ¹H NMR measurement, 10 ml of hydrolysis solution taken from the tube was first neutralized by adding NaOH solution. The mixture of inorganic salt and polymer was used for the NMR analysis after evaporating the water under reduced pressure.

Acid-triggered release of Nile red.

The acid-triggered release of Nile red was studied at three pH buffers, pH4, pH5 and pH7.4. In general, 0.1 ml of Nile red in ethanol (3mg/mL) was mixed with 0.9 mL of aqueous solution with 10 mg block copolymer at room temperature. After 1 min of incubation with stirring, the solution was put in a preheated water bath at 50 °C and incubated for 1 min. Then the solutions were transferred back to 37 °C water bath. 9 ml of pH buffer (preheated to 37 °C) was added to the solution to tune the polymer solution to be 1 mg/ml in different pH buffer, pH4, pH5 and pH7.4. The emission spectrum of the solution was recorded at 37 °C for every half an hour by fluorescence spectrophotometer.

Cell viability assay (MTT)

Dulbecco's Phosphate-Buffered Saline (DPBS), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), L-glutamine, sodium pyruvate, penicillin and streptomycin were purchased from Invitrogen. Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were commercially available at Sigma Aldrich. Acetone was purchased from Fischer Chemical. Membrane filters (0.22µm) were obtained from Whatman. HCA2-hTERT human fibroblasts were kindly provided by C. Jones (Cardiff University, UK). RAW 264.7 mouse macrophages were obtained from ATCC. The MTT assay was executed in 96-well titer plates purchased from TPP. Absorbance was measured on a Perkin Elmer microplate reader.

HCA2-hTERT human fibroblasts and RAW264.7 mouse macrophages were cultured in DMEM, supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and antibiotics (50 units/mL penicillin and 50 µg/mL streptomycin). Cells were incubated at 37 °C in a controlled, sterile environment of 95% relative humidity and 5% CO₂.

Polymer stock solutions 5mg/ml were prepared by dissolving 20mg of polymer in 4ml cold (4°C) culture medium. Acetone was added to stock solutions of fully hydrolyzed polymers (polymer 1, 5 and 6) in accordance to the amount that would be released after hydrolysis of the corresponding non-hydrolyzed polymer. Stock solutions were sterilized by membrane filtration. With these obtained stock solutions, two dilutions in culture medium were made to reach a concentration of 0.5 and 0.05 mg/mL, respectively.

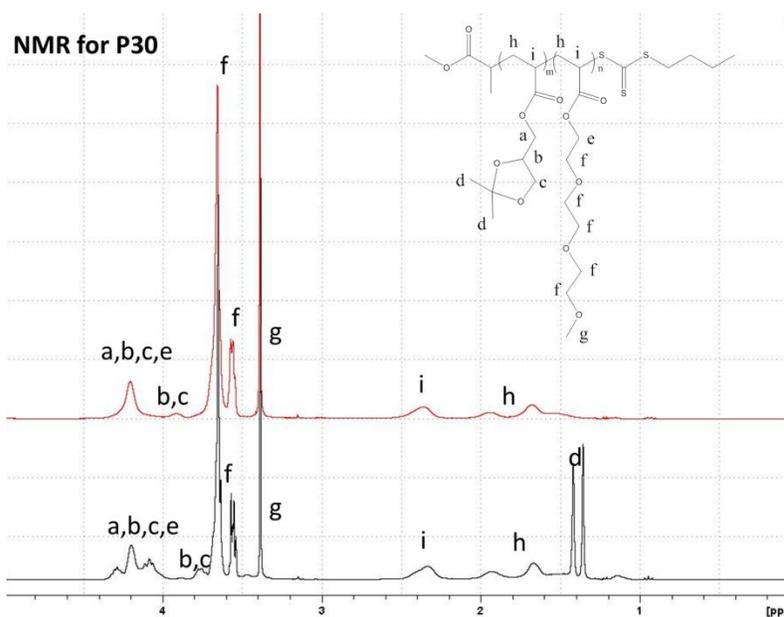
MTT stock solution consisted of 250mg MTT, dissolved in 50ml of PBS. For MTT assay, MTT solution was prepared by a fivefold dilution of MTT stock solution in culture medium. The MTT assay was performed

according to established procedures. Briefly, HCA2-hTERT human fibroblasts and RAW264.7 mouse macrophages were seeded into 96-well titer plates (10 000 cells per well, suspended in 200µl of culture medium) and incubated for 24h. Thereupon, 50µl of polymer solution (5, 0.5 or 0.05 mg/mL), DMSO (positive control = 0% viability) or culture medium (negative control = 100% viability) was added. After 24h of incubation, medium was aspirated and cells were washed with 250 µL PBS. After aspiration, 200 µL of MTT solution was added for 4h. MTT solution was removed and the purple formazan crystals were dissolved in 50µl of DMSO. Absorbance was determined at 590nm on a microplate reader. The absorbance of the positive control was subtracted from all values. Cell viability (%) was defined as absorbance of the test polymer divided by absorbance of negative control times 100% . Experiments were carried out in quintuplicate.

Protein bioconjugation

PAGE experiments were conducted using the Bio-Rad mini-PROTEAN tetra set-up. A typical conjugation procedure for the was as follows. A lysozyme (from hen egg white, Sigma) solution in DI water (0.25 mL , $8.56 \times 10^{-5}\text{ mmol}$, 5 mg mL^{-1}) was added to different volumes of a NHS-terminated p(AcA28-MTEGA56) solutions in DI water (10 mg mL^{-1} for the 7 kDa polymer and 20 mg mL^{-1} for the 17 kDa polymer), respectively in a 1:1, 1:10 and 1:20 molar ratio. The total volume was brought to 2 mL with a 0.1 M sodium bicarbonate buffer of pH 8.3. The solutions were kept at room temperature with gentle shaking overnight. Polymer solution without lysozyme was included as a control. Moreover half of the conjugation mixture was brought to pH 3 with 0.1 M HCl and kept in a heating block at 56°C overnight, in order to hydrolyze the polymer under accelerated conditions. The undiluted conjugation mixtures were analyzed by SDS-PAGE. To confirm LCST behavior, native page was performed at room temperature and at 45°C by placing the PAGE set-up in an oven.

Supplemental figures



SEC trace of P30

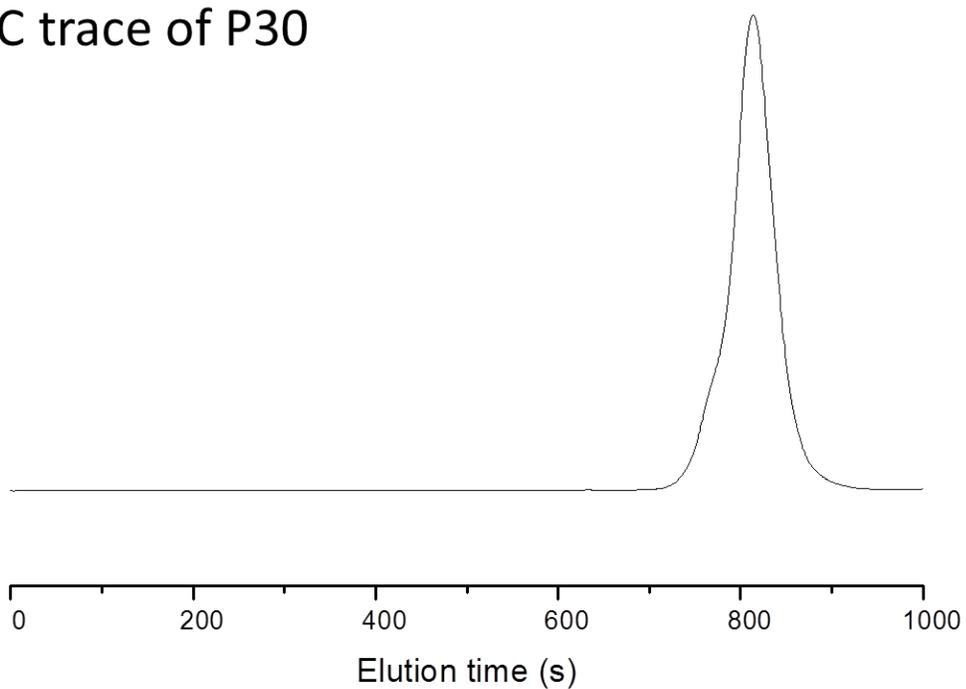
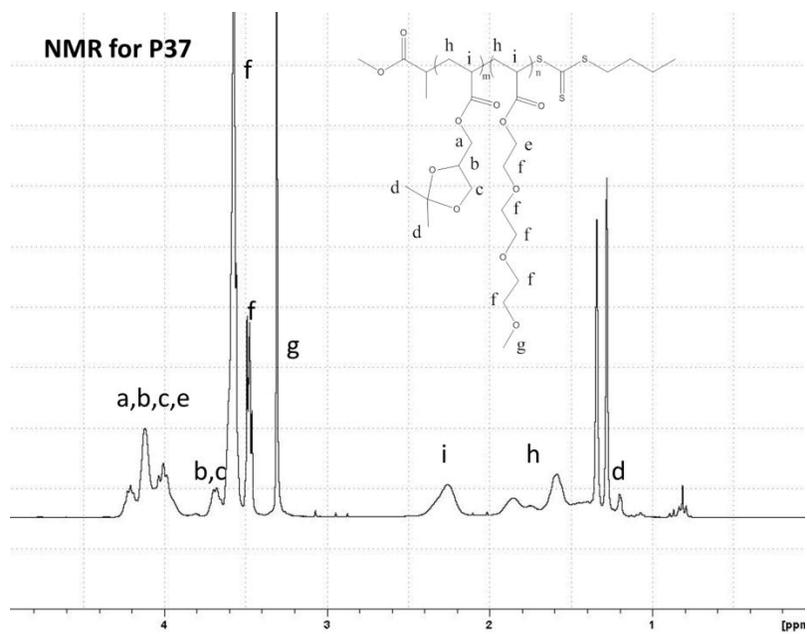


Fig. S3 Top: ^1H NMR of P30 before (black line) and after (red line) hydrolysis in CDCl_3 . Bottom: THF SEC trace of P30.



