Electronic supplementary information

Influence of charged groups on the cross-linking efficiency and release of guest molecules from thiol-ene cross-linked poly(2-oxazoline) hydrogels

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Methods:

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Fourier 300 with 300 MHz. Deuterated solvents DMSO-d_6 and CD_3CN, with the solvent peak as internal reference, were used.

Raman spectroscopy was measured on a RAMAN spectrometer from Thermo Scientific equipped with a DXR 780 nm laser and DXR Raman microscope.

Microwave synthesis was performed in a microwave synthesizer Discover SP from CEM GmbH (Kamp Lintfort, Germany).

UV-LED cubes purchased from Polymerschmiede (Aachen, Germany), with a wavelength of 365 nm and a power of 11 W each, were used to carry out UV mediated reactions.

Cloud point measurements and Zeta potential measurements were performed on a Zetasizer Nano-ZSP from Malvern (Herrenberg, Germany) equipped with a 633 nm HeNe laser and the measurement angles 13° and 173°. For cloud point measurement, the polymer solution was heated in a glass cuvette in 0.5 °C steps with an equilibration time of 3 min at each time point. At each point, 3 measurements of 3 times 10 s each were performed. The cloud point was reached when the count rate increased drastically, usually 10'000 kcps. The cloud point measurement was stopped at 85.5 °C as the instrument itself is limited to 90 °C and the boiling point of water was approached. For zeta potential measurements, a DTS2070 cuvette was filled with a 5 mg/mL solution of cryo-milled, freeze-dried and redispersed hydrogel and measured four times. Each measurement consisted of three measurements with a maximum of 100 runs.

Size exclusion chromatography (SEC) was measured on an OMNISEC RESOLVE combined with an OMNISEC REVEAL from Malvern Panalytical. The system is equipped with an autosampler, pump, degasser and column oven at 45 °C. A refractive index detector, viscosity detector, right angle light scattering detector and low angle light scattering detector at 45 °C were used for calibration with narrow poly(methyl methacrylate) (PMMA) standards. A pre-column (Dguard), a D2000 and a D3000 column from Malvern were used in series. The flow was 1 ml/min.

Ellman assay was performed using Ellman's reagent 5,5'-Dithiobis(2-nitrobenzoic acid) for the quantification of thiols. A dilute series of mercaptoethanol was used for calibration. The

assay was performed as follows. First, the thiol containing compound (polymer or mercaptoethanol) was dissolved in 0.8 M phosphate buffer (NaH_2PO_4 and Na_2HPO_4), then a 1 M $NaBH_4$ (in 0.01 M NaOH) was added. After 1 h of incubation at rt, 1 M HCl was added during which a strong bubble formation was observed. The solution was incubated for 30 min followed by the addition of 0.8 M phosphate buffer and Ellman's reagent. The samples were then analyzed via UV absorption at 412 nm.

UV absorption and fluorescence was measured with a Tecan Spark® 20 M multimode microplate reader from Tecan Group Ltd. (Männedorf, Swiss) with Nunclon 96 wellplates (Thermo Fisher Scientific). The measurement for the Ellman assay was performed at a wavelength of 412 nm and for the release study of methylene blue at 660 nm. The fluorescence intensity of fluorescein was measured in flat black 96 well plates (Greiner) at an excitation wavelength of 460 nm and an emission wavelength of 515 nm.

Stereomicroscopy pictures were taken on Carl Zeiss Discovery V.20 (Oberkochen, Germany), equipped with a 5 MP, 12 bit, color Camera (Zeiss icc5) and lenses (0.63x and 1.5x Plan Apo).

Materials:

Acetonitrile (anhydrous, 99.8%, Sigma-Aldrich), CaH_2 (92 % abcr GmbH), Chloroethylamin HCl (99.8 %, Alfa Aesar), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid-hydrochlorid (CarboSynth),5,5'-Dithiobis(2-nitrobenzoic acid) (99%, Sigma-Aldrich), N-Hydroxysuccinimide (98 %, Sigma-Aldrich), Pentenoic acid (≥98 %, Sigma-Aldrich), Dichloromethane (reagent grade, VWR International), Sodium hydroxide (ACS, Reag. Ph Eur, Merck), Potassium hydroxide (85 %, Fisher Scientific), 2-Mercaptoethanol (≥ 99.0%, Sigma-Aldrich), Methanol (99.8 %, anhydrous, Sigma-Aldrich), 2-Ethyl-2-oxazoline (≥99 %, Aldrich), 2-Methyl-2-oxazoline (≥99 %, Aldrich), Methyl tosylate (98 %, Sigma-Aldrich), Piperidine (≥ 99.5 %, purified by redistillation, Aldrich), Hydrogen chloride (32 %, Merck), Methanol (reagent, grade, Fisher Scientific), 2,2-Dimethoxy-2-phenylacetophenone (99 %, Aldrich), 2-Hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone (Irgacure 2959, Sigma-Aldrich), L-Cysteine hydrochloride monohydrate (≥ 98%, Sigma-Aldrich), Fluorescein sodium salt (extra pure, Merck, Darmstadt), Methylene blue B (for microscopy, Merck, Darmstadt), Acetone (≥ 99.5 %, Sigma-Aldrich), Sodium formiate (≥ 99%, Sigma), Formic acid (98/100 %, Bernd Kraft), Acetic acid anhydride (98 %, VWR ProLABO), 1,1'-Carbonyldiimidazole (CDI) (reagent grade, Aldrich), Cysteamine*HCl (BioChemica, AppliChem), Pyridine (> 99%, Merck), Chloroform

(reagent, grade, Fisher Scientifc), Ethyl acetate (reagent grade, Fisher Scientific), Magnesium sulfate (≥ 99.5 %, Sigma-Aldrich), Sodium borhydride (99 %, Acros Organics), Thioacetic acid (96 %, Aldrich), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (≥ 98 %, Carl Roth), Dimethylformamide (99.8 %, anhydrous, Sigma-Aldrich), Fluorescein isothiocyanate-dextran (average mol wt 4000, 40000 and 500000) (Sigma-Aldrich)

Synthesis

Cysteine Linker

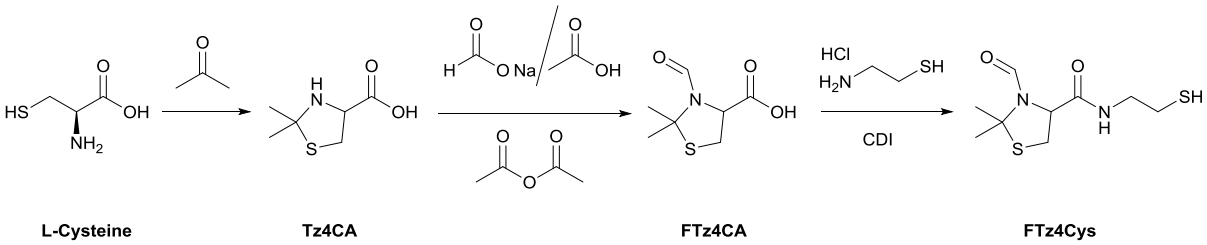


Fig. S1 Synthesis of Cysteine Linker.

The synthesis of 3-formyl- *N* -(2-mercaptoethyl)-2,2-dimethylthiazolidine-4-carboxamide (FTz4Cys) is a three step synthesis starting with 2,2-Dimethylthiazolidine-4-carboxylic acid (Tz4CA) which was synthesized according to Woodward *et al*.[1] The second step involving the formylation of the amine leading to 2,2-dimethylthiazolidin-3-(*N*-formyl)-4-carboxylic acid (FTz4CA)[2] and the following final step was performed according to Kuhlmann *et al.*[3] using chloroform instead of dimethylformamide.[4]

^{1}H NMR of FTz4CA (300 MHz, DMSO-d_6, ppm): δ = 12.95 (br-s, 1H, H-**a**), 8.40-8.23 (2s, 1H, H-**c**), 5.07-4.81 (dd/dq, 1H, H-**b**), 3.46-3.16 (m, 2H, H-**e**), 1.76 (s, 6H, H-**d**).

^{1}H NMR of FTz4Cys (300 MHz, $CDCl_3$, ppm): δ = 8.31 (s, 1H, H-**f**), 7.02 (br-s, 1H, H-**d**), 5.03/5.01 (m, 1H, H-**e**), 3.71-3.14 (m, 4H, H-**h**, **e**, **h'**), 2.67 (m, 2H, H-**b** 1.84-1.79 (2xs, 6H, H-**g**), 1.35 (s, 1H, H-**a**).

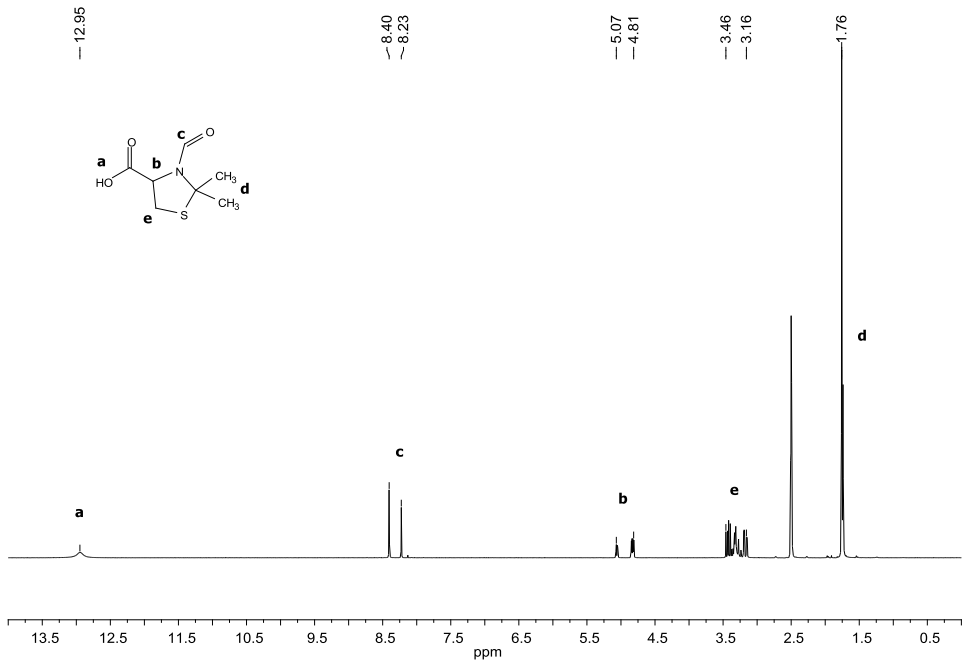


Fig. S2 ^{1}H NMR of Tz4CA in DMSO-d_6

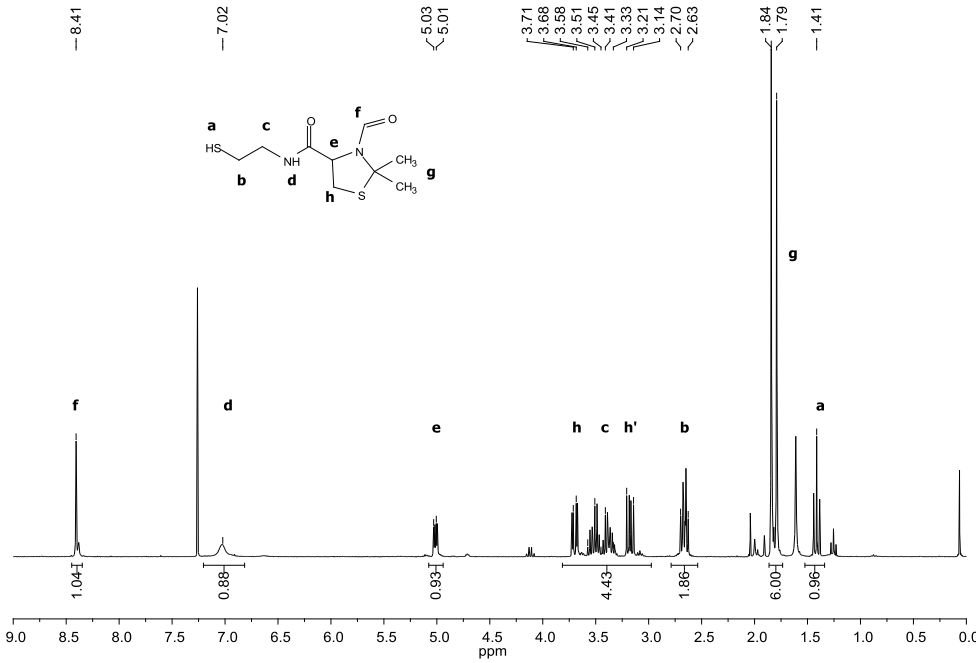


Fig. S3 ^{1}H NMR of FTz4Cys in $CDCl_3$.

