Supporting Information

Versatile, Multifunctional Block-Copolymers for the Self-Assembly of Well-Defined, Non-Toxic pDNA Polyplexes

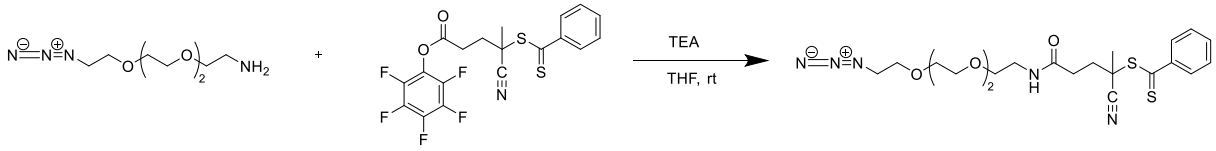
Nicolas Ritt,[†] Simone Berger,[‡] Ernst Wagner[‡] and Rudolf Zentel[*,†]

[†]Johannes Gutenberg University Mainz, Department of Chemistry, Duesbergweg 10-14, 55128 Mainz, Germany

[‡]Ludwig-Maximilians-University Munich, Department of Pharmacy, Butenandtstraße 5-13, Haus D, 81377 Munich, Germany

Small molecule synthesis

Synthesis of 1-azido-16cyano-13-oxo-3,6,9-trioxa-12-azaheptadecan-16-yl benzothioate (azide-CTA).



Scheme S1. Synthesis of 1-azido-16cyano-13-oxo-3,6,9-trioxa-12-azaheptadecan-16-yl benzothioate (azide-CTA).

In a 100 ml flask, 1184.0 mg (2.6582 mmol) 4-cyano-4-(phenylcarbonotioylthio)pentanoic acid pentafluorophenyl ester (PFP-CTA) and 310.4 mg (3.0675 mmol) triethylamine (TEA) were dissolved in 30 ml tetrahydrofuran (THF) under argon atmosphere. 446.7 mg (2.0467 mmol) 11-azido-3,6,9-trioxaundecan-1-amine were dissolved in 30 ml THF and added via a dropping funnel over the cause of 25 minutes, while cooling the reaction mixture in an ice/water mixture. The solution was stirred for 17 h at room temperature. After that, the solvent

was evaporated, and the crude product was purified using liquid chromatography. 867.1 mg (1.8079 mmol, 88,3% yield) of the viscous, red product was isolated via rotary evaporation.

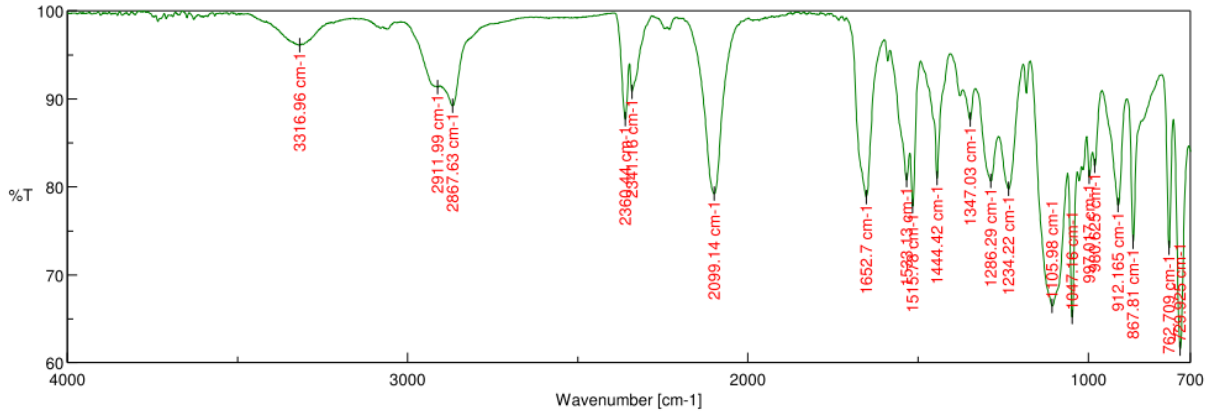


Figure S1. FT-IR of 1-azido-16cyano-13-oxo-3,6,9-trioxa-12-azaheptadecan-16-yl benzothioate (azide-CTA)

FT-IR: v [cm-1] = 3317 w; 2912 m; 2868 m; 2369 m; 2099 s; 1653 s; 1516 s; 1286 m; 1234 m; 1106 ss; 912 m; 868 m; 763 m; 730 s