SEC trace of P37

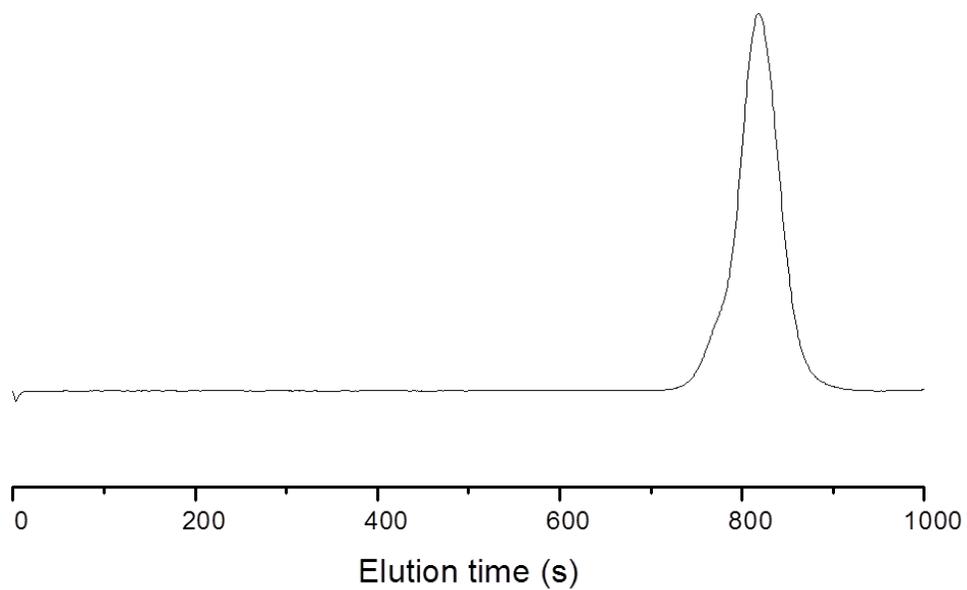
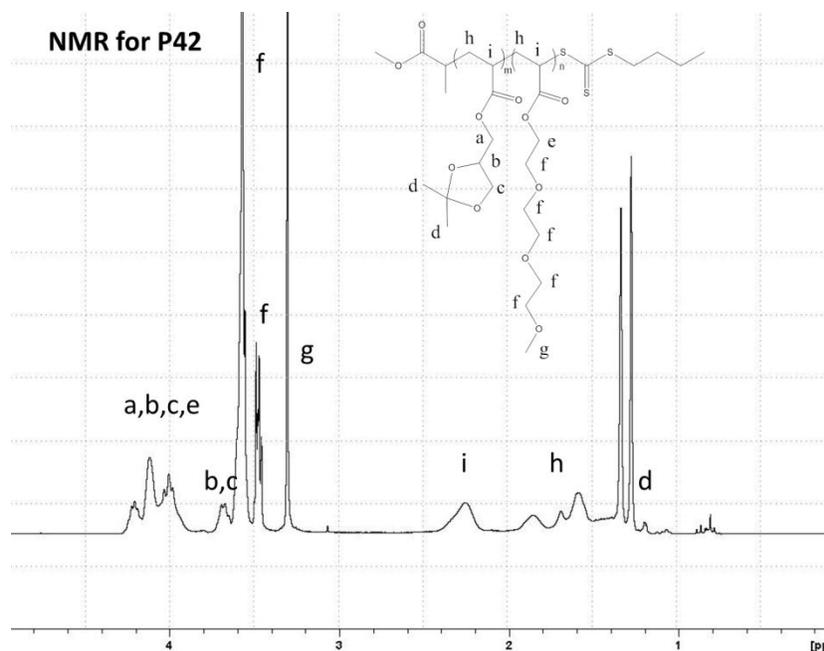


Fig. S4 ¹H NMR (top, in CDCl₃) and THF SEC trace (bottom) of P37



SEC trace of P42

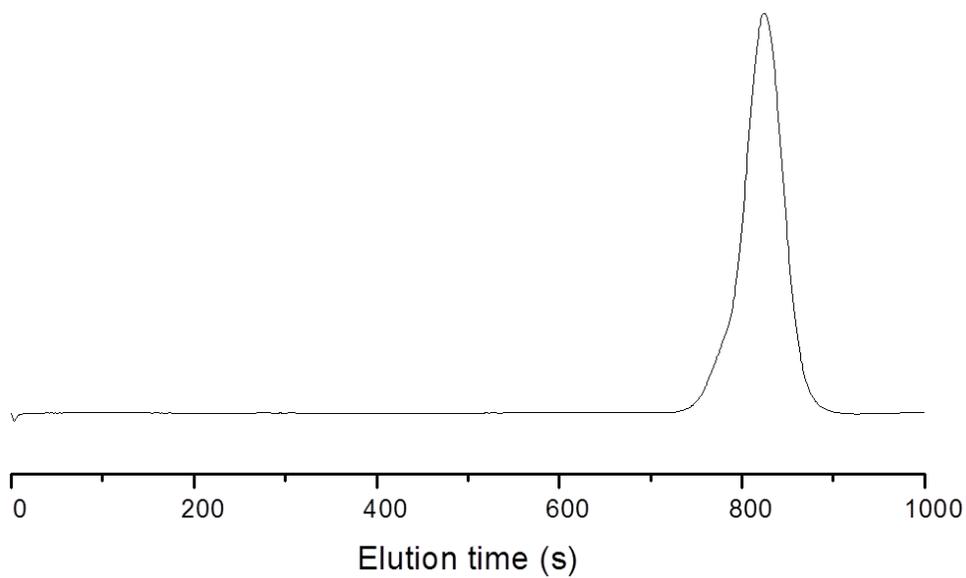
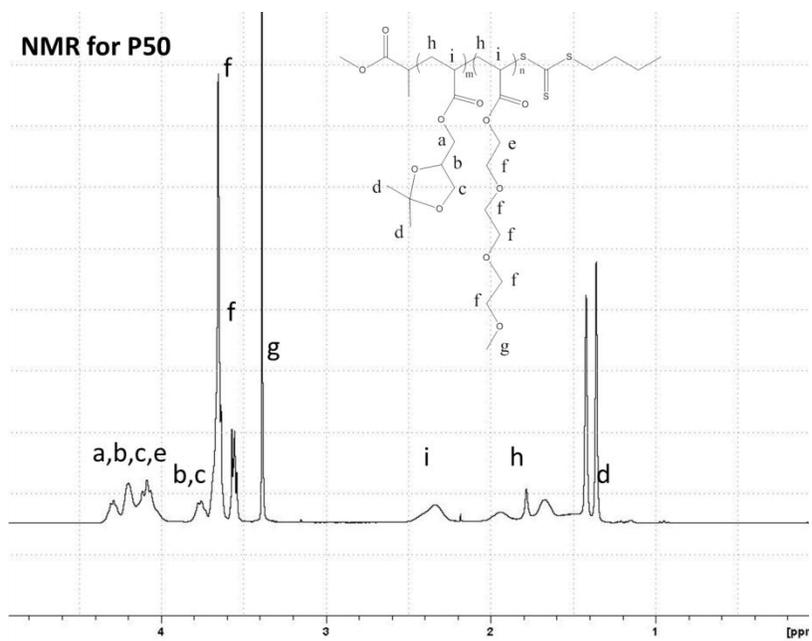