Synthesis of 2-(3-Butenyl)-2-oxazoline

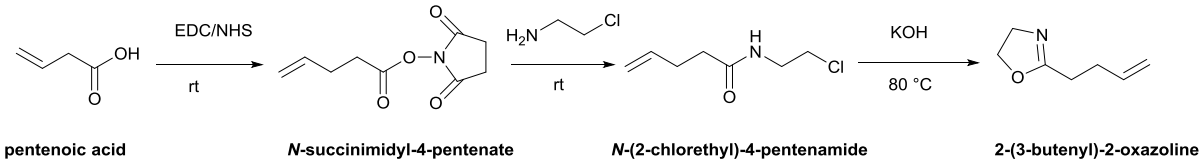


Fig. S4 Synthesis of 2-(3-Butenyl)-2-oxazoline (ButEnOx).

The three step synthesis of 2-(3-Butenyl)-2-oxazoline (ButEnOx) was performed according to Gress *et al.*[5] A slight adjustment was made as follows. After ring closure in methanol at 80 °C in the final synthesis step, the solvent methanol was evaporated. The monomer was redissolved in dichloromethane and washed 5 times with dist. water. The solution was then dried over $MgSO_4$ and the solvent was evaporated. After this cleaning step, the monomer was fractionally distilled at $3 \cdot 10^{-3}$ mbar at 45 °C.

^{1}H NMR of ButEnOx (300 MHz, $CDCl_3$, ppm): δ = 5.83 (m, 1H, H-**b**), 5.03 (m, 2H, H-**a**), 4.21 (t, 2H, H-**e**), 3.81 (t, 2H, H-**f**), 2.36 (s, 4H, H-**c, d**).

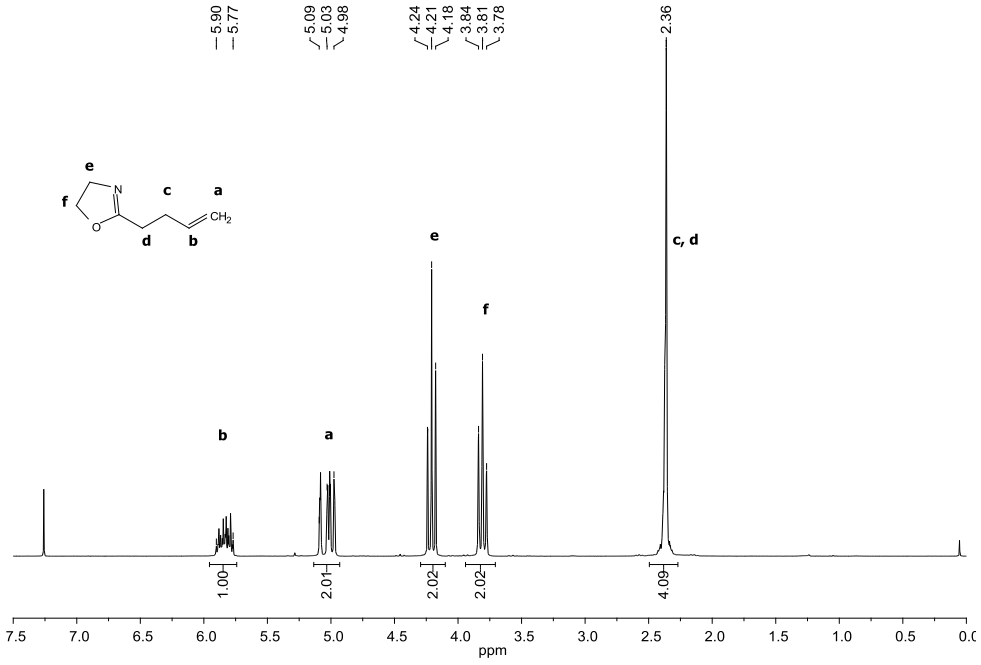


Fig. S5 ^{1}H NMR of ButEnOx in $CDCl_3$.

Copolymer synthesis

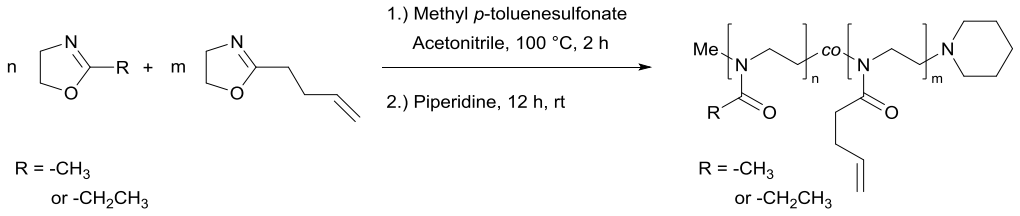


Fig. S6 Synthesis of P(MeOx-co-ButEnOx) or P(EtOx-co-ButEnOx).

The commercially available monomers 2-Methyl-2-oxazoline (MeOx) and 2-Ethyl-2-oxazoline (EtOx) as well as the synthesized ButEnOx were dried and distilled over CaH_2 prior to polymerization.

The random copolymers were synthesized by microwave heating known from literature.[5-7] In short, the initiator methyl *p*-toluenesulfonate was weighed in a microwave vial under dry and inert conditions in the glovebox. The appropriate molar amount of either MeOx or EtOx and ButEnOx was added and diluted with acetonitrile (concentration of monomers = 4 M). The solution was placed in the microwave synthesizer at 100 °C for 2 h and afterwards quenched with 3 eq. of piperidine at room temperature for 12 h. A small volume of chloroform, or chloroform/methanol (1:1 v/v) for polymers containing MeOx, was added and the polymer was precipitated in ice-cold diethyl ether. The solvent was evaporated and a white powder was received.

^{1}H NMR of P(MeOx-*co*-ButEnOx) (300 MHz, CD_3CN, ppm): δ = 5.87 (m, 1H, H-**g**), 5.01 (m, 2H, H-**h**), 3.39 (m, 2H, H-**b**, H-**c**; 2H, H-**b'**, H-**c'**), 2.99 (d, 2H, H-**a**), 2.85 (s, 1H, H-**a**), 2.42 (m, 2H, H-**e**), 2.30 (m, 2H, H-**f**), 2.01 (m, 3H, H-**d**).

^{1}H NMR of P(EtOx-*co*-ButEnOx) (300 MHz, CD_3CN, ppm): δ = 5.87 (m, 1H, H-**i**), 5.01 (m, 2H, H-**h**), 3.40 (m, 2H, H-**b**, H-**c**; 2H, H-**b'**, H-**c'**), 2.96 (d, 2H, H-**a**), 2.86 (s, 1H, H-**a**), 2.34 (m, 6H, H-**d, f, g**), 1.03 (m, 3H, H-**e**).

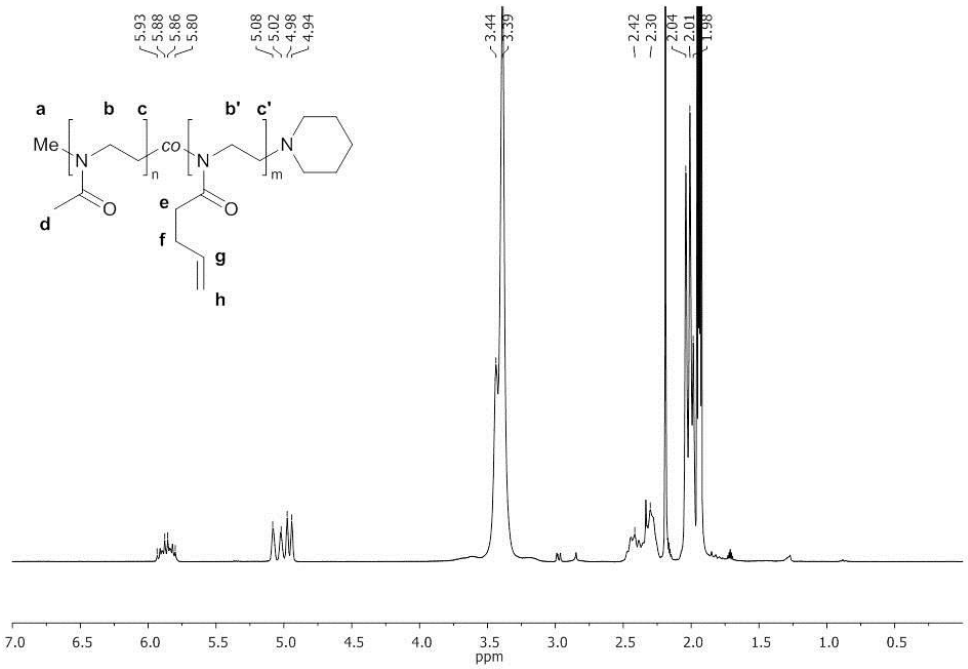


Fig. S7 ^{1}H NMR of P(MeOx-*co*-ButEnOx) in CD_3CN.

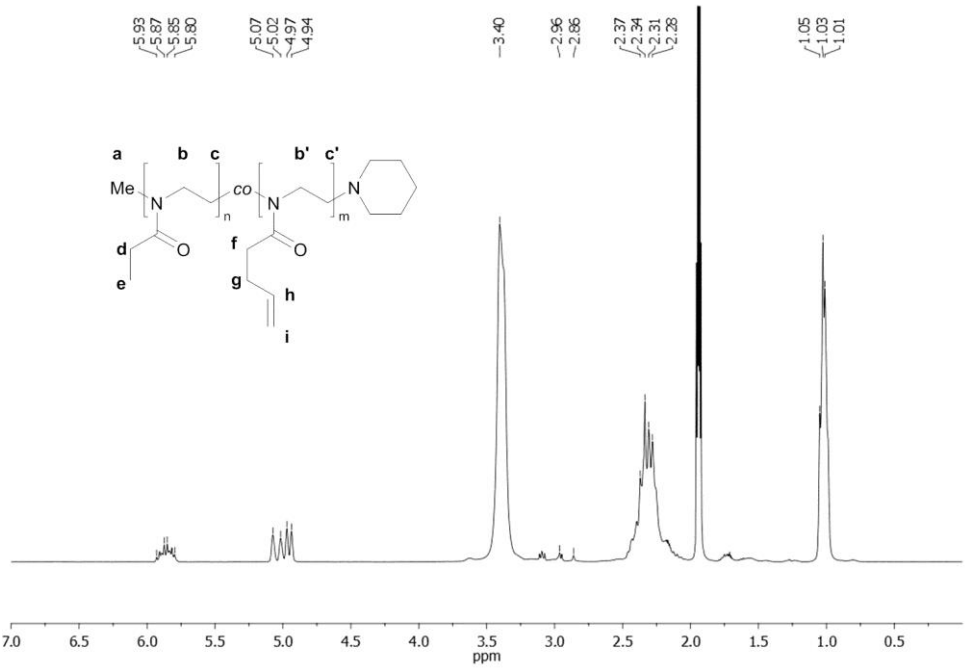


Fig. S8 ^{1}H NMR of P(EtOx-*co*-ButEnOx) in CD_3CN.