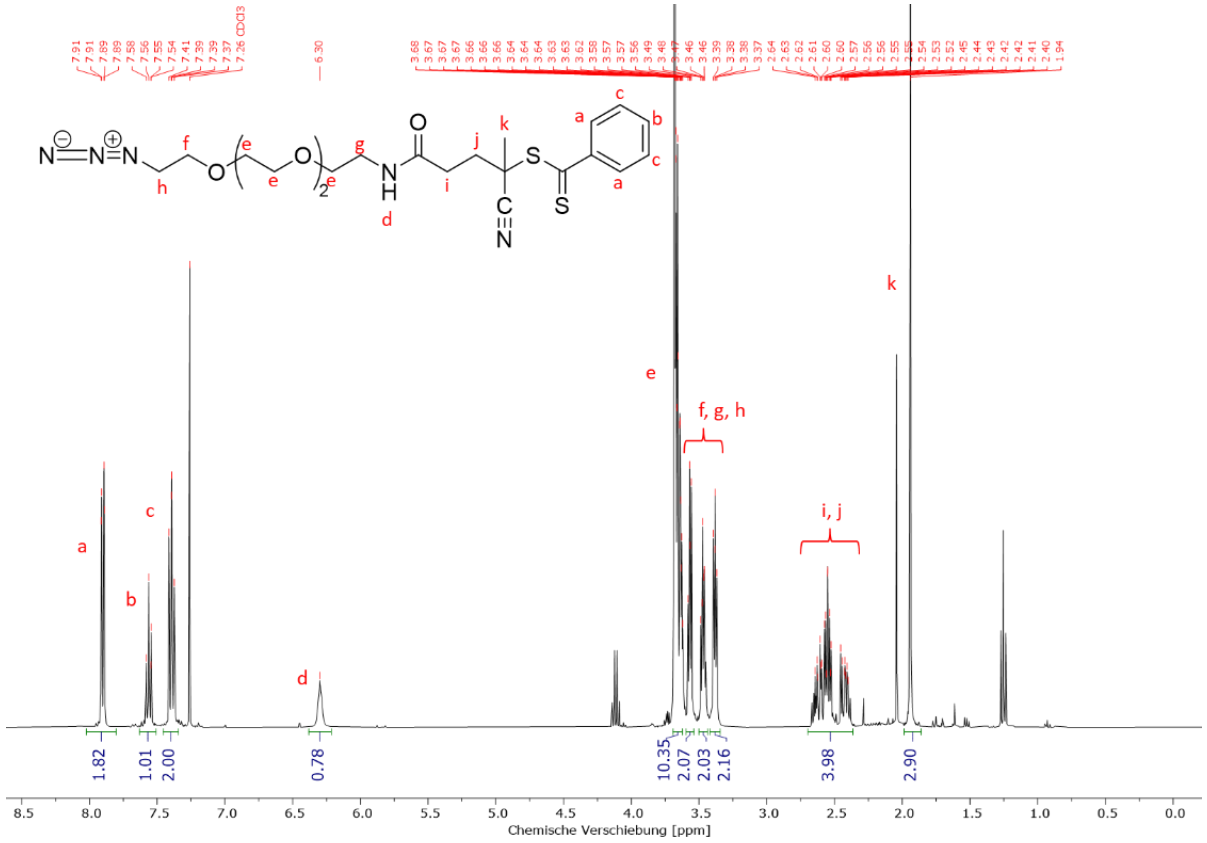
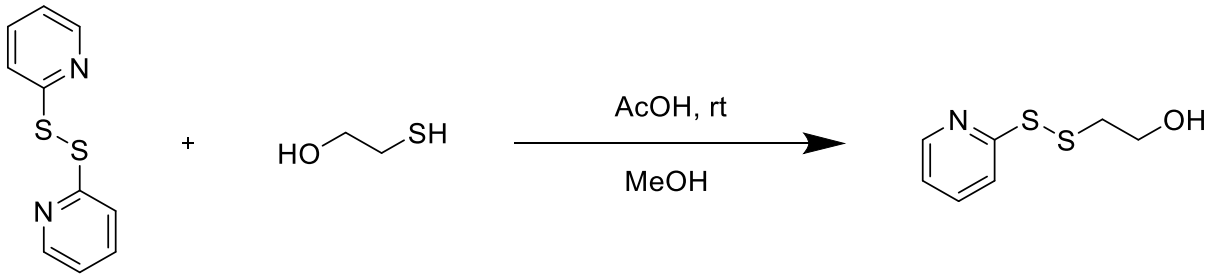


Figure S2. ^{1}H-NMR of 1-azido-16cyano-13-oxo-3,6,9-trioxa-12-azaheptadecan-16-yl benzothioate (azide-CTA)

^{1}H-NMR (400 MHz; CDCl3): δ [ppm] = 7.91-7.89 (m, 2 H); 7.58-7.54 (m, 1 H); 7.41-7.37 (m, 2 H); 6.30 (s, 1H); 3.68-3.56 (m, 10 H); 3.49-3.47 (m, 2H); 3.46-3.45 (m, 2 H); 3.39-3.37 (m, 2 H); 2.64-2.40 (m, 4 H); 1.94 (s, 3 H)

Synthesis of Hydroxyethylpyridyl disulfide (HPDS).



Scheme S2. Synthesis of hydroxyethylpyridyl disulfide (HPDS)

4.1967 g (19.0491 mmol) aldrithiol-2 was dissolved in 27 ml methanol under nitrogen atmosphere in a round bottom flask and 400 µl glacial acetic acid were added. Over 30 minutes 992.2 mg (12.6994 mmol) 2-mercaptoethanol in 13 ml methanol was added via a dropping funnel. Afterwards the reaction mixture was stirred for 18 h. The crude product was obtained by evaporation of the solvent. After purification by gradient column chromatography (mobile phase: cyclohexane / ethyl acetate; 3:1 to 2:1) and evaporation of the solvent, 1.7607 g (9.4014 mmol; 74% yield) hydroxyethylpyridyl disulfide were obtained as a slightly viscous yellow oil.

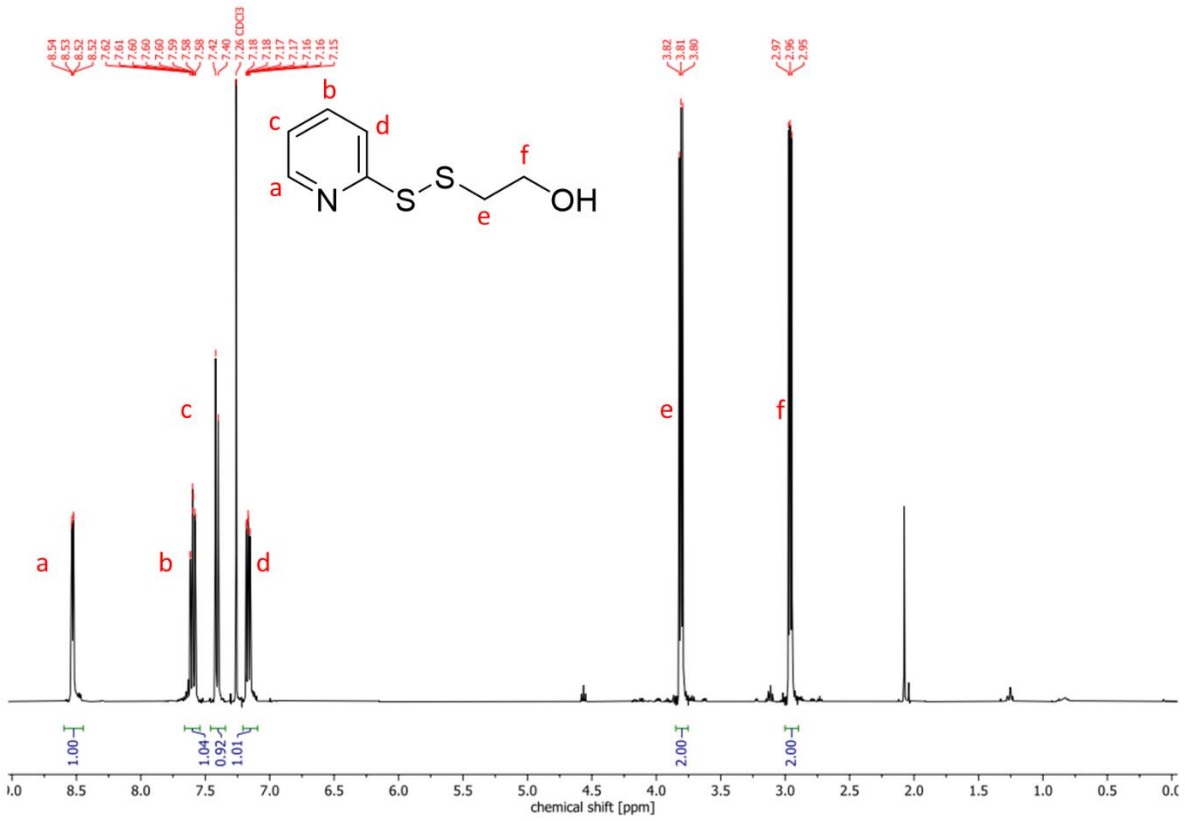
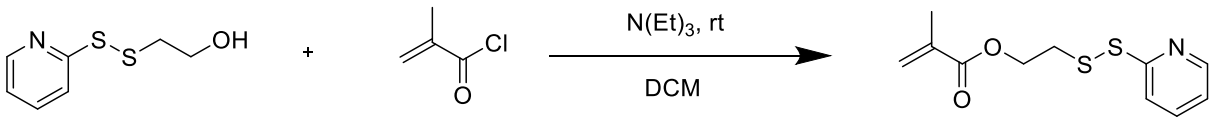