Fig. S5 ¹H NMR (top, in CDCl₃) and THF SEC trace (bottom) of P42



SEC trace of P50

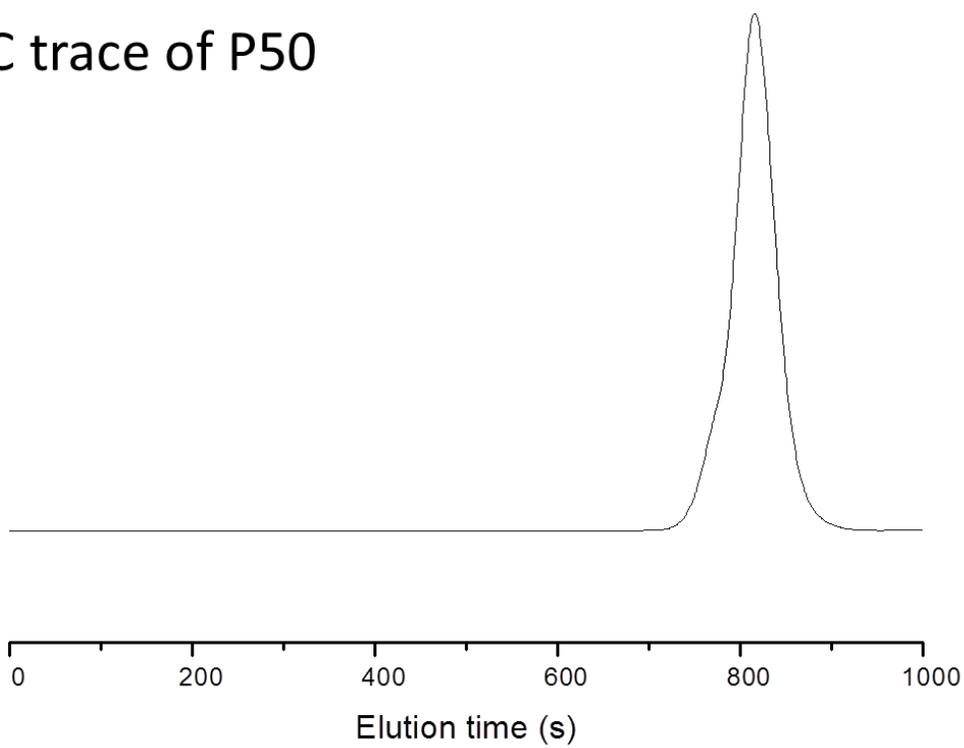
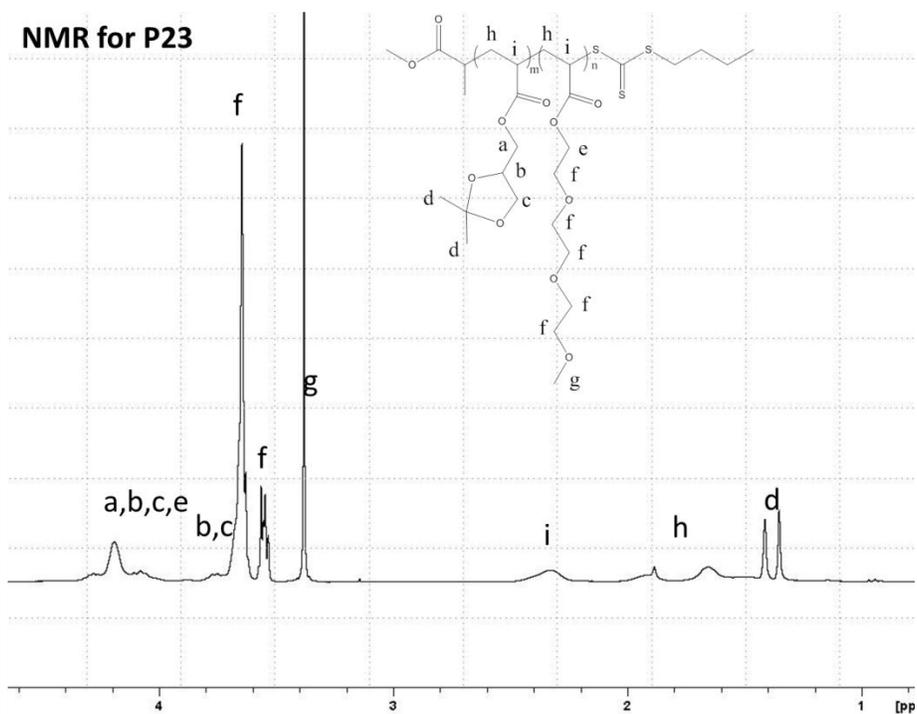


Fig. S6 ¹H NMR (top, in CDCl₃) and THF SEC trace (bottom) of P50



SEC trace of P23

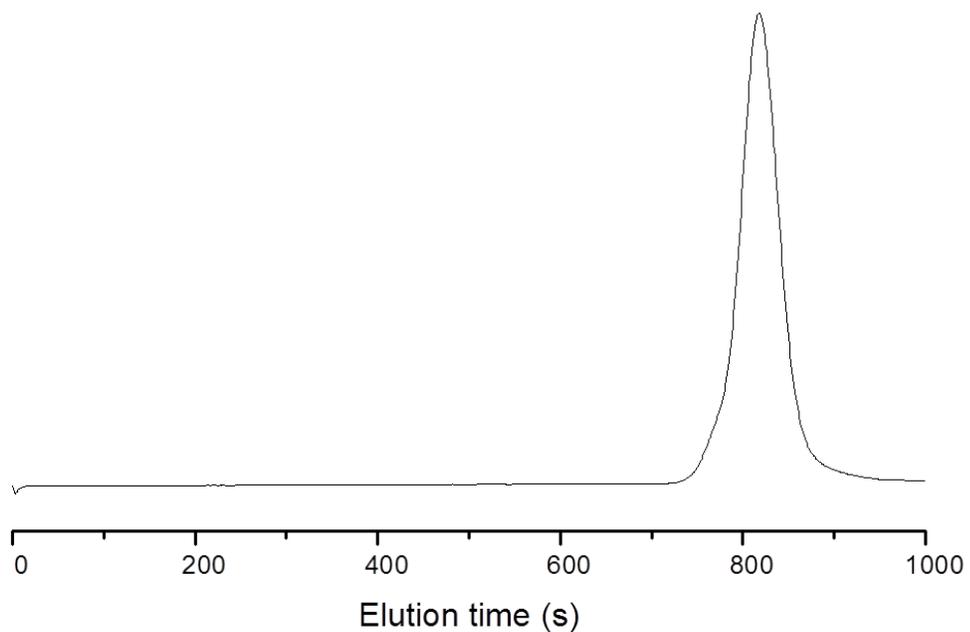
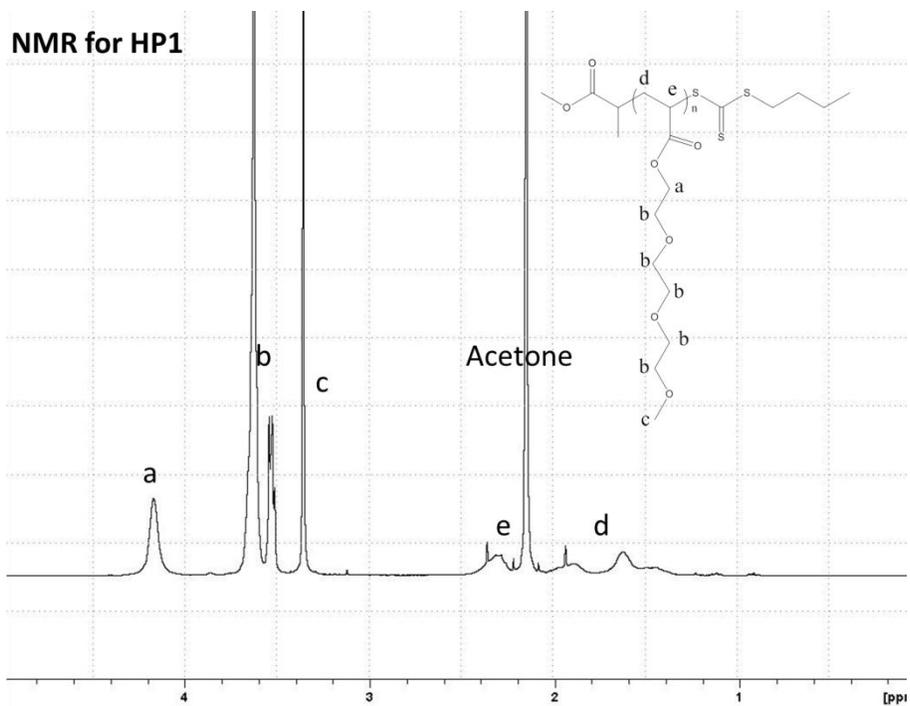