Tab. S1 Overview of all P(MeOx-*co*-ButEnOx) copolymers

Polymer	Repeating units°		Repeating units*		mol % vinyl	M_n (g/mol)*	M_n (g/mol)#	Đ#
	MeOx	ButEnOx	MeOx	ButEnOx				
PMeOx-*co*-En10.1	50	5	51	5	8.9	5065	8855	1.09
PMeOx-*co*-En10.2	45	5	39	5	11.4	4044	6760	1.23
PMeOx-*co*-En20.1	40	10	40	9	18.4	4629	6821	1.29
PMeOx-*co*-En20.2	40	10	42	12	22.2	5176	7370	1.21
PMeOx-*co*-En30	35	15	31	14	31.1	4890	6974	1.18

°theoretical *determined by ^{1}H NMR in CD_3CN, #determined by GPC in DMF

Tab. S2 Overview of all P(EtOx-*co*-ButEnOx) copolymers

Polymer	Repeating units°		Repeating units*		mol % vinyl	M_n (g/mol)*	M_n (g/mol)#	Đ#
	EtOx	ButEnOx	EtOx	ButEnOx				
PEtOx-*co*-En10	50	5	54.5	5.5	9.2	6190	6907	1.09
PEtOx-*co*-En20	40	10	42	11	20.8	5639	7557	1.11

°theoretical *determined by ^{1}H NMR in CD_3CN, #determined by GPC in DMF

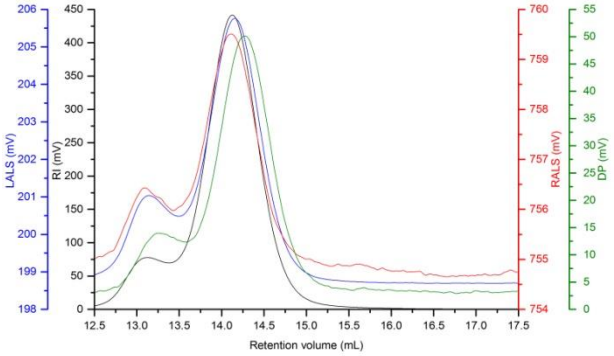


Fig. S9 GPC trace of PMeOx-*co*-En10.1 in DMF.

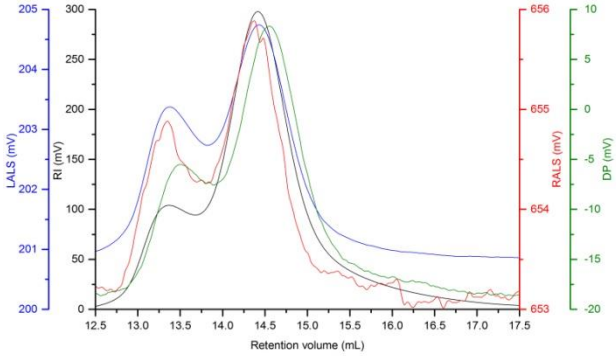


Fig. S10 GPC trace of PMeOx-*co*-En10.2 in DMF.

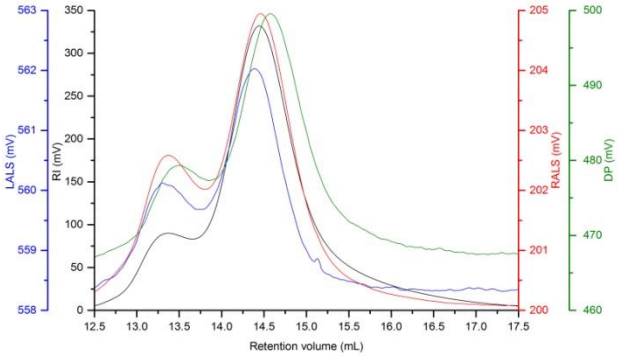


Fig. S11 GPC trace of PMeOx-*co*-En20.1 in DMF.

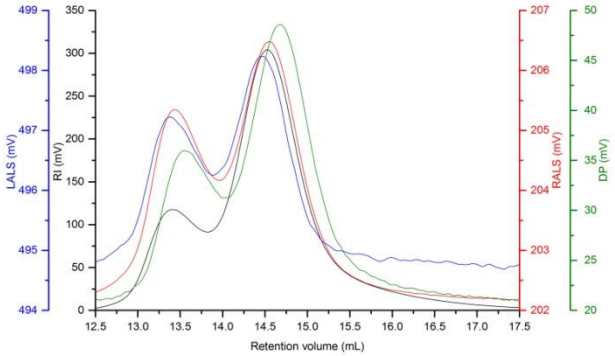


Fig. S12 GPC trace of PMeOx-*co*-En20.2 in DMF.

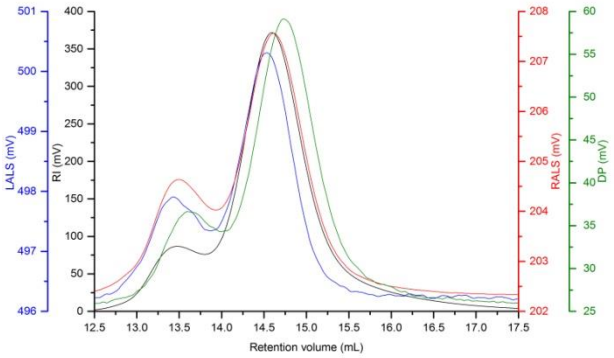


Fig. S13 GPC trace of PMeOx-*co*-En30 in DMF.

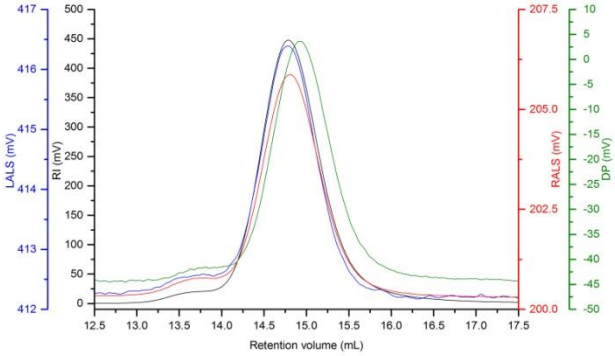


Fig. S14 GPC trace of PEtOx-*co*-En10 in DMF.

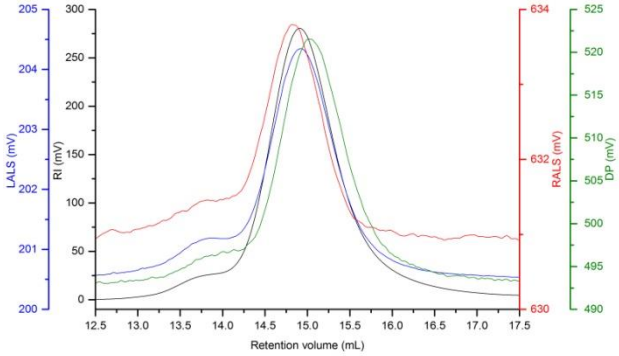


Fig. S15 GPC trace of PEtOx-*co*-En20 in DMF.

Thiol functionalization of POx copolymers

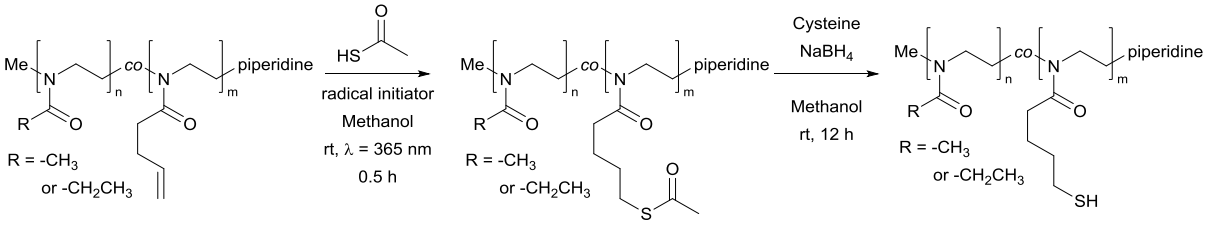


Fig. S16 Synthesis scheme of thiol functionalized POx.

The thiol functionalization is a two step synthesis starting by the addition of thioacetic acid to the butenyl side chain followed by the deprotection of the thioester.

The copolymer was dissolved in methanol and flushed with argon for 15 minutes. Afterwards, 3.5 eq. of thioacetic acid per vinyl functionality of the copolymer and 0.5 eq. of the UV-light initiator DMPA were added to the solution. The reaction mixture was stirred for 0.5 h under UV light (λ = 365 nm, UV LED-cubes, Polymerschmiede, Aachen). The solvent was evaporated under reduced pressure and the polymer was precipitated from chloroform for 3 times into ice-cold diethyl ether. After removal of residual solvent, the intermediate product was received as a white powder.

The deprotection of the thioester was accomplished by reacting 1.5 eq. of cysteine and 3 eq. of $NaBH_4$ per vinyl functionality in methanol under dry and oxygen-free conditions over night. The polymer and the cysteine were dissolved in methanol in separate flame-dried

Schlenk flasks. After argon had been bubbled through the solutions for 15 min, $NaBH_4$ was added to the cysteine solution under strong gas formation and a white precipitate formed. The polymer solution was added to the cysteine solution and stirred over night. A white precipitate formed which was filtered off and the solution was reduced to a small volume under reduced pressure. The polymer was then precipitated from this small volume in ice-cold diethyl ether. After removing all solvents under reduced pressure, the polymer was redissolved in water and 1 eq. of TCEP per vinyl unit was added and stirred for 2 h. The polymer was cleaned via dialysis (dialysis membrane Spectra/Por®, Carl Roth, MWCO 3.5 kDa) against degassed ultrapure water for 3 days with frequent water changes. PEtOx-*co*-SH20 had to be dialyzed in an ice-bath due to its low cloud-point. After freeze-drying, the polymer was received as a white powder in ~50 % yield.

^{1}H NMR of P(MeOx-*co*-ButOxSCOMe) (300 MHz, CD_3CN, ppm): δ = 3.39 (m, 2H, H-**b**, H-**c**; 2H, H-**b'**, H-**c'**), 2.99 (d, 2H, H-**a**), 2.85 (s, 1H, H-**a**, 2H, H-**h**), 2.34 (3H, Tosylate-CH_3), 2.29 (s, 3H, H-**i**), 2.01 (m, 3H, H-**d**), 1.58 (s, 2H, H-**f**, **g**).