Figure S3. [1]H-NMR of hydroxyethylpyridyl disulfide (HPDS)

[1]H-NMR (400 MHz; CDCl3): δ [ppm] = 8.52 (d, J = 5 Hz, 1 H); 7.62-7.58 (m, 1 H); 7.41 (d, J = 8 Hz, 1 H); 7.18-7.15 (m, 1 H); 3.81 (t, J = 5 Hz, 2 H); 2.96 (t, J = 5 Hz, 2 H).

Synthesis of Pyridyldisulfidethyl methacrylate (PDSM).



Scheme S3. Synthesis of pyridyldisulfidethyl methacrylate (PDSM).

1.7607 g (9.4014 mmol) hydroxyehtylpyridyl disulfide was dissolved in 15 ml dichloromethane (DCM). The mixture was cooled with an ice-water bath and 1.4792 g (14.1021 mmol; 1.50 eq) methacryloylchloride in 3 ml DCM was added dropwise. After 30 minutes a pinch of 2,6-di-tert-butyl-4-methylphenol was added and the reaction mixture was stirred for 17 h at room

temperature. After filtration, the filtrate was washed 3 times with 10 ml of water. The collected organic phases were dried with magnesium sulfate and concentrated by vacuum evaporation. The crude product was purified by column chromatography (cyclohexane / ethylacetate 10:1). After solvent evaporation 2.0907 g (8.1876 mmol; 87 % yield) of PDSM was obtained as slightly viscous, yellow oil.

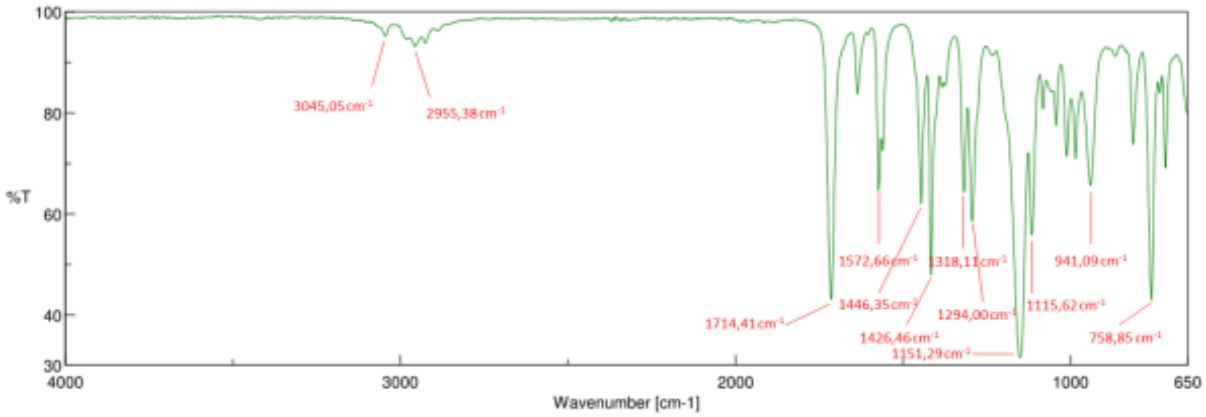


Figure S4. FT-IR of pyridyldisulfidethyl methacrylate (PDSM).

FT-IR: v [cm^{-1}] = 3045 w; 2955 w; 1714 s; 1573 m; 1446 m; 1426 s; 1318 m; 1294 m; 1151 ss; 1116 m; 941 m; 759 s.