Fig. S7 ¹H NMR (top, in CDCl₃) and THF SEC trace (bottom) of P23



SEC trace of PolymTEGA

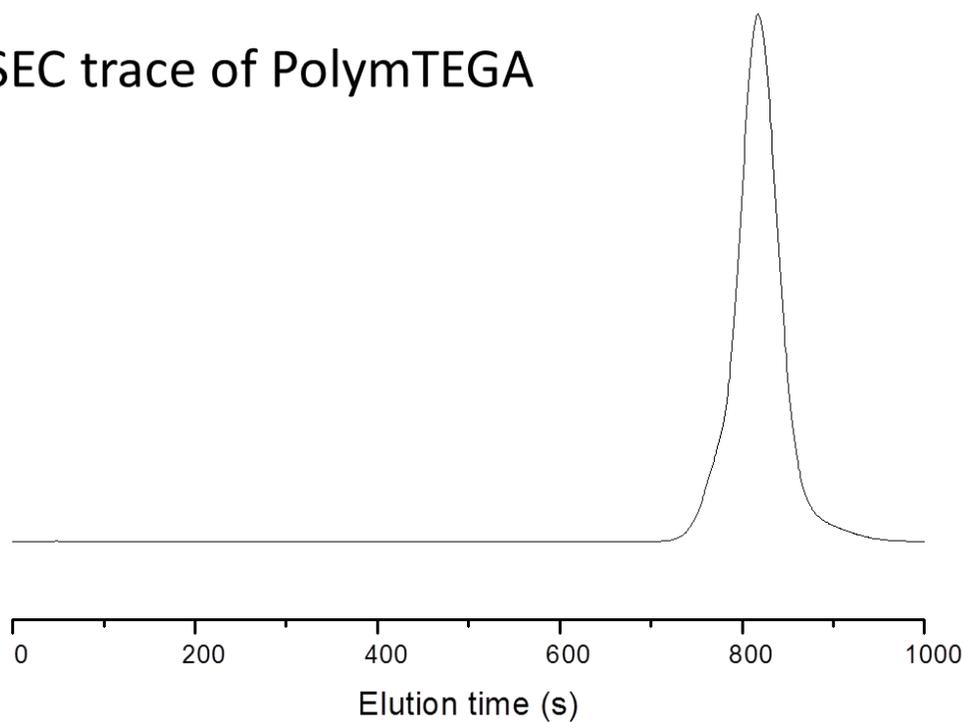


Fig. S8 ^1H NMR (top, in CDCl_3) and THF SEC trace (bottom) of HP1

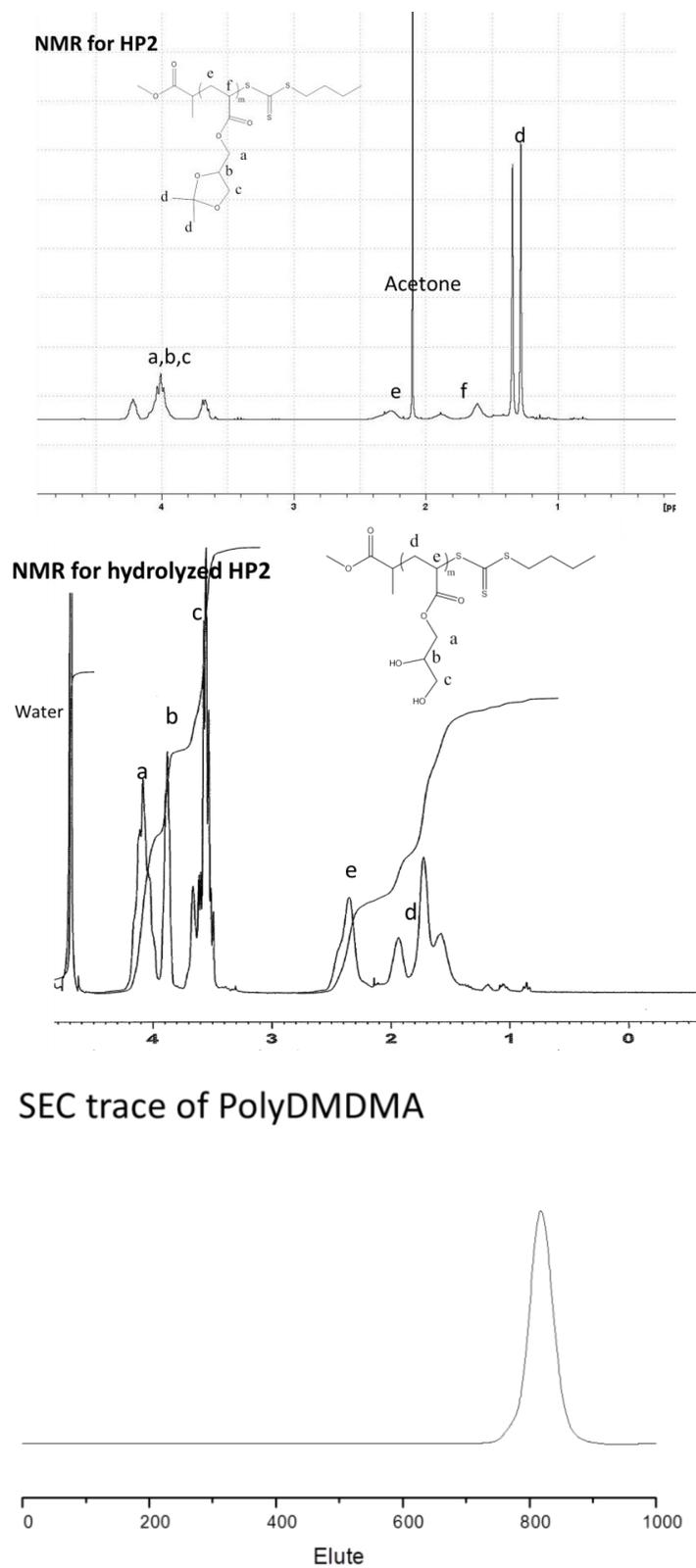


Fig. S9 Top: ^1H NMR of HP2 in CDCl_3 ; Middle: ^1H NMR of HP2 after hydrolysis in D_2O Bottom: THF SEC trace of HP2.

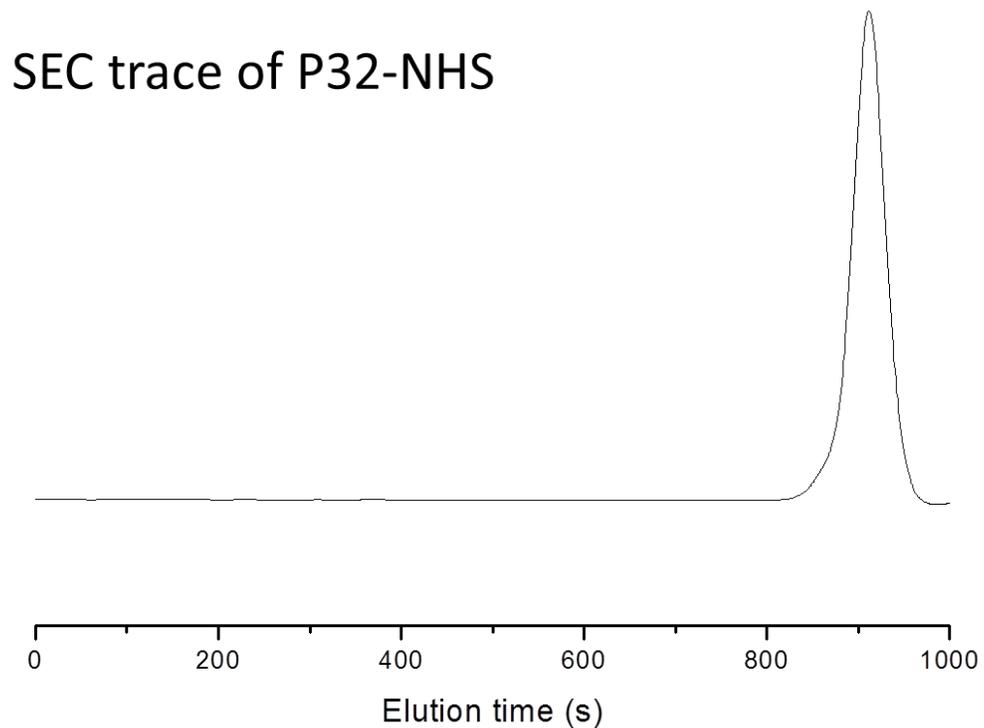
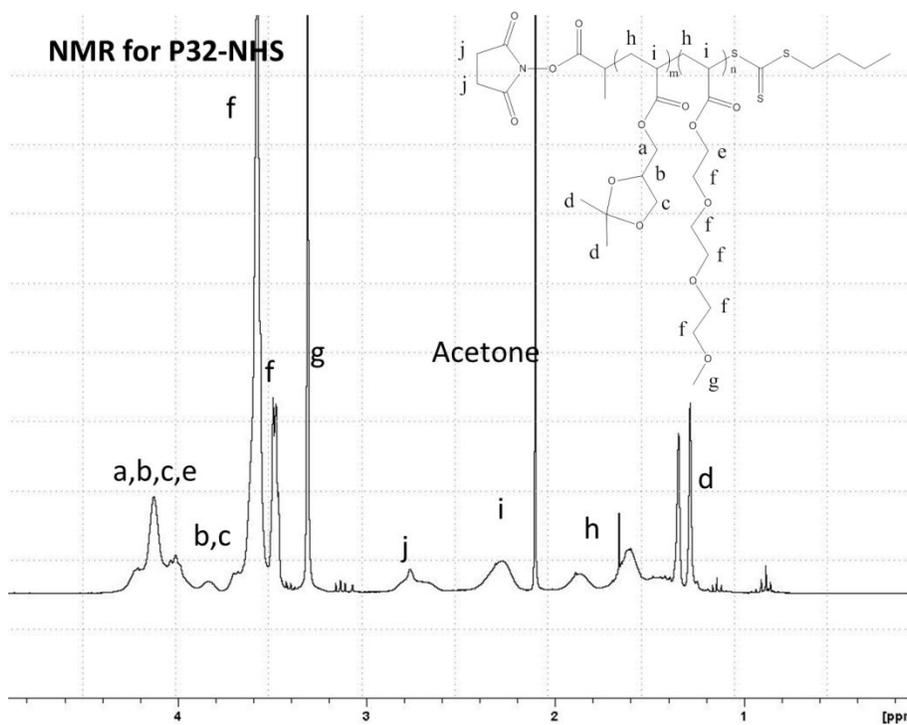
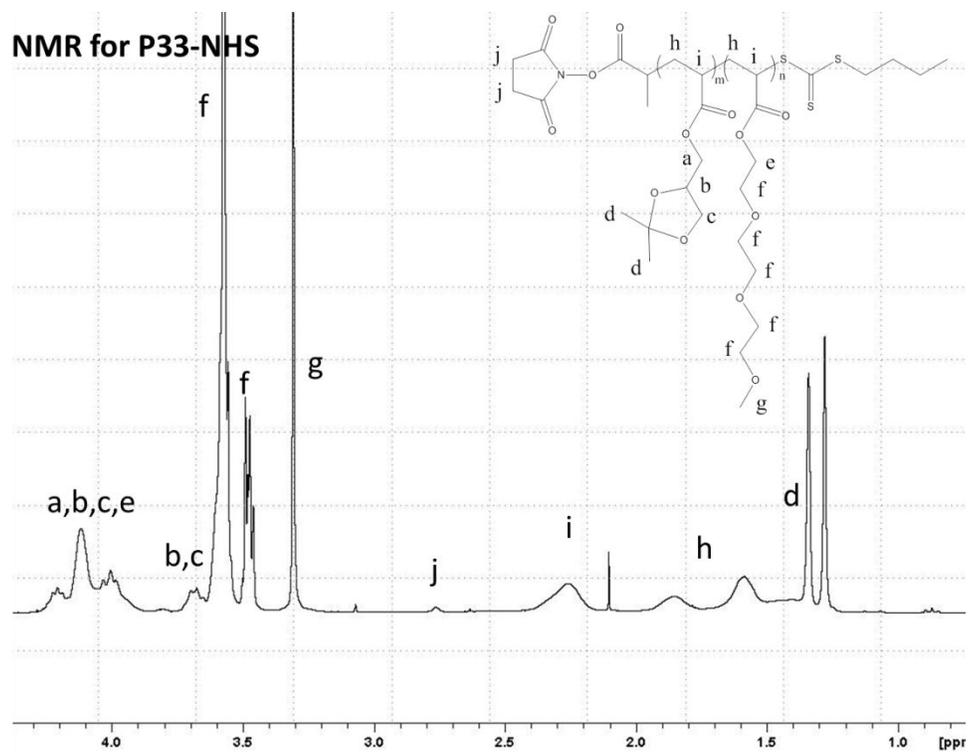