^{1}H NMR of P(EtOx-*co*-ButOxSCOMe) (300 MHz, CD_3CN, ppm): δ = 3.41 (m, 2H, H-**b**, H-**c**; 2H, H-**b'**, H-**c'**), 2.96 (d, 2H, H-**a**), 2.86 (s, 1H, H-**a**; 2H, H-**i**), 2.33 (m, 6H, H-**d**, **f**), 2.29 (s, 3H, H-**j**), 1.57 (s, 4H, H-**g**, **h**), 1.03 (m, 3H, H-**e**).

^{1}H NMR of P(MeOx-*co*-ButOxSH) (300 MHz, D_2O, ppm): δ = 3.6 (m, 2H, H-**b**, H-**c**; 2H, H-**b'**, H-**c'**), 3.10 (d, 2H, H-**a**), 2.95 (s, 1H, H-**a**), 2.60 (s, 2H, H-), 2.40 (m, 2H, H-), 2.11 (m, 3H, H-), 1.66 (s, 4H, H-.

^{1}H NMR of P(EtOx-*co*-ButOxSH) (300 MHz, D_2O, ppm): δ = 3.59 (m, 2H, H-**b**, H-**c**; 2H, H-**b'**, H-**c'**), 3.10 (d, 2H, H-**a**), 2.95 (s, 1H, H-**a**), 2.58 (d, 2H, H-**i**), 2.39 (m, 4H, H-**d**, **f**), 1.66 (s, 4H, H-**g**, **h**), 1.08 (m, 3H, H-**e**).

Raman spectra of P(MeOx-*co*-ButOxSH) and P(EtOx-*co*-ButOxSH): $\tilde{\nu}_{\max}$ = 2934 (CH, CH_2), 2716 (CH_2), 2566 (SH), 1636 (N-C=O), 1483 (CH_2, CH_3 asym.), 1432 (CH_2, CH_3 asym.), 699 (C-S) cm^{-1}.

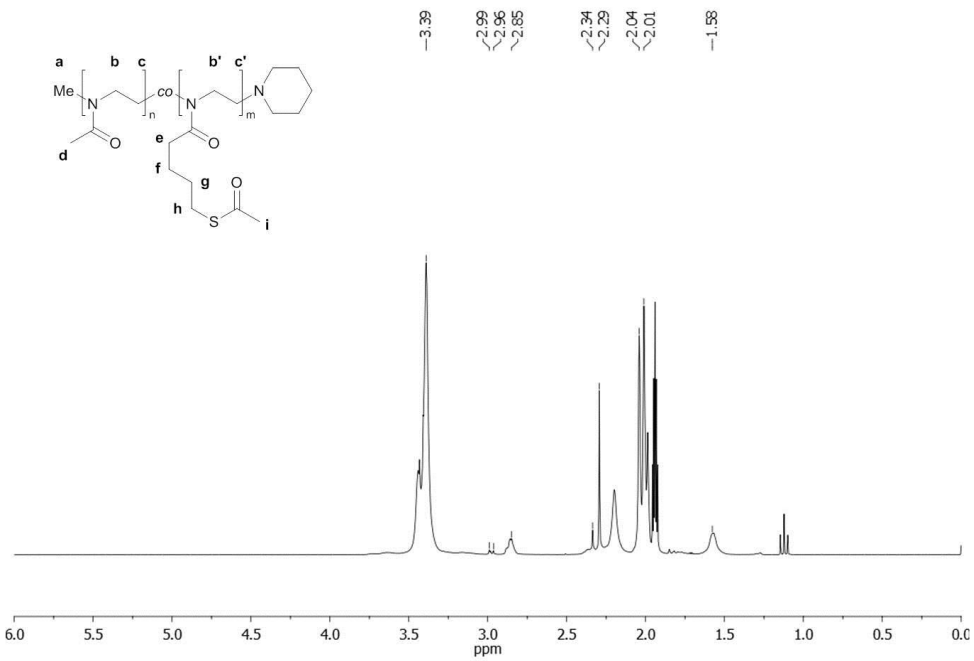


Fig. S17 ^{1}H NMR of P(MeOx-*co*-ButOxSCOMe) in CD_3CN.

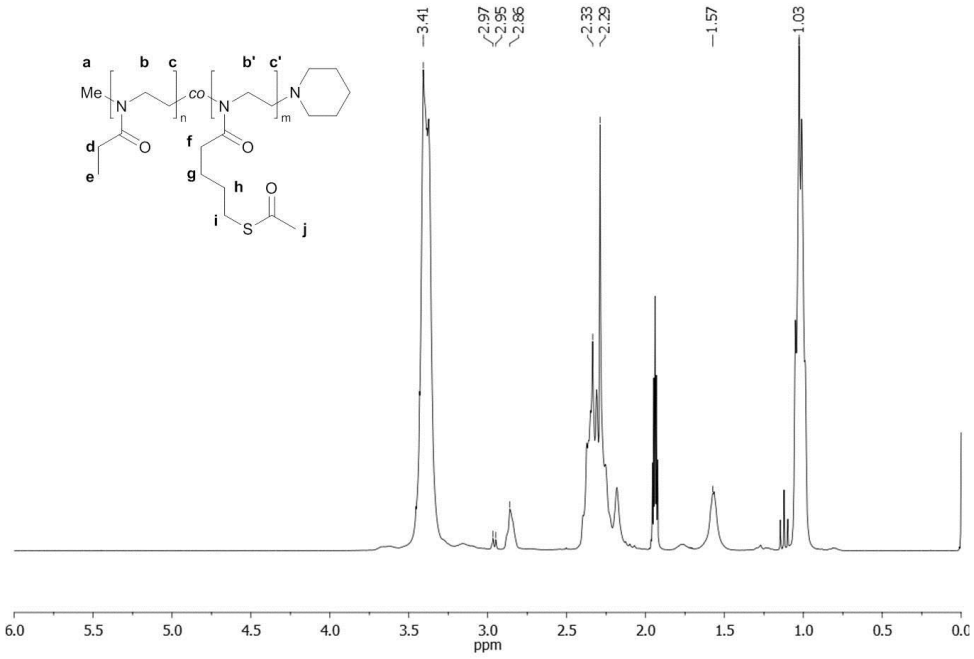


Fig. S18 ^{1}H NMR of P(EtOx-*co*-ButOxSCOMe) in CD_3CN.

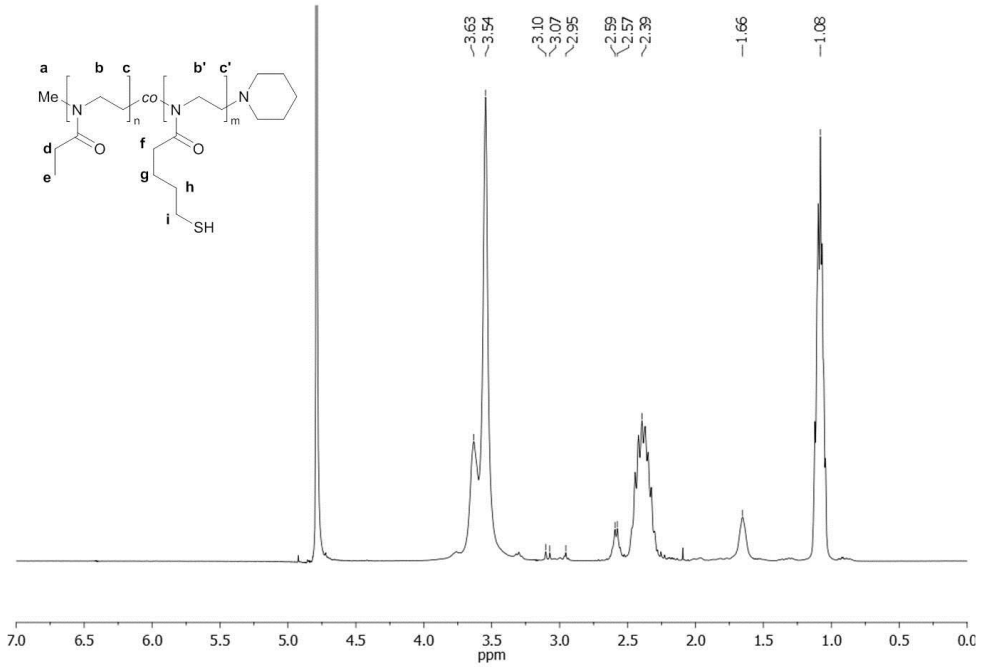


Fig. S19 ^{1}H NMR of P(EtOx-*co*-ButOxSH) in D_2O.

Tab. S3 Overview of thiol functionalized P(MeOx-*co*-ButOxSH)

Polymer	Repeating units*		mol % thiol	M_n (g/mol)*	M_n (g/mol)#	Đ#	mol % thiol§
	MeOx	ButOxSH					
PMeOx-*co*-SH10	50	5	9.1	5145	7729	1.19	6.65 ± 0.54
PMeOx-*co*-SH20	42.5	10.5	19.8	5373	8258	1.19	17.03 ± 2.25

*determined by ^{1}H NMR in D_2O, #determined by GPC in DMF, §determined by Ellman Assay

Tab. S4 Overview of thiol functionalized P(EtOx-*co*-ButOxSH)

Polymer	Repeating units*		mol % thiol	M_n (g/mol)*	M_n (g/mol)#	Đ#	mol % thiol§
	EtOx	ButOxSH					
PEtOx-*co*-SH10	50	5.3	9.6	5894	11880	1.12	9.83 ± 0.68
PEtOx-*co*-SH20	42	11.9	22	6146	12990	1.19	16.71 ± 1.25

*determined by ^{1}H NMR in D_2O, #determined by GPC in DMF, §determined by Ellman Assay

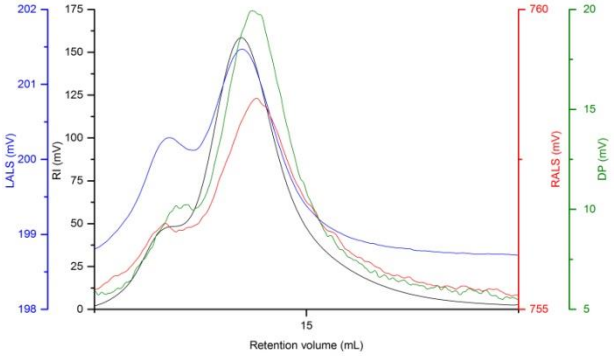


Fig. S20 GPC trace of PMeOx-*co*-SH10 in DMF.

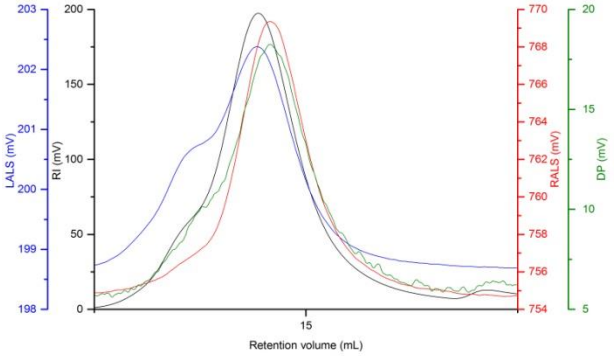


Fig. S21 GPC trace of PMeOx-*co*-SH20 in DMF.

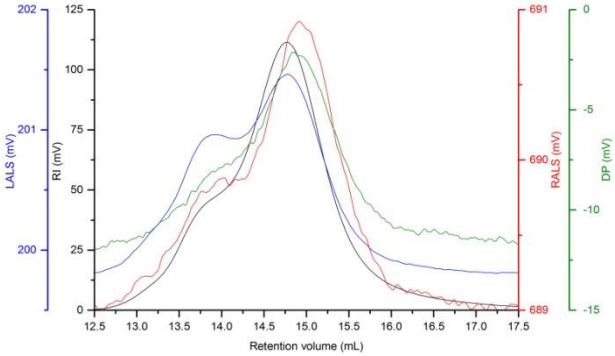


Fig. S22 GPC trace of PEtOx-*co*-SH10 in DMF.