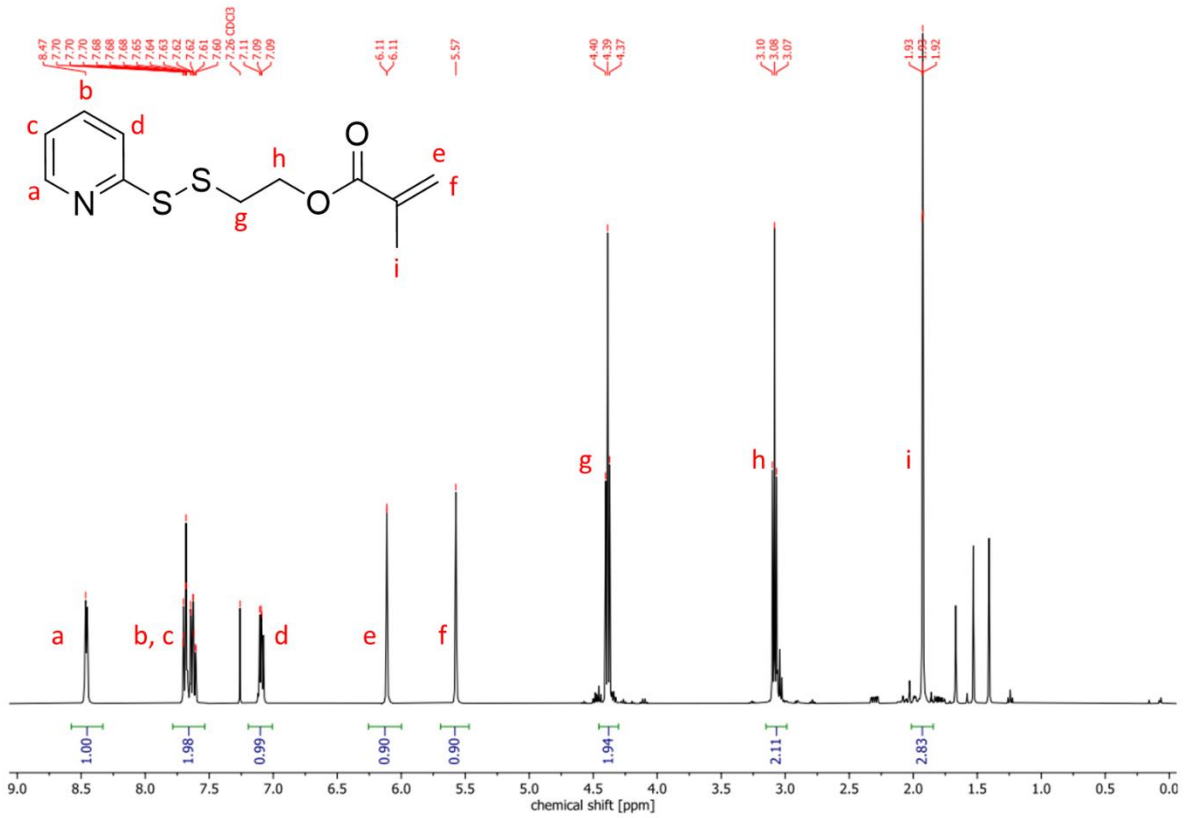
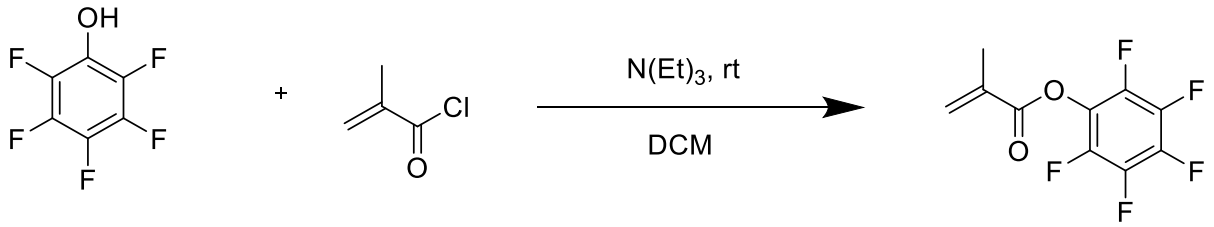


Figure S5. ^{1}H-NMR of pyridyldisulfidethyl methacrylate (PDSM).

^{1}H-NMR (400 MHz; CDCl3): δ [ppm] = 8.47 (m, 1 H); 7.70-7.68 (m, 1 H); 7.65-7.60 (m, 1 H); 7.11-7.09 (m, 1 H); 6.11 (m, 1 H); 5.57 (m, 1 H); 4.39 (t, J = 6 Hz, 2 H); 3.08 (t, J = 6 Hz, 2 H); 1.93 (m, 3 H).

Synthesis of Pentafluorophenyl methacrylate (PFPMA).



Scheme S4. Synthesis of pentafluorophenyl methacrylate (PFPMA).

In a 250 ml flask 10100.0 mg (54.872 mmol) pentafluorophenol and 5530.0 mg (54.648 mmol) triethylamine (TEA) were dissolved in 65 ml dichloromethane (DCM) under argon atmosphere. Over the cause of 30 minutes, 5107.0 mg (48.855 mmol) methacryloylchloride in 35 ml DCM was added via a dropping funnel. The solution was stirred vigorously and cooled in an ice / water bath. After further stirring for 17 h at room temperature the formed triethylammonium chloride residue was separated via filtration, washed with DCM and discarded. The combined organic phases were dried with magnesium sulfate and the solvent was removed by rotary evaporation. The crude product was purified by fractionated vacuum distillation. 7264.0 mg (28.807 mmol, 59% yield) of the colorless product were obtained..