Fig. S10 ^1H NMR (top, in CDCl_3) and THF SEC trace (bottom) of P32-NHS



SEC trace of P33-NHS

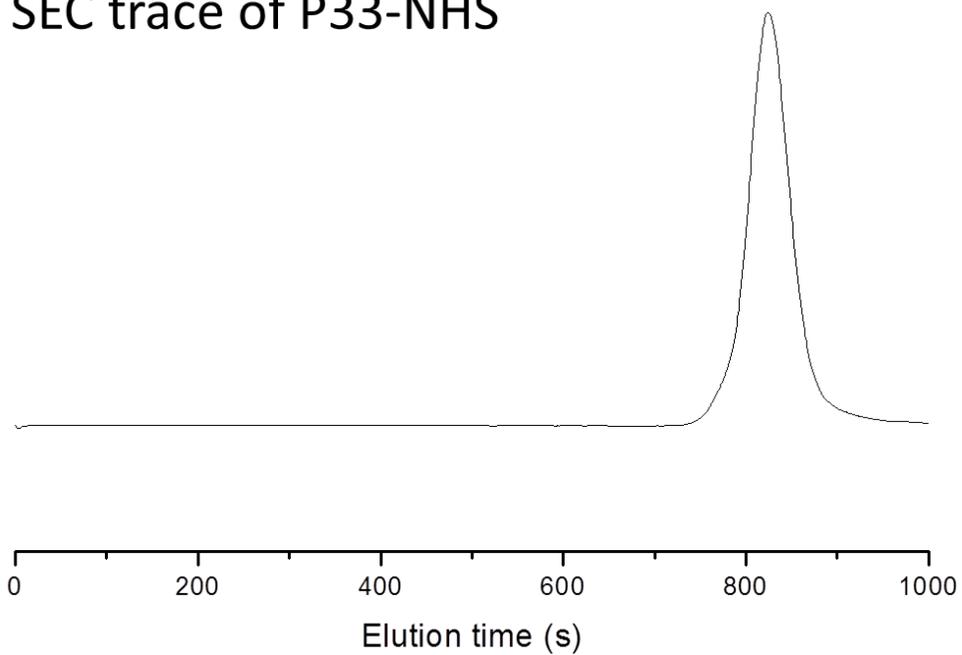
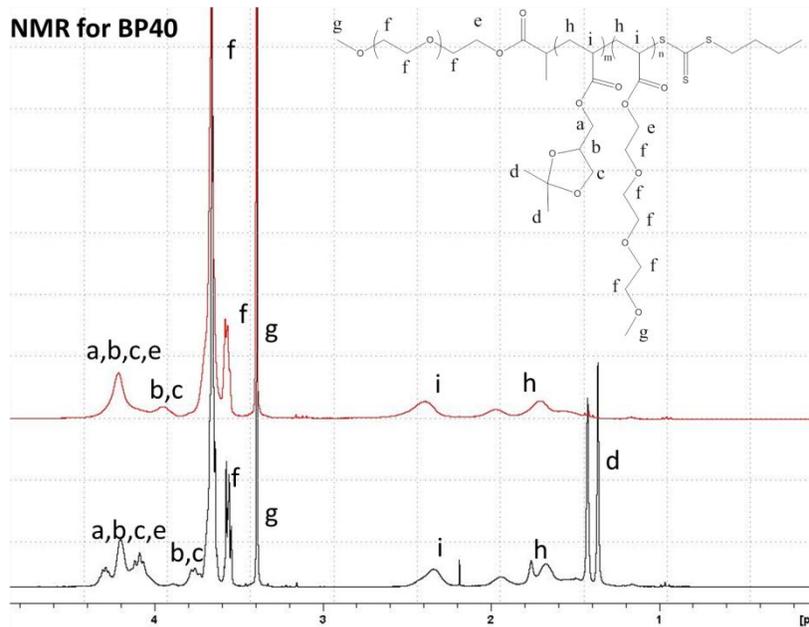


Fig. S11 ¹H NMR (top, in CDCl₃) and THF SEC trace (bottom) of P33-NHS



SEC trace of BP40

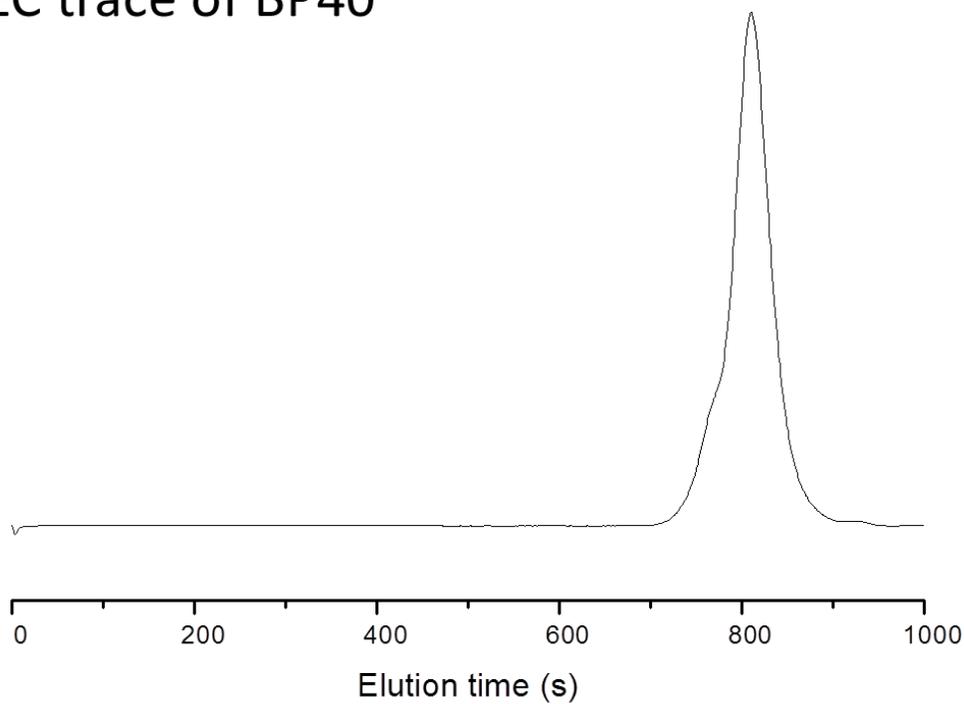
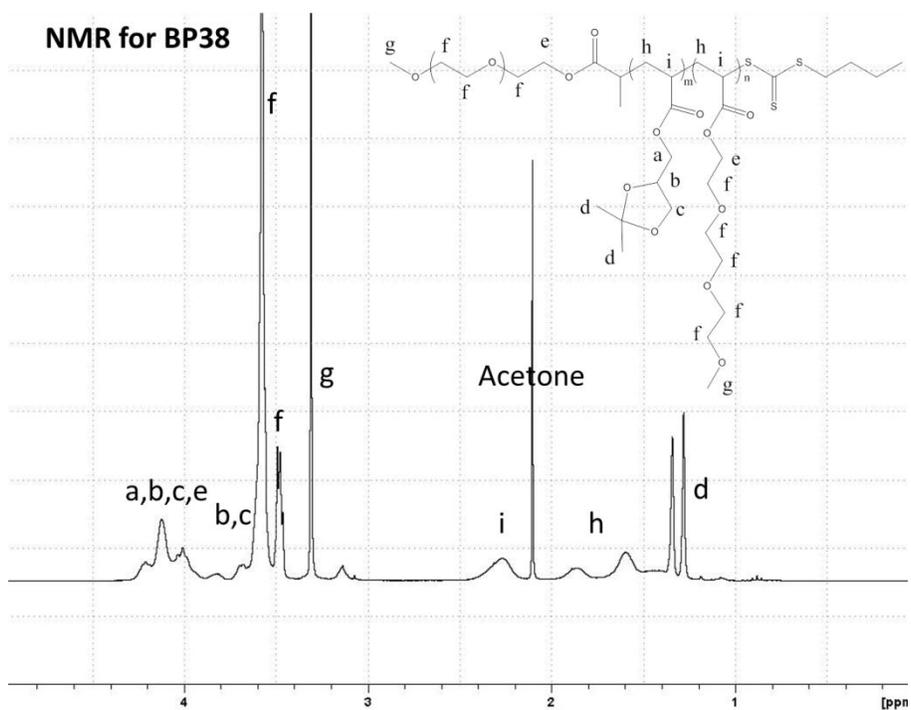