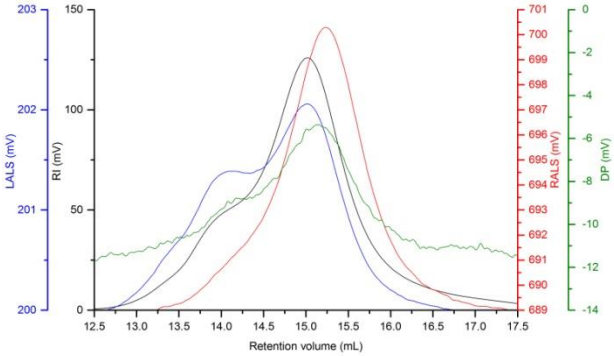


Fig. S23 GPC trace of PEtOx-*co*-SH20 in DMF.

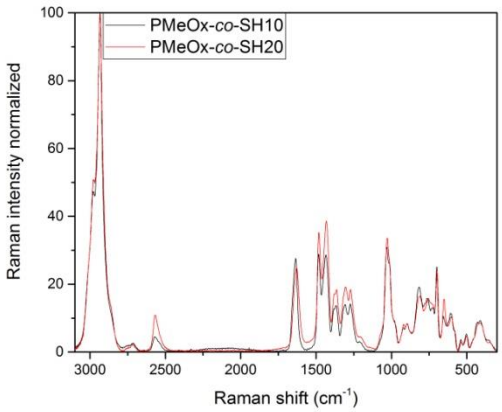


Fig. S24 Raman spectra of PMeOx-*co*-SH.

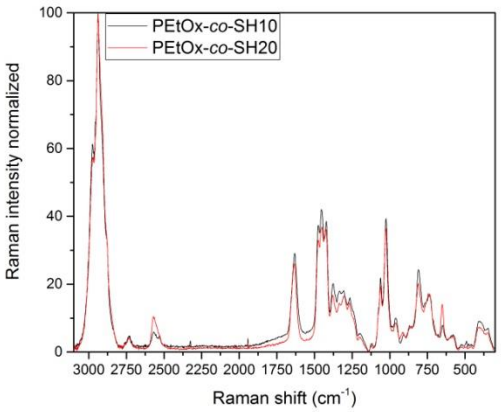


Fig. S25 Raman spectra of PEtOx-*co*-SH.

Functionalization of P(MeOx-co-ButEnOx) with FTZ4Cys

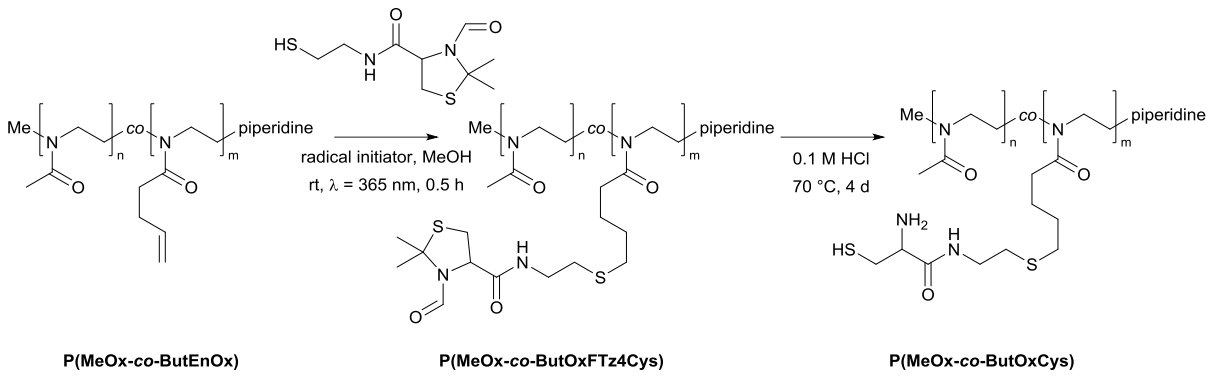


Fig. S26 Synthesis scheme of cysteine side-functionalized P(MeOx-*co*-ButEnOx).

The procedure to synthesize cysteine side-functionalized from the starting polymer P(MeOx-*co*-ButEnOx) has already been described in detail by Schmitz *et al*.[4]

The cleaning procedure of the polymer was modified as disulfide formation between the cysteine thiols was observed during dialysis against 0.01 M acetic acid, especially for higher degrees of functionalization. Hence, TCEP (1 eq. per functional cysteine group) was added after the hydrolysis and the solution was stirred for 2 h under neutral conditions before it was dialyzed (dialysis membrane Spectra/Por®, Carl Roth, MWCO 1 kDa) for 3 days against degassed water. Afterwards, the polymer was freeze-dried and a white powder was received with a yield of ~50 %.

The copolymer P(EtOx-*co*-ButEnOx) with a mol % of ButEnOx higher than 10 % could not be functionalized with FTz4Cys as the deprotection step involves temperatures of 70 °C which is far above the cloud point of the polymer. Hence, no series of different functionality degrees could be synthesized.

All cysteine side-functionalized copolymers did not display a cloud temperature below 85.5 °C. All P(MeOx-*co*-ButOxCys) were only soluble in water and it was tried to measure GPC with water as eluent. However, we will not show any data as the polymer interacted very strongly with the column material and no molecular weight could be determined.

^{1}H NMR of P(MeOx-*co*-ButOxFTz4Cys) (300 MHz, $CDCl_3$, ppm): δ = 8.35 (s, 1H, H-**m**), 7.59 (tosylate counterion), 7.18-7.15 (tosylate counterion), 4.96 (t, 1H, H-**l**), 3.45 (m, 2H, H-**b**, H-**c**; 2H, H-**b'**, H-**c'**), 3.22 (m, 2H, H-**j**), 3.00 (t, H 3, H-**a**), 2.60 (m, 4H, H-**h**, H-**i**), 2.33-2.28 (br, 4H, H-**k**, tosylate counterion), 2.10 (t, 3H, H-**d**), 2.07 (d, 4H, H-**g**), 1.80 (s, 6H, H-**n**, H-**n'**), 1.58 (s, 4H, H-**e**, H-**f**).

^{1}H NMR of P(MeOx-*co*-ButOxCys) (300 MHz, D_2O, ppm): δ = 4.19 (t, 1H, H-**l**), 3.57 (br, 2H, H-**b**, H-**c**; 2H, H-**b'**, H-**c'**), 3.31 (br, 2H, H-**j**), 3.10-3.08 (d, 2H, H-**k**), 2.94 (br, 3H, H-**a**), 2.79 (m, 2H, H-**i**), 2.77-2.38 (m, 4H, H-**g**, H-**h**), 2.15 (t, 3H, H-**d**), 1.65 (s, H 4H, H-**e**, H-**f**).
Raman spectra of P(MeOx-*co*-ButOxCys): $\tilde{\nu}_{\max}$ = 2932 (CH, CH_2), 2567 (SH), 1628 (N-C=O), 1483 (CH_2, CH_3 asym.), 1423 (CH_2, CH_3 asym.) cm^{-1}.

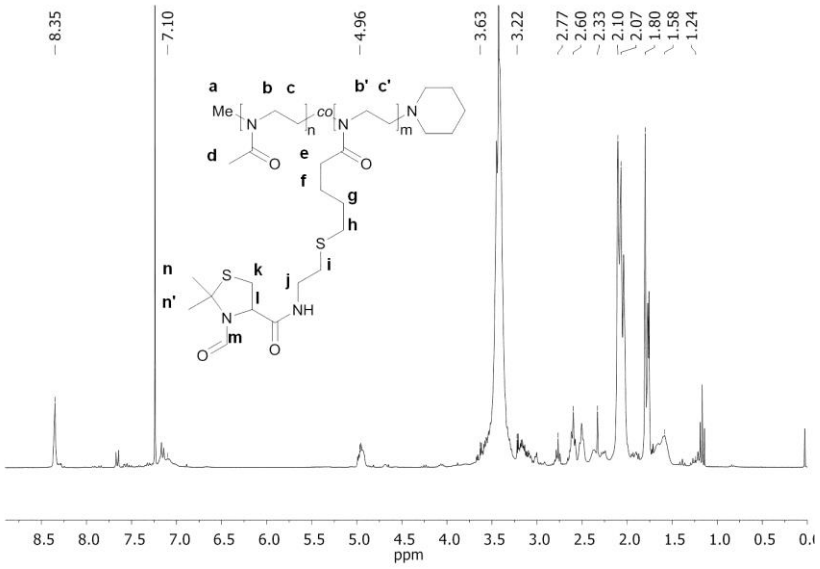


Fig. S27 ^{1}H NMR of P(MeOx-*co*-ButOxFTz4Cys) in $CDCl_3$.

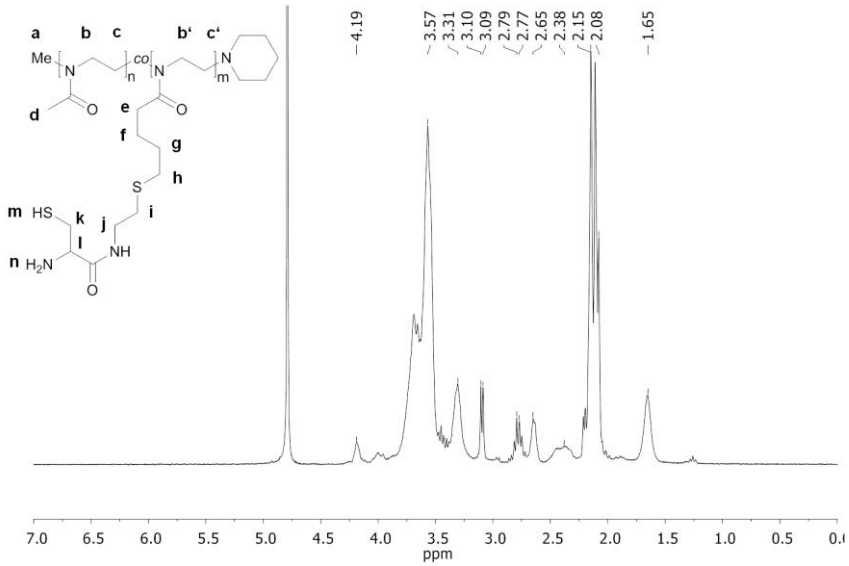


Fig. S28 ^{1}H NMR of P(MeOx-*co*-ButOxCys) in D_2O.

Tab. S5 Overview of all polymers with side-functionalized cysteine

Polymer	Repeating units*		mol % cysteine	M_n (g/mol)*
	MeOx	ButOxCys		
PMeOx-*co*-Cys10	39	5	11.4	4950
PMeOx-*co*-Cys20	42	12	22.2	7351
PMeOx-*co*-Cys30	31	14	31.1	7028

*determined by ^{1}H NMR in D_2O,

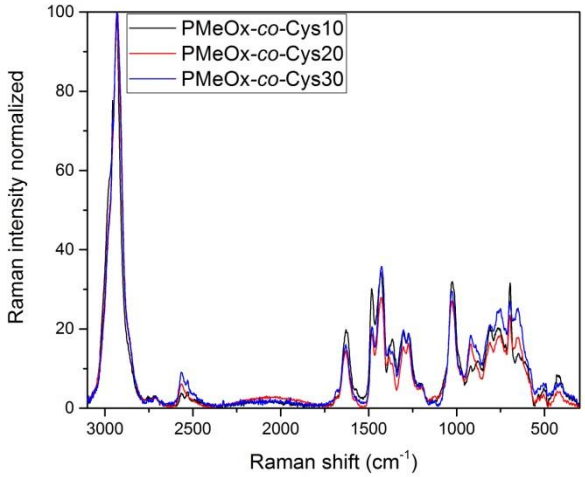


Fig. S29 Raman spectra of PMeOx-*co*-ButOxCys.