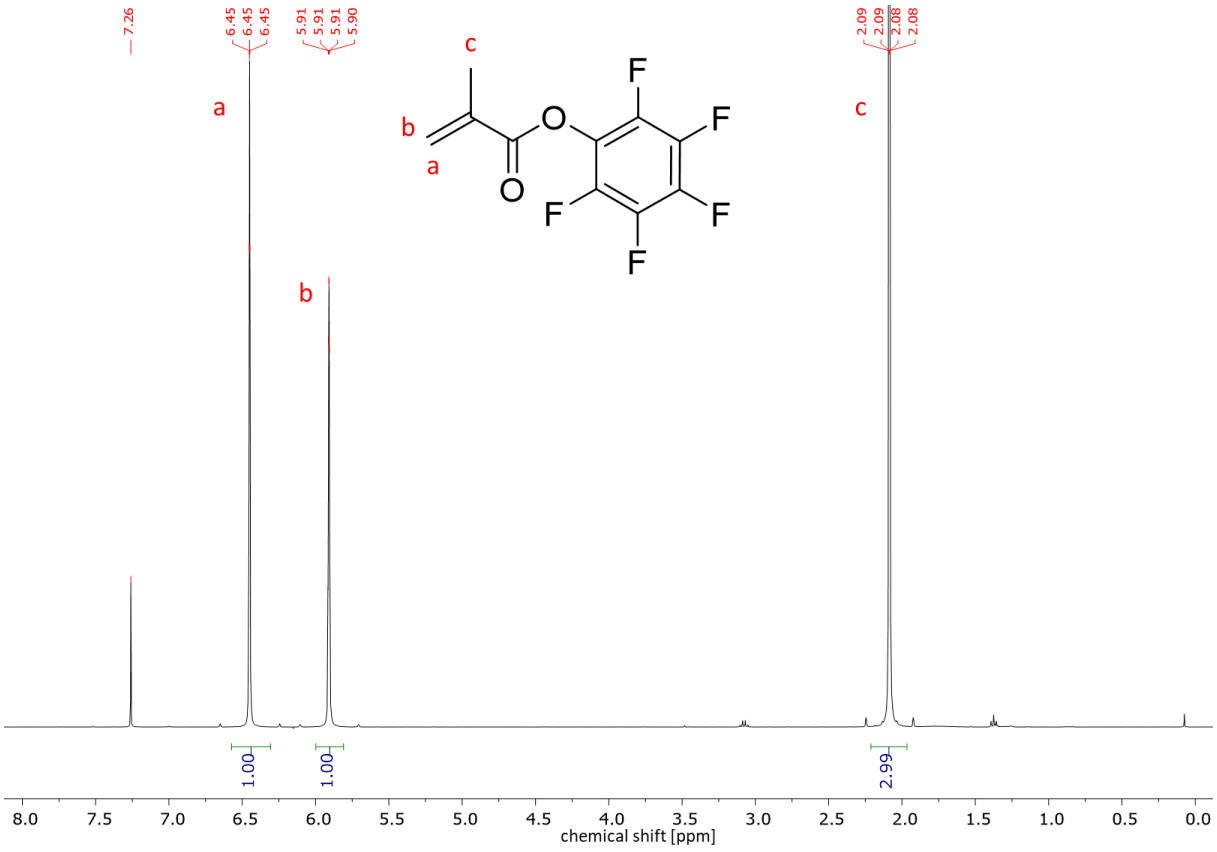


Figure S6. [1]H-NMR of pentafluorophenyl methacrylate (PFPMA).

[1]H-NMR (400 MHz; CDCl3): δ [ppm] = 6.45 (s, 1 H); 5.91 (s, 1 H); 2.09 (s, 1 H).

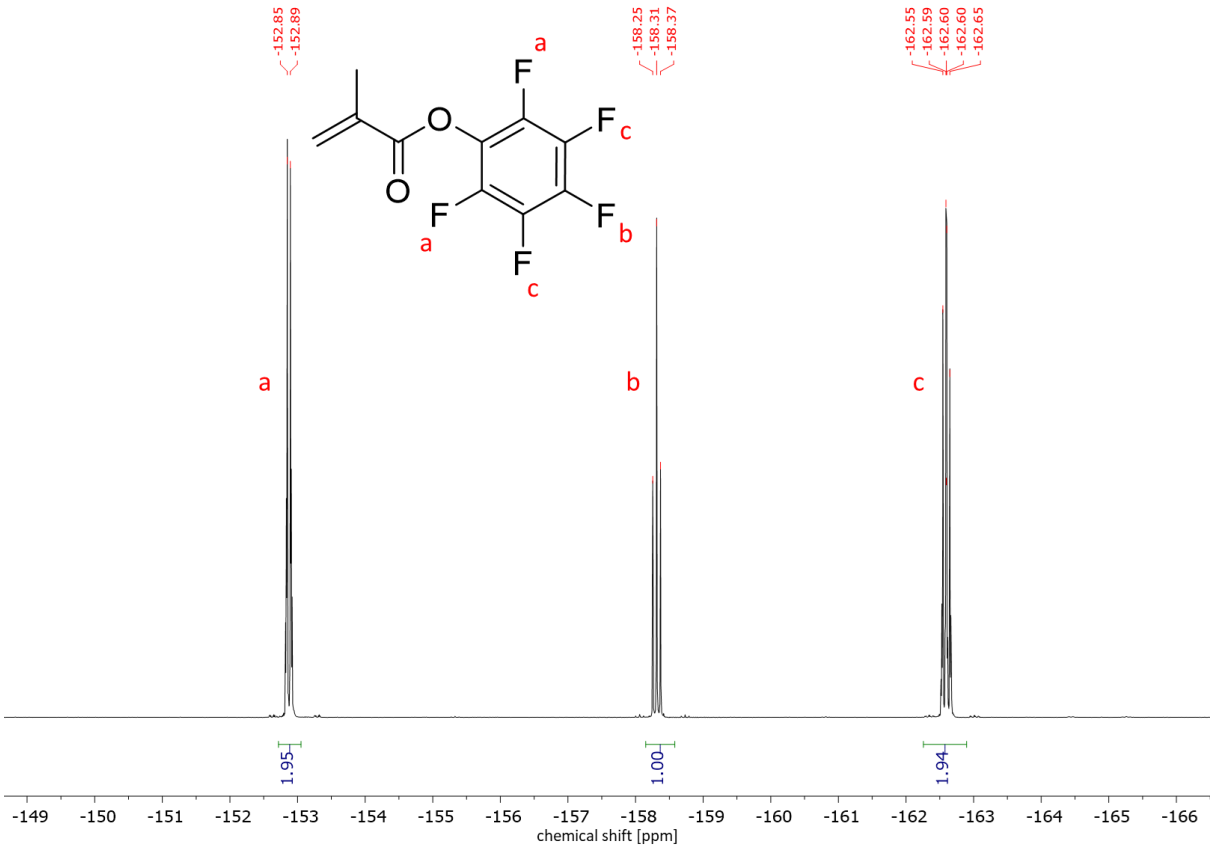
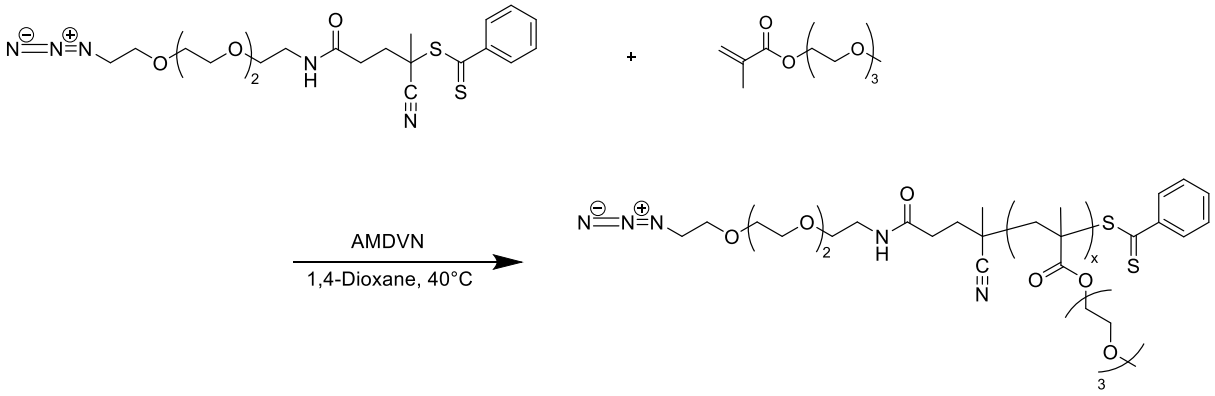


Figure S7. ^{19}F-NMR of pentafluorophenyl methacrylate (PFPMA).