Fig. S12 ^1H NMR (top, in CDCl_3) and THF SEC trace (bottom) of BP40



SEC trace of BP38

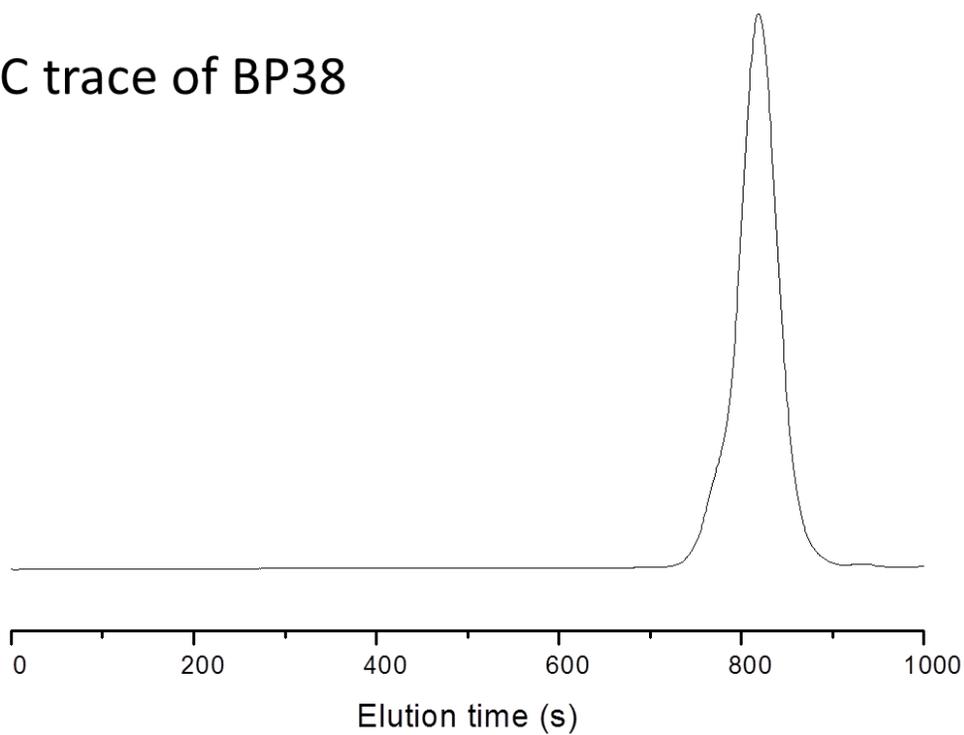


Fig. S13 ^1H NMR (top, in CDCl_3) and THF SEC trace (bottom) of BP38

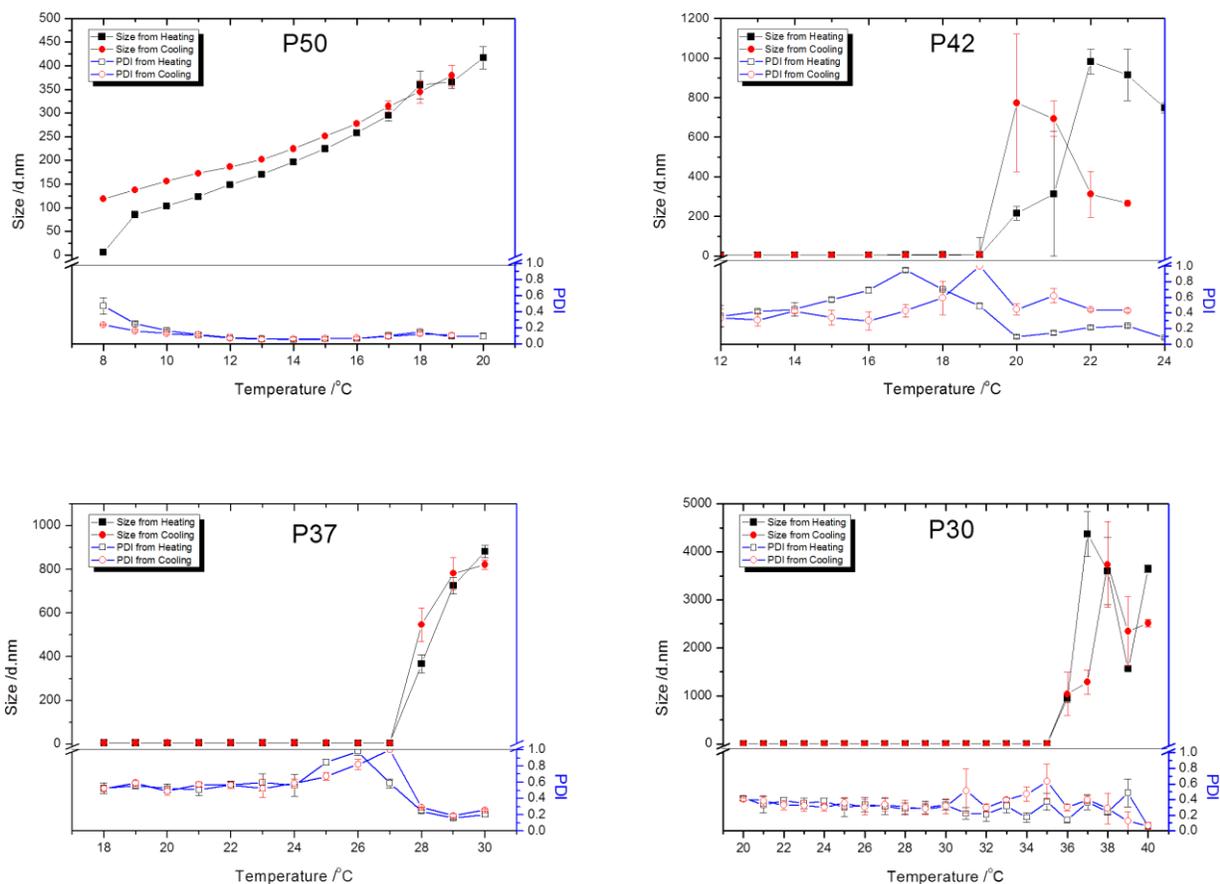


Fig. S14 Hydrodynamic diameter of P50, P42, P37 and P30 versus temperature, measured by dynamic light scattering. The concentrations of polymers were 1 mg/ml. Error bars correspond to standard deviations based on 5 measurements.

The T_{CP} s measured here were found to be 2-3 °C higher than measured by UV-vis, which can be ascribed to the lower concentration. P50 was found to aggregate into rather small and defined aggregates at 9 °C and this initial aggregates grow in size upon further heating. As such the T_{CP} determined by turbidimetry (Fig. S1b) is higher as this technique only detects aggregates big enough to scatter 600 nm light. This different behavior of P50 compared to the other copolymers, that agglomerate into micrometer-sized agglomerates at the transition temperature, can be ascribed to the higher hydrophobic DMDMA content leading to the formation of a hydrophobic core that is stabilized by a mTEGA corona. In other words, P50 acts more like an amphiphilic polymer while the other polymers with less DMDMA behave like statistical random copolymers that full collapse upon heating.