Cellcompatibility tests

Cell Culture Materials: Mouse fibroblasts (L 929 CC1) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) contained 10% fetal bovine serum (Sigma-Aldrich) in the presence of 1 % Penicillin-Streptomicin (ThermoFisher-Scientific).

Cytotoxicity test of the copolymers

Cytotoxicity was determined via the CellTiter-Glo Luminescent
Cell Viability Assay (Promega Corporation, Madison, WI, USA). The assay reagent produces luminescence in the presence of ATP from viable cells.
Mouse fibroblasts were cultivated in a T75 cell culture bottle (BD Falcon) in cell culture medium at 37 °C and 5 % CO_2. The cells were washed two times with 1xPBS and detached with 1 mL Accutase (Sigma-Aldrich) for 5 min. The detached cells were suspended in 10 mL cell medium to inactivate the accutase and for dilution. The cell number was determined and the concentration was diluted to 50 000 cells/mL. 500 µL of the cell suspension was seeded per well in a 48 well plate and cultivated for 24 h at 37 °C/5 % CO_2.

All polymer combinations, except for PEtOx-*co*-SH20 due to the low cloud point, were tested. A 15 mg/mL stock solution of each polymer in cell media was prepared and diluted to concentrations 5 mg/ml, 1 mg/mL and 0.1 mg/mL. The polymer eluate and controls were tested in fourfold. The supernatant was carefully sucked away and 0.5 mL of the appropriate eluate was added. The cells were incubated for 48 h at 37 °C/5 % with the eluate.
As negative control, polystyrene (Nunc, ThermoFisher Scientific) was used and fresh media was added. As positive control, the eluate of 1 mL per 1.25 cm^2 of a Vekoplan KT PVC plate (König GmbH, Wendelstein, Germany), eluated for 24 h, was used.
The well plates as well as the assay reagent were tempered at room temperature for 30 min before the assay. The needed volume of the reagent was diluted in a 1:1 ratio with media. The media supernatant was carefully sucked away and 0.5 mL of the diluted assay reagent was added. The blank was the diluted assay reagent. The well plates were mixed on an orbital shaker for 2 min and incubated for 10 min at rt to stabilize the luminescence signal. Of each well, 2x0.2 mL were pipetted into a white 96 well plate to measure duplicates. The luminescence was measured on a Tecan Spark® 20 M multimode microplate reader from Tecan Group Ltd. (Männedorf, Swiss). The cell viability was referenced to polystyrene (100 % viability).

Cytotoxicity of hydrogels in direct cell contact
To determine cell proliferation, a WST Assay was used (WST-1 reagent by Roche purchased from Sigma-Aldrich). Hydrogels (15 wt%) were prepared in triplicates under sterile conditions in PBS in silicon molds with a diameter of 6 mm and a height of 1 mm (volume = 30 µL) and UV cross-linked for 10 min.
Mouse fibroblasts were cultivated as described for the copolymer cytotoxicity test. The cell number was determined, and the concentration was diluted to 40 000 cells/mL (approximately 18000 cells/cm^2). 500 µL of the cell suspension was seeded per well in a 48 well plate and cultivated for 24 h at 37 °C/5 % CO_2.
After 24 h, the media supernatant was sucked away and the hydrogel specimen was placed on the top of the cells in the middle of the well. New media was added and the hydrogels were incubated for 7 days, with cell culture media change after 3 days.
For the cell proliferation test, the WST reagent was diluted 1:10 with cell culture media. The hydrogels were removed from the well and the cell culture media was sucked away. 0.5 mL of the diluted WST reagent was added per well and afterwards incubated for 30 min at 37 °C. Two times 0.2 mL of each hydrogel well was pipetted into a 96 well plate so that each well was measured in duplicates. The absorption was measured on a Tecan Spark® 20 M multimode microplate reader from Tecan Group Ltd. (Männedorf, Swiss) at a wavelength of 450 nm and a reference wavelength at 620 nm was subtracted. As positive control, the eluate of 1 mL per 1.25 cm^2 of a Vekoplan KT PVC plate (König GmbH, Wendelstein, Germany), eluated for 24 h, was used. As negative control, polystyrene and 2 wt% agarose hydrogels were used, to which cell viability was referenced to exclude any effect caused by the hydrogel covering the cells.

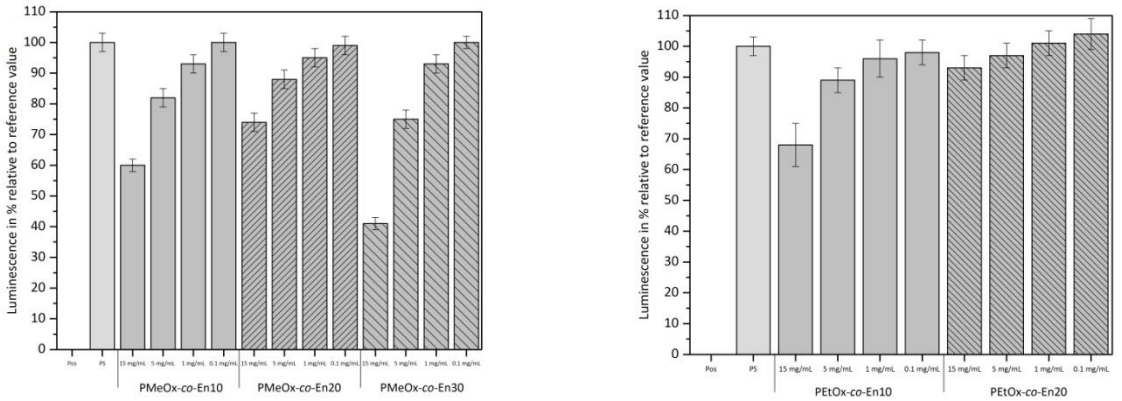


Fig. S 30 CellTiter-Glo Luminescent cell viability assay of PMeOx-*co*-En (left) and PEtOx-*co*-En (right) of concentrations 15 mg/mL, 5 mg/mL, 1 mg/mL and 0.1 mg/mL.

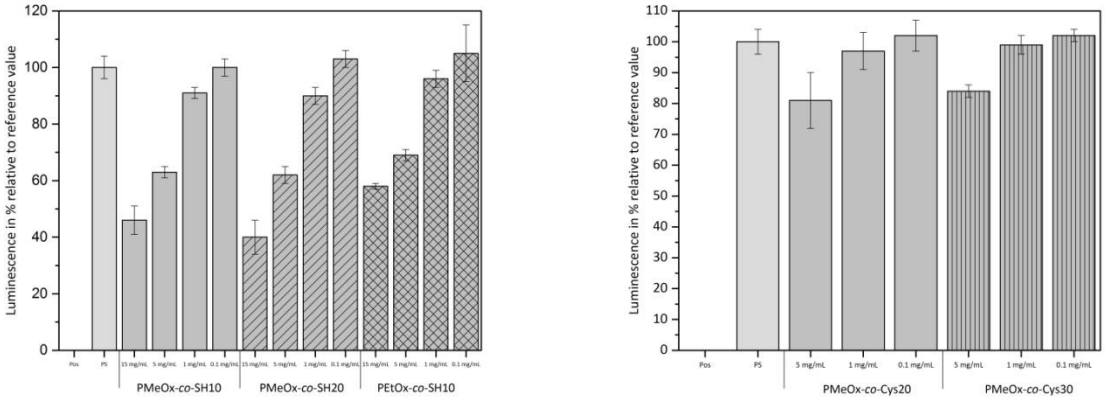
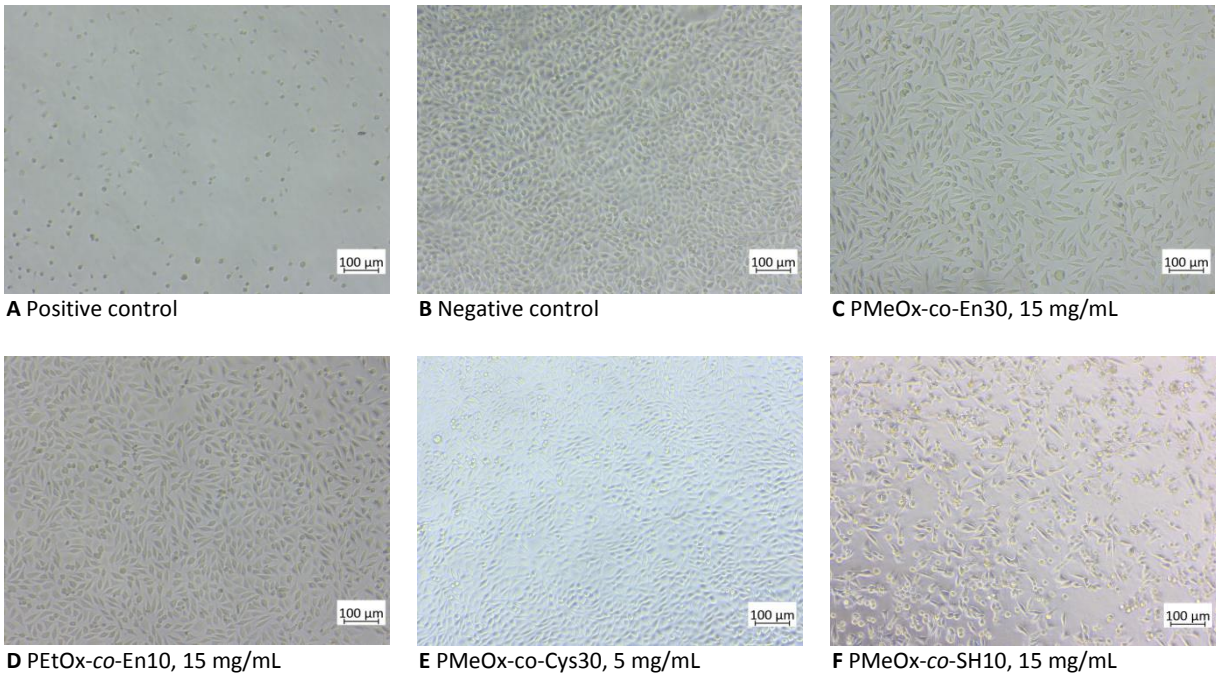


Fig. S 31 CellTiter-Glo luminescent cell viability assay of PMeOx-*co*-SH and PEtOx-*co*-SH (left) and PMeOx-*co*-Cys (right) of concentrations 15 mg/mL, 5 mg/mL, 1 mg/mL and 0.1 mg/mL.



A Positive control **B** Negative control **C** PMeOx-co-En30, 15 mg/mL

D PEtOx-*co*-En10, 15 mg/mL **E** PMeOx-co-Cys30, 5 mg/mL **F** PMeOx-*co*-SH10, 15 mg/mL

Fig. S 32 Stereomicroscopy pictures of cells with copolymers at different concentrations after incubation for 48 h.