^{19}F-NMR (400 MHz; CDCl3): δ [ppm] = -152.85-(-152.89) (d, J = 17.2 Hz, 2 F); -158.31 (t, J = 21.72 Hz, 1 F); -162.6 (dd, J_1 = 21.98 Hz, J_2 = 16.81 Hz, 2 F)

Polymer synthesis

Synthesis of azide-functionalized poly-(tri(ethyleneglycol)-methylether methacrylate) (azide-p(MEO$_3$MA)).



Scheme S5. Synthesis of azide-p(MEO3MA)

1000.00 mg (4.3053 mmol) of the MEO$_3$MA monomer were dissolved in 1.5 ml 1,4-dioxane and transferred into a 25 ml Schlenk-tube. 44.84 mg (0.0935 mmol) of the azide CTA in 0.5 ml 1,4-dioxane and 5.77 mg (0.0187 mmol) AMDVN in 0.5 ml 1,4-dioxane were added. The reaction vessel was dipped into liquid nitrogen until the solution was frozen. After that, the solution was degassed via the freeze-pump-thaw method the vessel was transferred to an oil bath to start the polymerization. After 20 h the conversion was controlled by ^{1}H-NMR.

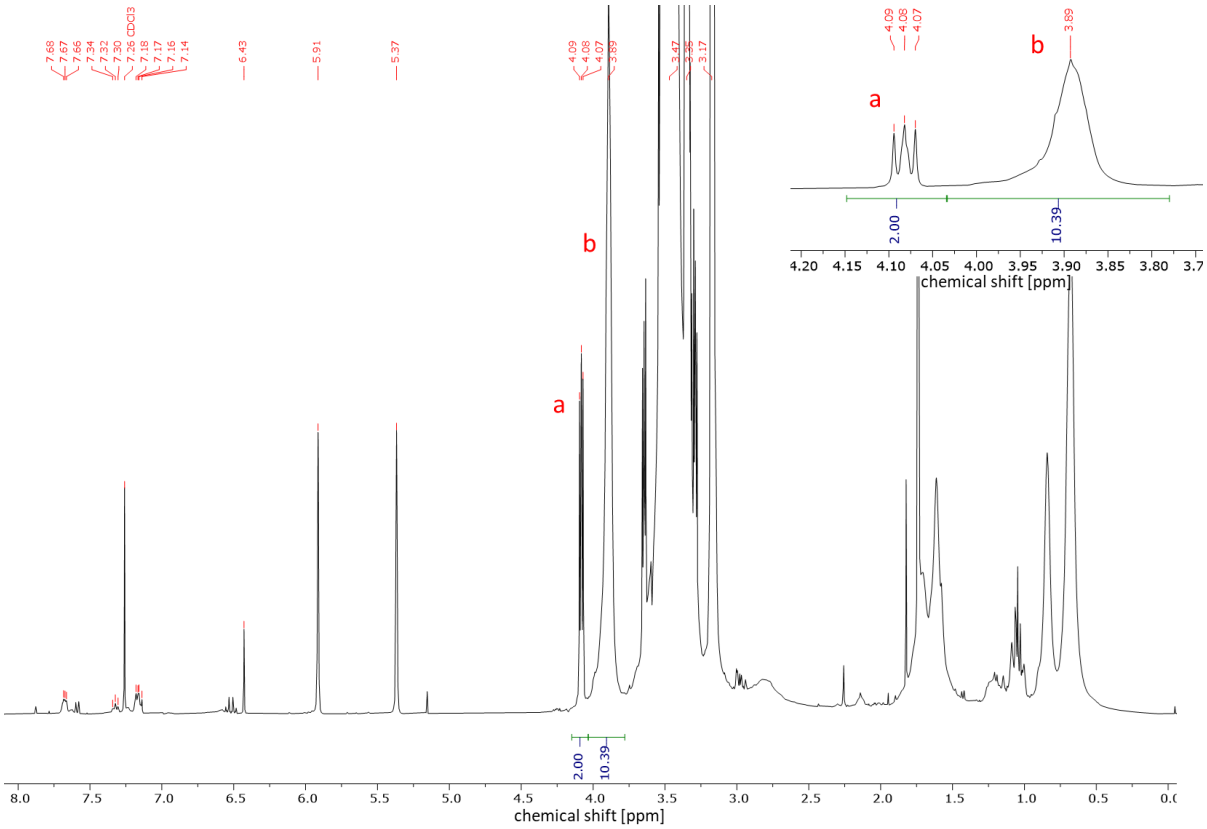


Figure S8. ^{1}H-NMR for determining the conversion of the polymerization reaction

For determining the monomer conversion, The signals a and b were compared:

$$P = \frac{I(b)}{I(a) + I(b)} = \frac{10.39}{2.00 + 10.39} = 0.8386$$

At about 84% conversion the reaction was quenched and the product was isolated by precipitation against n-hexane and dried in vacuo. 927.40 mg (0.0906 mmol, 97% yield) of the resulting highly viscous, red oil was collected and used as macro-CTA for following polymerizations.