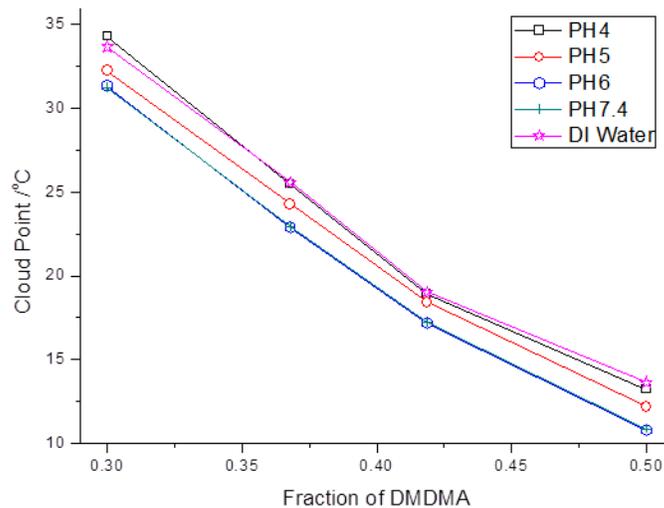


Fig. S15 Dependence of cloud point on the percentage of DMDMA in different pH buffer and Milli Q water (5mg/ml)

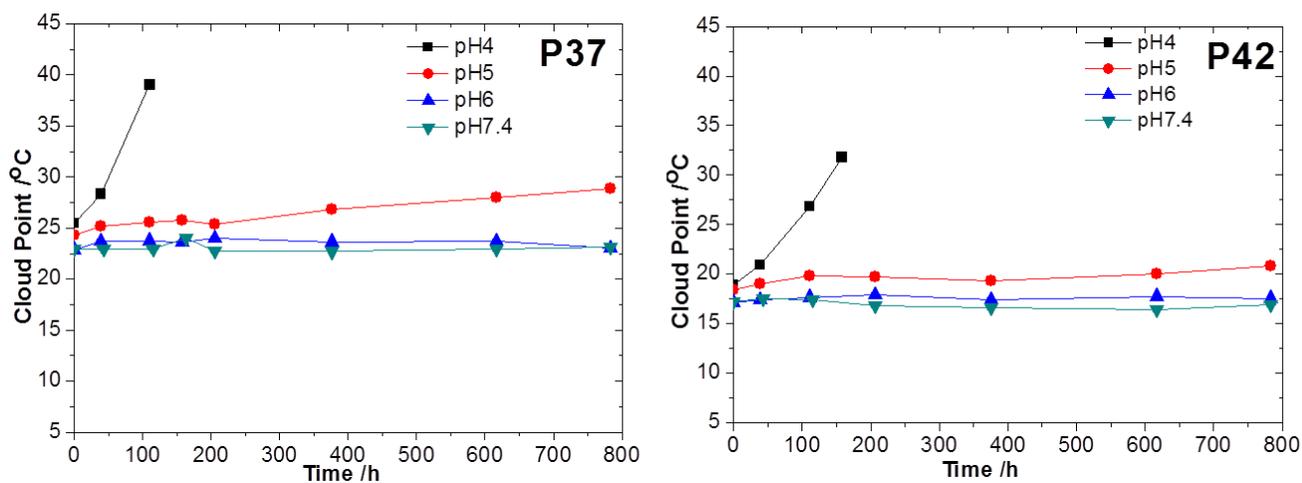


Fig. S16 Plot of T_{CP} versus hydrolysis time for copolymers with different mTEGMA:DMDMA in a long time period.