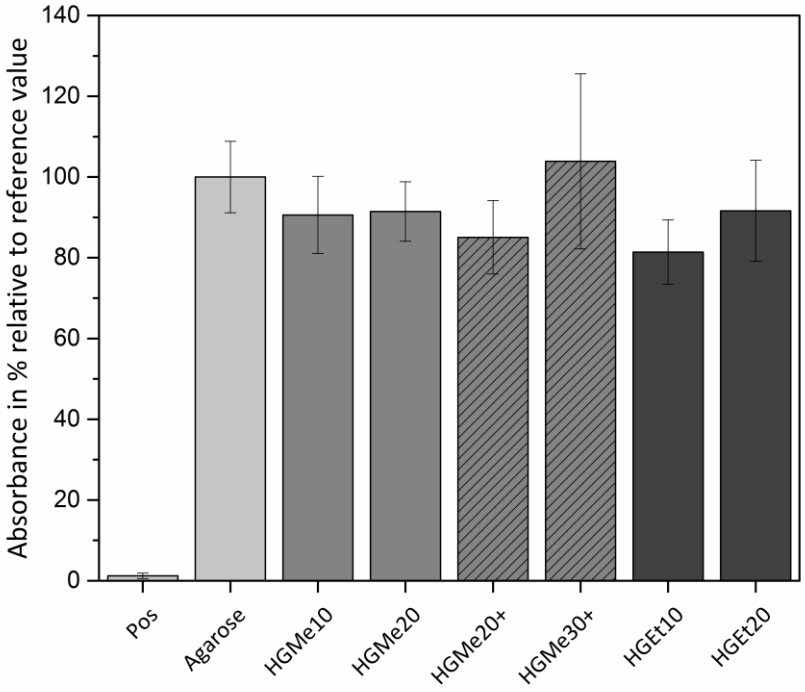


Fig. S 33 WST cell viability assay of all hydrogels after 7 d in direct cell contact

Model reaction of Cysteine methyl ester/mercaptoethanol with butenoic acid

A deuterated phosphate buffer was prepared in D_2O. L-Cysteine methyl ester (3.49 mg, 44.7 µmol) or mercaptoethanol (7.68 mg, 44.7 µmol) were mixed with 3-butenoic acid (3.85 mg, 44.7 µmol) in 1 mL of deuterated PBS buffer. The pH was measured and if needed adjusted to the intended pH with deuterated NaOH or HCl. 10 µL of an Irgacure 2959 solution (0.044 g/mL) was added and the solution was irradiated for 30 min with LED UV cubes. Afterwards, the solution was analyzed via ^{1}H NMR and the conversion was determined via the signal intensity of the alkene groups of butenoic acid.

Influence of the cysteine group on cross-linking behavior

The fact that no stable hydrogels could be formed with 10 mol% cysteine and a relatively low gel fraction of the hydrogel formed with 20 % cysteine (gel fraction = 68 %) indicates that the

close proximity of the primary amino group and the thiol group of the cysteine interferes with the thiol-ene reaction. The effect of different functional groups on the thiol-ene reaction under biological relevant conditions has been extensively studied by Colak *et al*.[8] They examined carboxylic acid, alkene, ester, alcohol and amine groups in the close neighborhood of the thiol and found that the molecular environment and the pH play an important role for the reaction efficiency. The latter is quite logic as the thiol radical cannot be formed if the thiol is deprotonated. They also tested two peptides with a terminal cysteine and used ^{1}H NMR spectroscopy to quantify that the conversion was only around 60 % at neutral pH. These results corroborated with their findings of the reaction efficiency of the small molecule cysteamine, where the reaction efficiency dropped dramatically when the pH was increased above pH 7. We believe that the same effect hinders complete cross-linking in our hydrogel system.

In order to study this effect, cysteine methyl ester was chosen as a small model molecule to investigate the cross-linking efficiency at different pH. In addition, a pH titration curve was measured (see Fig. S 34), which showed that the pH range in which the cysteine functionality exists as a zwitter ion (S^-–NH_3^+ ⟷ HS–NH_2) is between pH 4.2 and pH 7.4. This means that the ideal case where all thiols can form a radical and take part in the thiol-ene reaction is only given at very acidic pH below values of 4.2. This assumption was also confirmed by model experiments between cysteine methyl ester and butenoic acid and their analysis via ^{1}H NMR. In the case of a 1:1 equivalency, no reaction occurred at pH 7.2 and at pH 3.3. Only by raising the equivalency to 3:1 SH:En, a 100 % conversion of the double bond at pH 5 was achieved, which was not yet the case for an equivalency of 2:1. When the same conditions were tested for a model reaction between mercaptoethanol and butenoic acid in a 1:1 molar ratio, 100 % conversion was reached at acidic pH. These results confirm the findings by Colak *et al*.

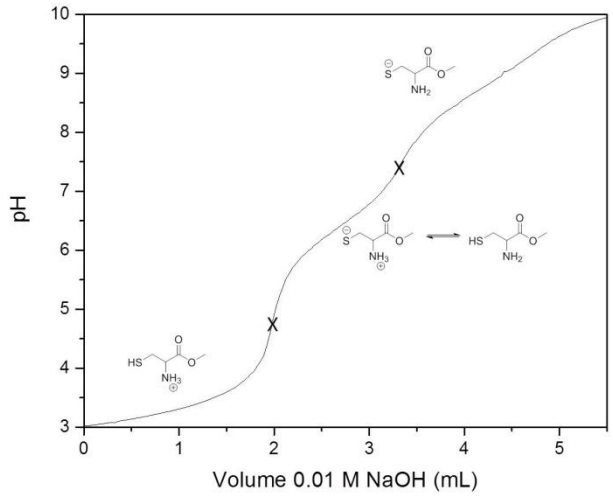


Fig. S 34 pH curve of cysteine methyl ester.

LCST type behavior of HGEt20

We assessed if the hydrogel exhibits the same Type I LCST behavior as it was observed for PEtOx hydrogels synthesized by radical network formation by Christova *et al*.[9] For this purpose, the weight of the hydrogel with the strongest thermo-responsive behavior, HGEt20, was weighed over a temperature range starting from 10 °C, referencing the weight at 5 °C as m_0. Fig. S 35 shows the gradual and continuous deswelling with each temperature increase comparable to the pure poly(2-ethyl-2-oxazoline) (PEtOx) hydrogel.[9] This indicates that this hydrogel system also underlies a Type I LCST behavior.

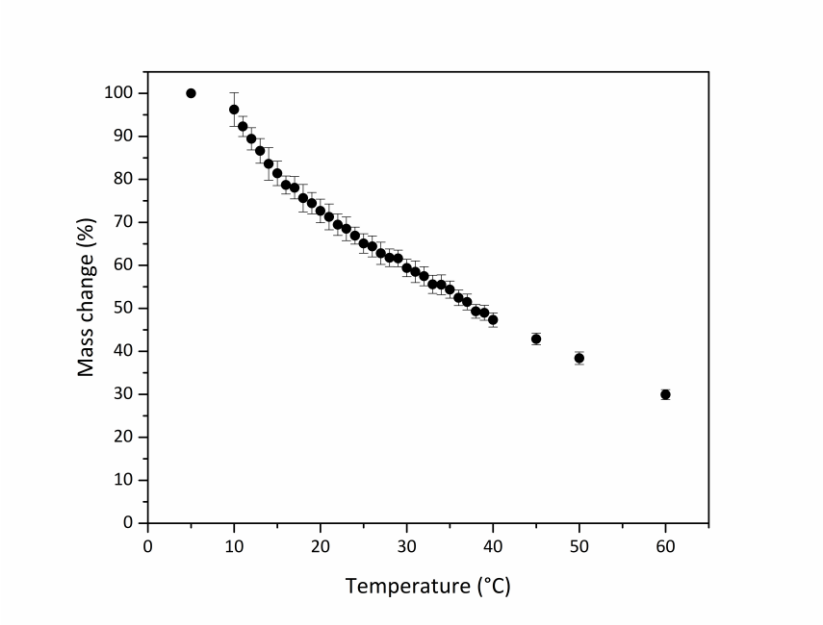


Fig. S 35 Mass change of hydrogel HGEt20 over a temperature range of 10 °C to 50 °C in PBS. Meand and standard deviation shown (n = 3).

Volume change of hydrogels

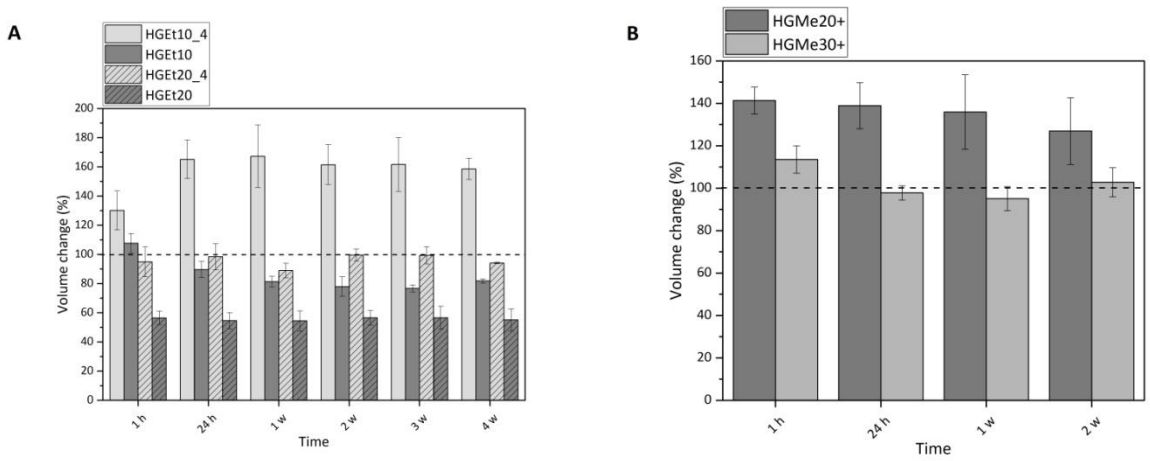


Fig. S 36 Volume change of hydrogels in PBS over a time course of two weeks.