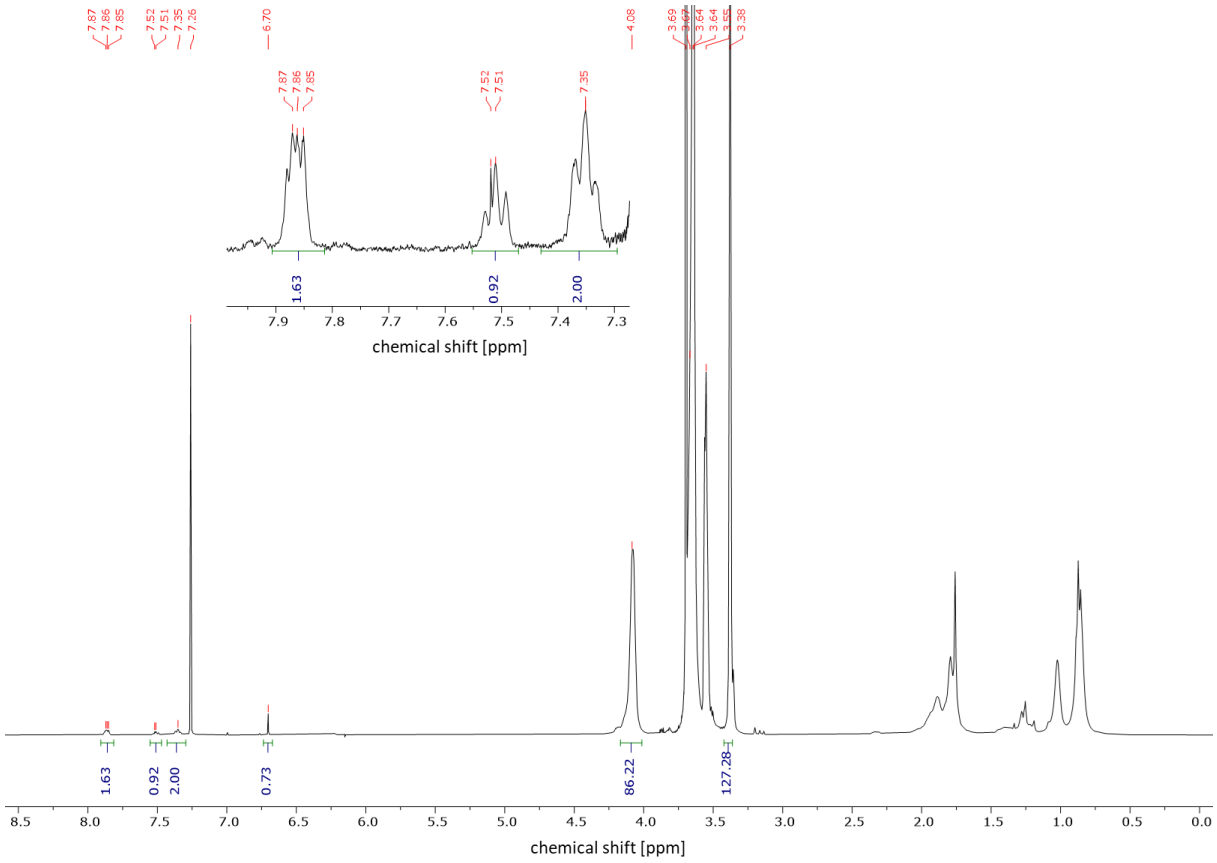


Figure S9. ^{1}H-NMR of azide-(MEO3MA)$_{42}$

^{1}H-NMR (400 MHz; CDCl3): δ [ppm] = 7.88-7.85 (m, 2 H); 6.70 (s, 1 H); 4.08 (m, X_n(x)·2 H); 3.69-3.64 (m, X_n(x)·8 H); 3.55 (m, X_n(x)·2 H); 3.38 (m, X_n(x)·3 H).