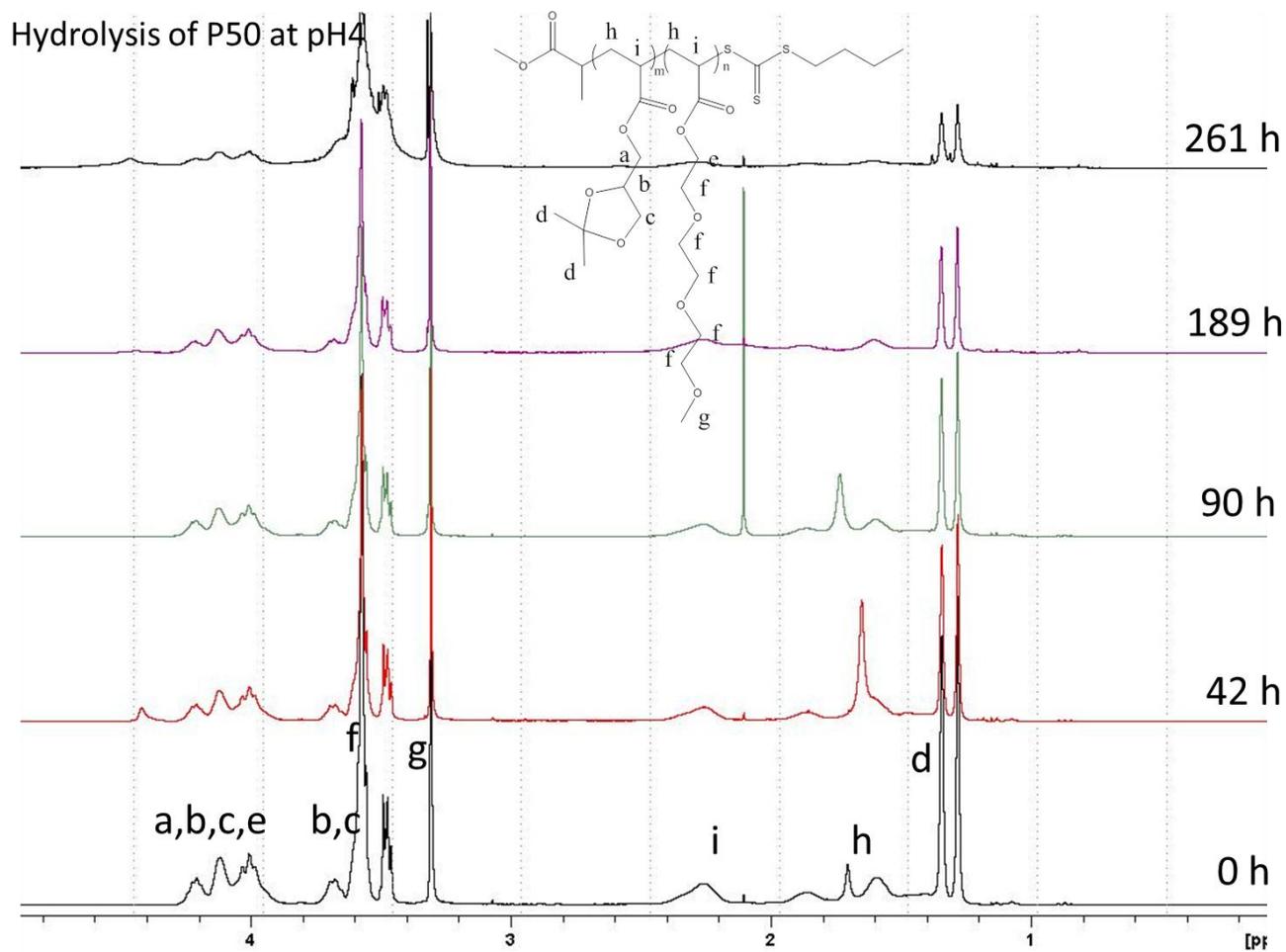


Fig. S17 ^1H NMR spectra showing the hydrolysis of P50 at pH4

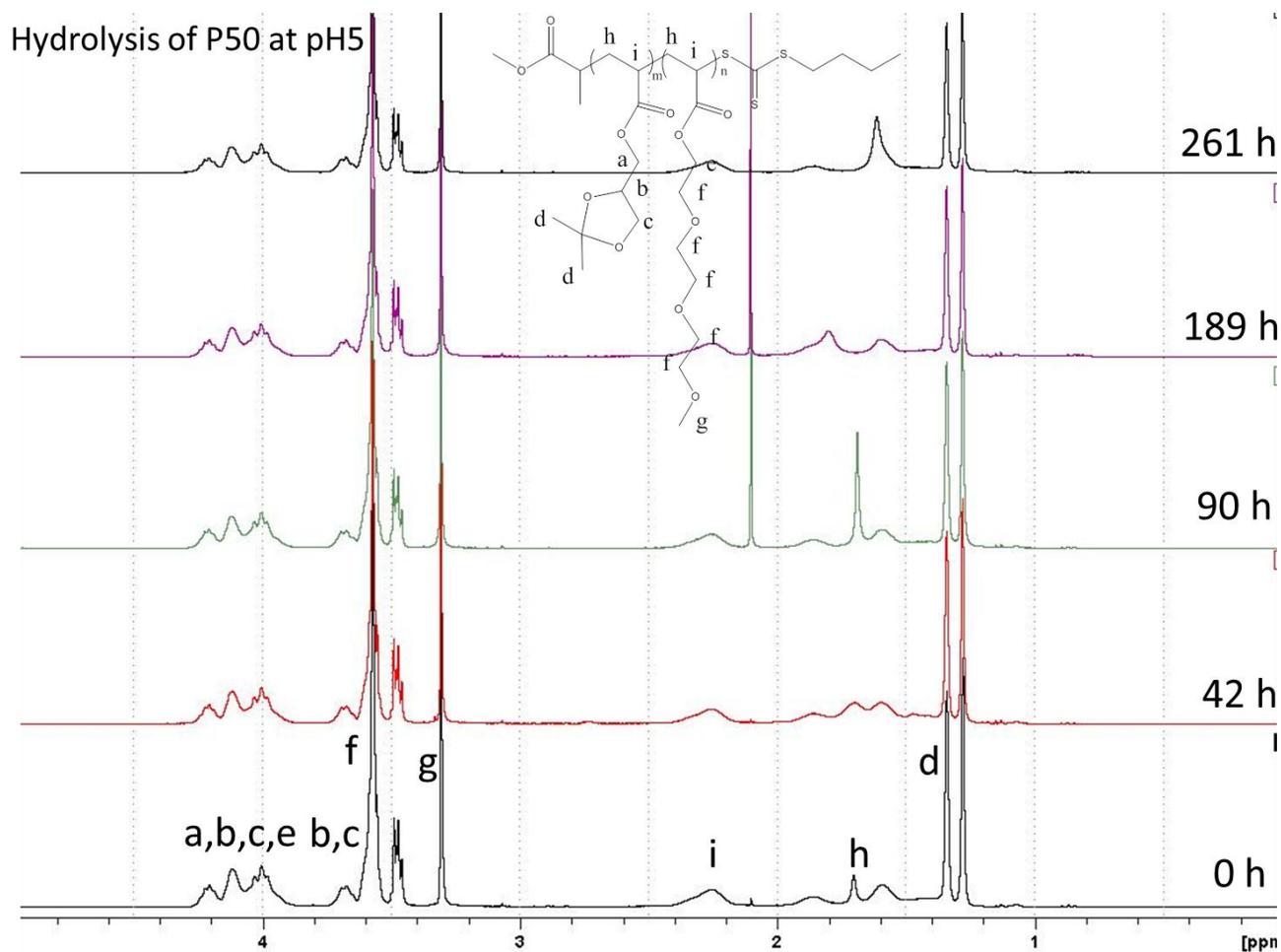


Fig. S18 ^1H NMR spectra showing the hydrolysis of P50 at pH5

Hydrolysis of P30 at pH4

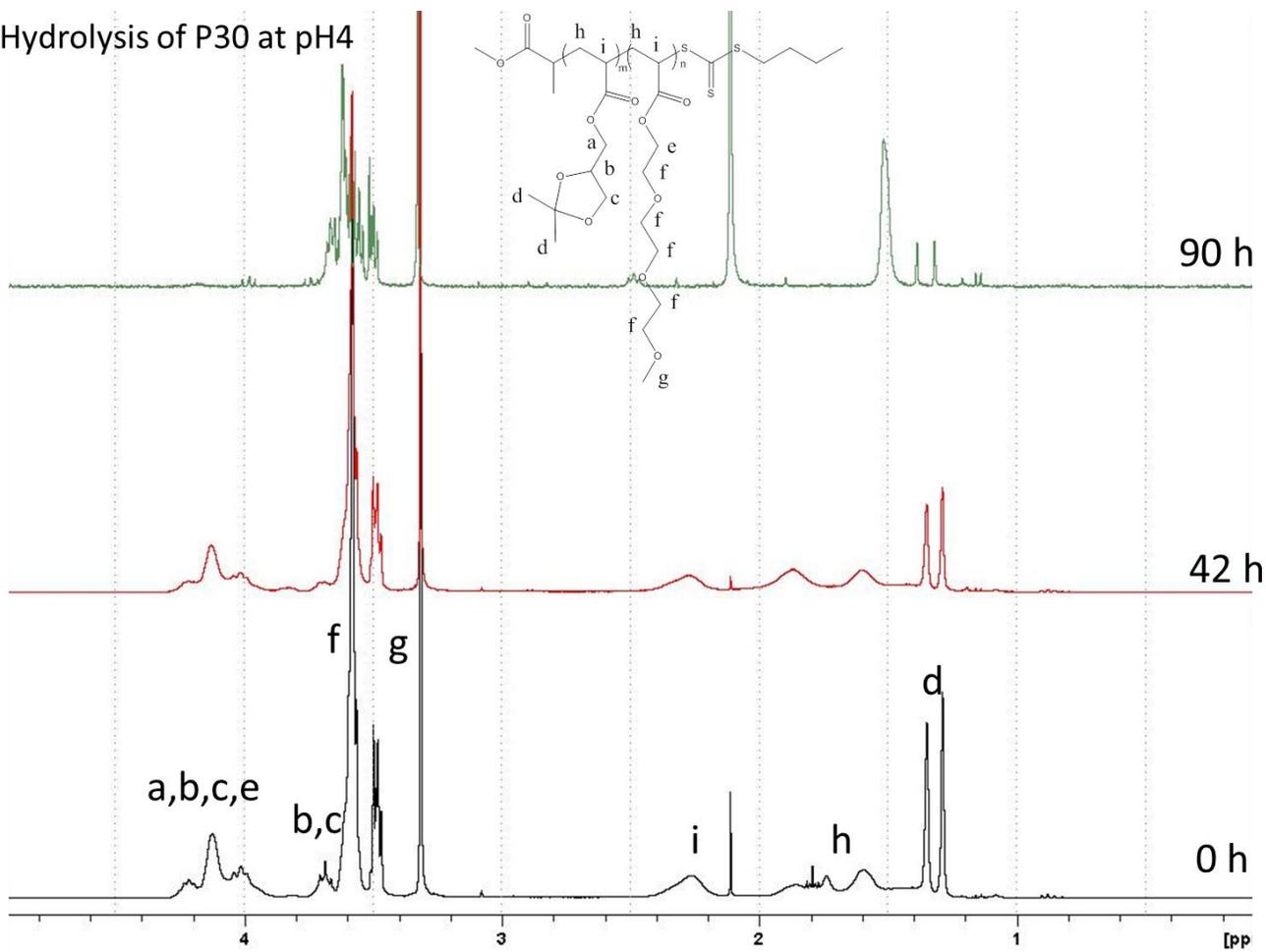


Fig. S19 ¹H NMR spectra showing the hydrolysis of P30 at pH4

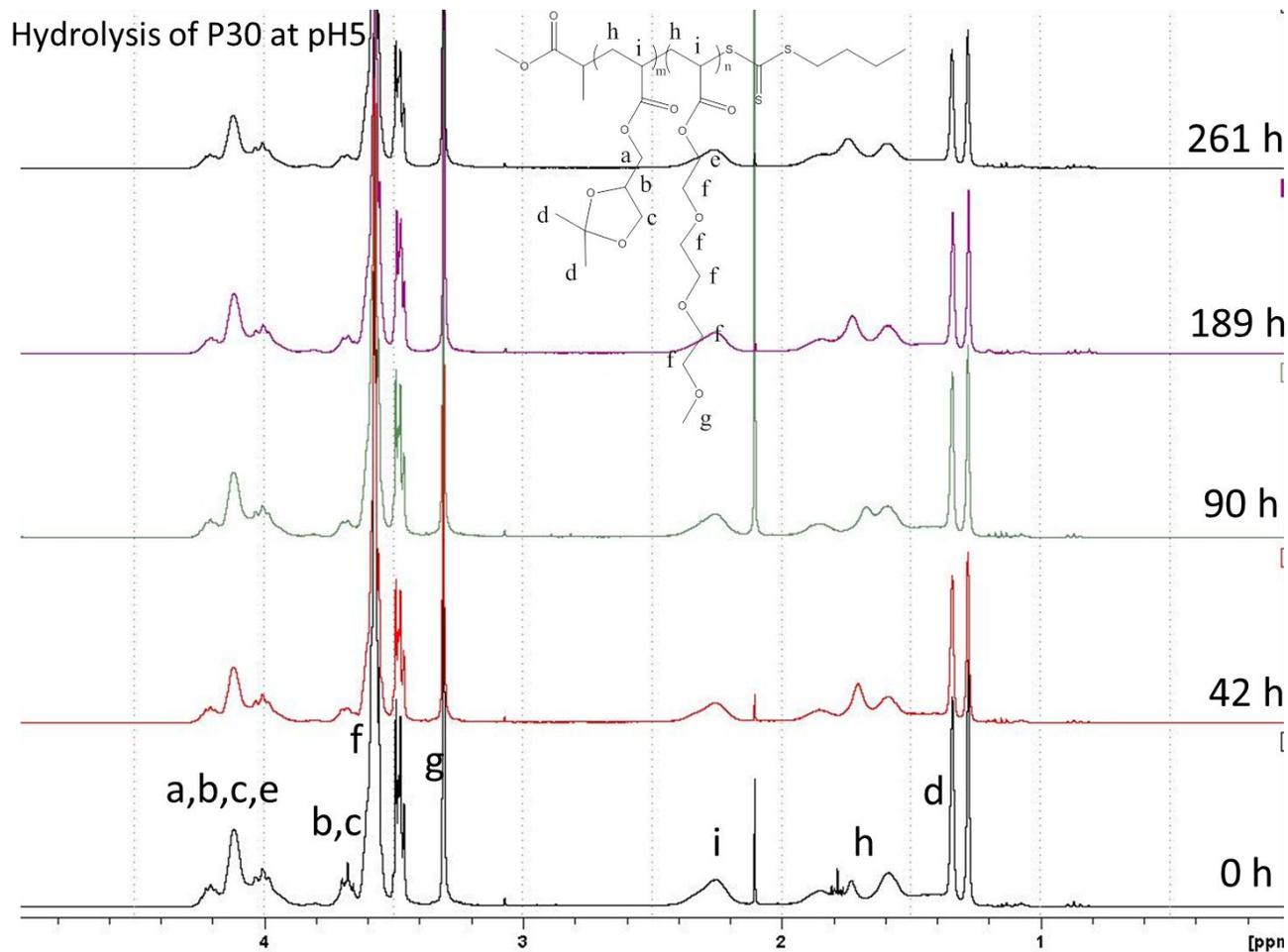


Fig. S20 ^1H NMR spectra showing the hydrolysis of P30 at pH5

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- (2) Zhang, D.; Zhang, H.; Nie, J. Yang, J. *Polym. Int.* **2010**, *59*, 967.
- (3) Ryu, J.-H.; Roy, R.; Ventura, J. Thayumanavan, S. *Langmuir* **2010**, *26*, 7086.
- (4) Gody, G.; Rossner, C.; Moraes, J.; Vana, P.; Maschmeyer, T. Perrier, S. *J. Am. Chem. Soc.* **2012**, *134*, 12596.