Flory-Rehner analysis

The determination of the mesh size by Flory-Rehner analysis was adapted by Dargaville *et al.*[10]

Example calculation for HGMe10: Dry mass (w_d) = 5.37 mg; equilibrium swelling mass (w_s): 48.72 mg

The swelling ratio (SD) is calculated as follow: $SD = \frac{w_s - w_d}{wd} = \frac{48.72 - 5.37}{5.37} = 8.07$

To determine the polymer volume fraction in the swollen state ($v_{2,s}$) the density of poly(2-ethyl-2-oxazoline) 1.14 g/mL was used as an approximation to the copolymers used for this hydrogel. The density of water is 1.008 g/mL, so that $v_{2,s}$ can be calculated as follows:

$$v_{2,s} = \frac{\frac{1}{\rho_{polymer}}}{Q_m/\rho_{solvent} + \frac{1}{\rho_{polymer}}} = \frac{\frac{1}{1.14}}{8.07/1.008 + \frac{1}{1.14}} = 0.099$$

The effective molar mass between crosslinks, $\bar{M}_c$, can be further calculated by using the following equation. The specific volume of the polymer ($\bar{v}$), which is the reciprocal of the polymer density, has a value of 0.88 mL/g. The molar volume of water (V_1) is 18.016 g/mol and the polymer solvent interaction parameter (χ_{12}) of POx and water has been reported to be 0.485. The average molar mass of the polymer chains ($\bar{M}_n$) of PMeOx-*co*-En10 and PMeOx-*co*-SH10 is 5105 g/mol.

$$\frac{1}{\bar{M}_c} = \frac{2}{\bar{M}_n} - \frac{(\bar{v}/V_1)\left[\ln\left(1 - v_{2,s}\right) + v_{2,s} + \chi_{12}{v_{2,s}}^2\right]}{\left({v_{2,s}}^{1/3} - v_{2,s}/2\right)}$$

$$\frac{1}{\bar{M}_c} = \frac{2}{5105} - \frac{\left(\frac{0.88}{18.016}\right)\left[\ln(1 - 0.099) + 0.099 + 0.485 * 0.099^2\right]}{(0.099^{1/3} - 0.099/2)}$$

$$\frac{1}{\bar{M}_c} = 0.00045$$

$$\bar{M}_c = 2222.15$$

With the average molar mass of the repeating units of the polymer, M_r, which is 90.107 g/mol for PMeOx-*co*-En10 and PMeOx-*co*-SH10, $v_{2,s}$ and $\bar{M}_c$ the distance between macromolecular chains (ξ) can be estimated with the Flory characteristic ratio (C_n), which has been reported to be 1.67 for POx, and the bond length l, which is 1.39 Å, as determined

by Dargaville *et al.* for the average length of carbon-nitrogen and carbon-carbon single bonds.

$$\xi = v_{2,s}{}^{-\frac{1}{3}}\left[l\left(\frac{C_n * 2\bar{M}_c}{M_r}\right)\right]^{\frac{1}{2}} = 0.099^{-\frac{1}{3}}\left[1.39\left(\frac{1.67 * 2 * 2222.15}{90.107}\right)\right]^{\frac{1}{2}}$$

$$\xi = 23.15\ \text{Å} = 2.32\ \text{nm}$$

Tab. S 6 Distance between macromolecular chains for all hydrogels

Hydrogel	ξ (nm)	Degree of cross-linking (X)
HGMe10	2.32	0.0203
HGMe20	1.71	0.0277
HGMe20+	1.87	0.0254
HGMe30+	1.46	0.0359
HGEt10	1.74	0.0263
HGEt10_4	2.54	0.0186
HGEt20	0.99	0.0593
HGEt20_4	1.89	0.0252

Solid-state NMR of thiol-ene cross-linked hydrogels

In addition, ^{13}C solid-state NMR spectra were recorded by direct excitation (DE) choosing the delay between the scans to be very short. This acts as a filter for units with large T1 relaxation times and thus, only mobile parts of the molecule appear in the resulting spectrum. As expected, the groups from the chains in the original polymers show highest mobility. Also, the vinyl functional polymer shows less mobility than the thiol functionalized polymer due to the different number of CH_2 moieties present in the side chain. A comparison with the original CP experiments is shown in Fig. S 37. To compare DE to CP, we used the hydrogel with the highest functionality degree, HGEt20. It could be observed from this comparison, see Fig. S 37B, that the cross-linked polymer still displays some mobility in the chains. The mobility observed is in-between the one observed for the vinyl and the thiol polymer precursor. To compare the two hydrogels with a different amount of cross-linking, HGEt10 and HGEt20, the signal intensities of the two spectra were scaled according to the number of scans (1005 vs. 1400) so that they can be directly correlated. The amount of impurities in both samples is almost identical. The only differences in intensity can be observed for the carbonyl carbon signal at 175.8 ppm and for the signal at 34.1 ppm. This latter signal corresponds to the CH_2 groups adjacent to the carbonyl moieties and in the vicinity of the sulfur atom. As the carbon spectrum does not show any indications of leftover vinyl groups (around 137 and 115 ppm), this increase of the signal intensity is caused mainly

by the formation of more cross-linking units (grey spectrum) with a small contribution of the CH_2 units next to the carbonyl group (most likely due to lower mobility), Fig. S 37C.

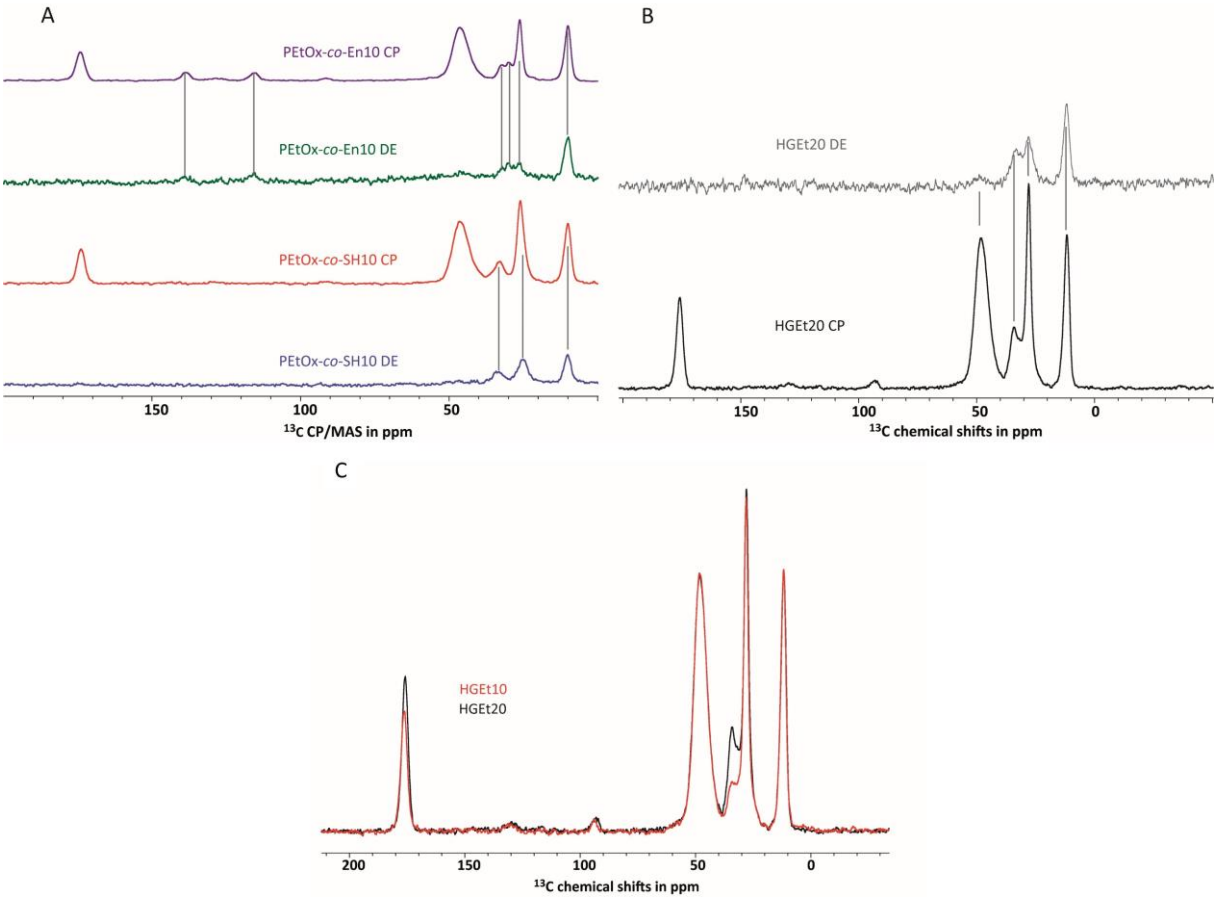


Fig. S 37 A) Comparison of the ^{13}C NMR spectra of the two polymer precursors used for the later cross-linking step. For each polymer, the ^{1}H-^{13}C CP/MAS experiment from figure 7 and the data obtained by direct excitation (DE) are shown. B) Comparison of the ^{13}C NMR spectrum obtained by direct excitation (DE) with the corresponding spectrum obtained from cross-polarization (CP) for the sample HGEt20. C) Comparison of the ^{1}H-^{13}C CP/MAS NMR spectra of the two polymers HGEt10 and HGEt20 with different amount of cross-linking (black > red). All experiments were recorded at 14.1 T and 12.5 kHz MAS. For the CP / MAS experiments, a contact time of 2 ms was used.

Release of FITC-dextran

A B

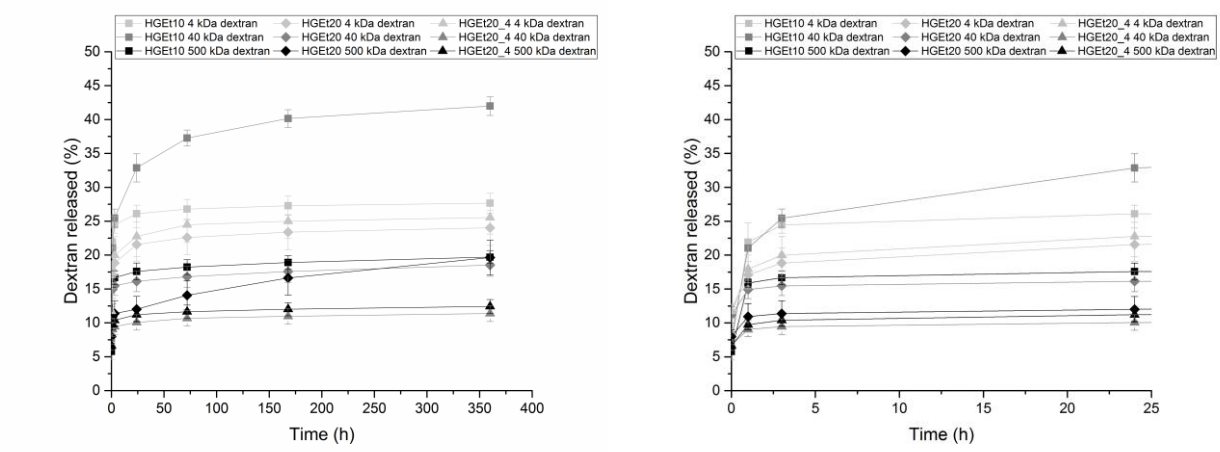


Fig. S 38 A) Release of FITC-dextran from HGEt10 at 37 °C and HGEt20 at 4 °C and 37 °C in PBS for 15 d and B) zoom on the first 24 h

A B

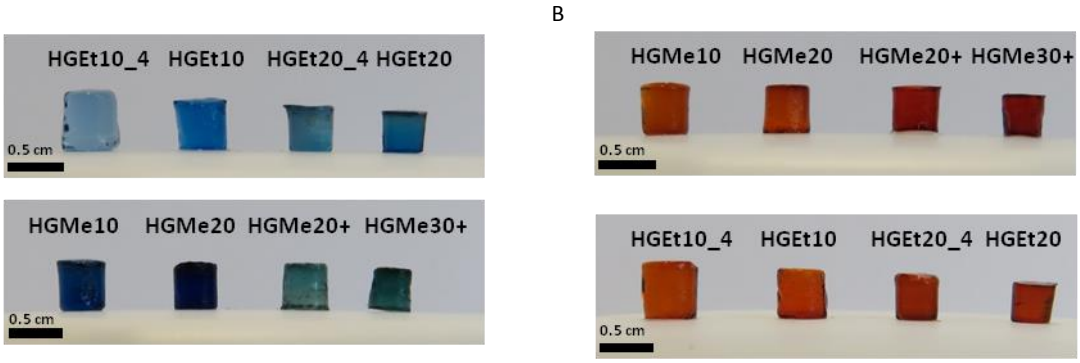


Fig. S 39 A) Visual appearance of the hydrogels loaded with methylene blue and B) loaded with fluorescein sodium after 72 h

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