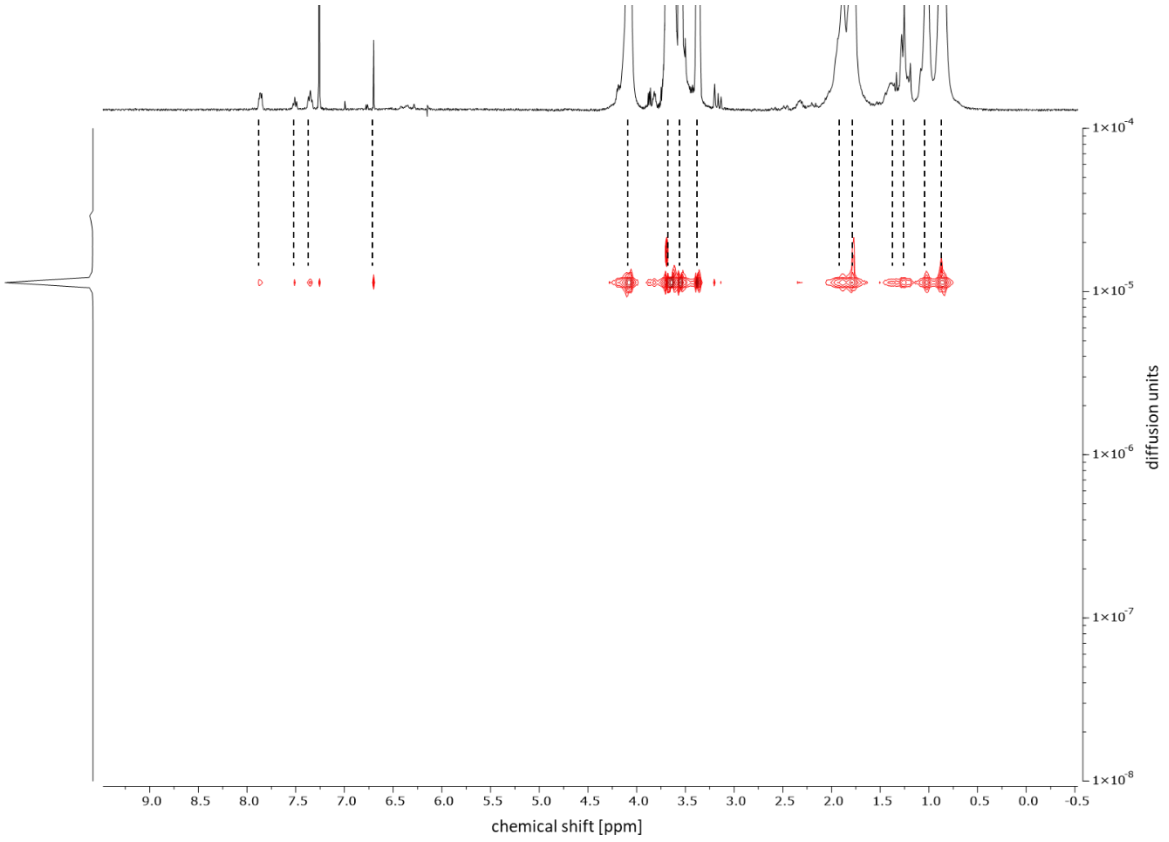


Figure S10. DOSY-NMR of azide-$(MEO_3MA)_{42}$

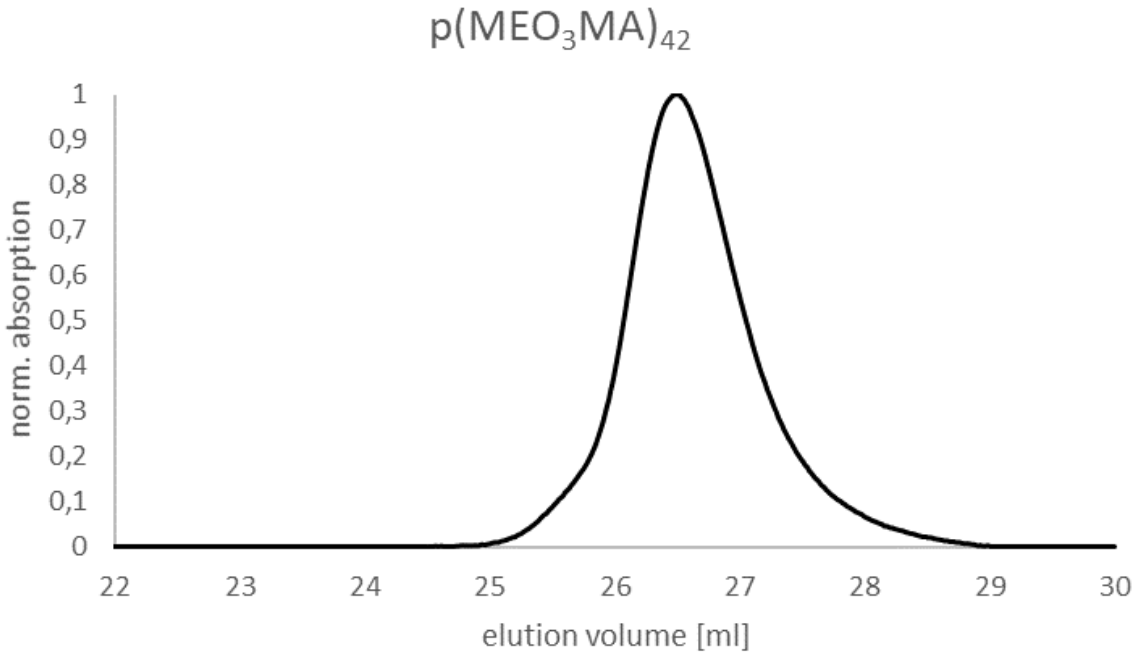


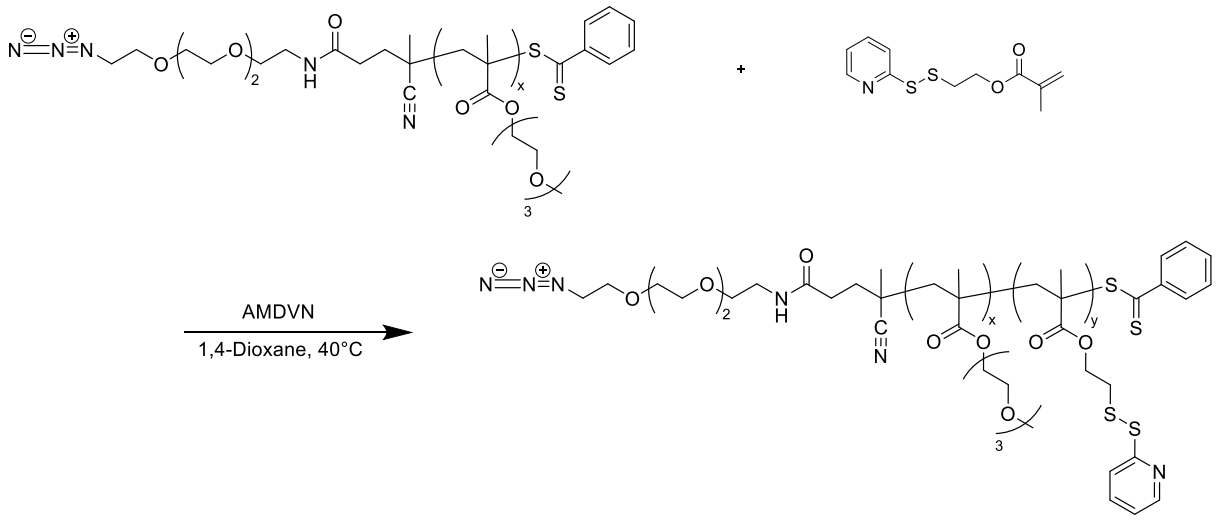
Figure S11. SEC of azide-$p(MEO_3MA)_{42}$.

Table S1. Characterization of p(MEO_3MA) homopolymers P5-1 – P5-4.

polymer	Composition	Mono: CTA: AMDVM	p [%][a]	M_n[b] [g/mol]	M_n[c][g/mol]	M_w[d] [g/mol]]	Đ[e]
P1-1	$P(MEO_3MA)_{42}$	46:1:0.2	85	10240	7650	9390	1.23
P1-2	$P(MEO_3MA)_{42}$	46:1:0.2	84	10240	7620	9240	1.21
P1-3	$P(MEO_3MA)_{52}$	62:1:0.2	83	12560	9410	11280	1.20
P1-4	$P(MEO_3MA)_{52}$	62:1:0.2	83	12560	9510	11350	1.19

[a]conversion of the MEO_3MA monomer. [b]number average molecular weight calculated from NMR data. [c]number average molecular weight as detrermined by SEC. [d]weight average molecular weight as determined by SEC. [e]polydispersity as determined by SEC.

Synthesis of azide-poly-(tri(ethlyenglycol)-methylether methacrylate)-*block*-(pyridyldisulfidethyl methacrylate) (azide-p(MEO_3MA)-*b*-p(PDSM)).



Scheme S6. Synthesis of azide-p(MEO3MA)-*b*-p(PDSM)

For the synthesis of the azide bearing diblock-copolymer, 927.4 mg (0.091 mmol) of the respective macro-CTA were dissolved in 1.0 ml 1,4-dioxane and transferred to a 25 ml Schlenk tube. After that, 356.0 mg (1.394 mmol) pyridyldisulfidethyl methacrylate in 1.0 ml 1,4-dioxane and 5.552 mg (0.018 mmol) AMDVN were added. The solution was then degassed using the

freeze-pump-thaw method and heated to 40°C to initialize the polymerization. After 23 h the conversion was controlled via ^{1}H-NMR.

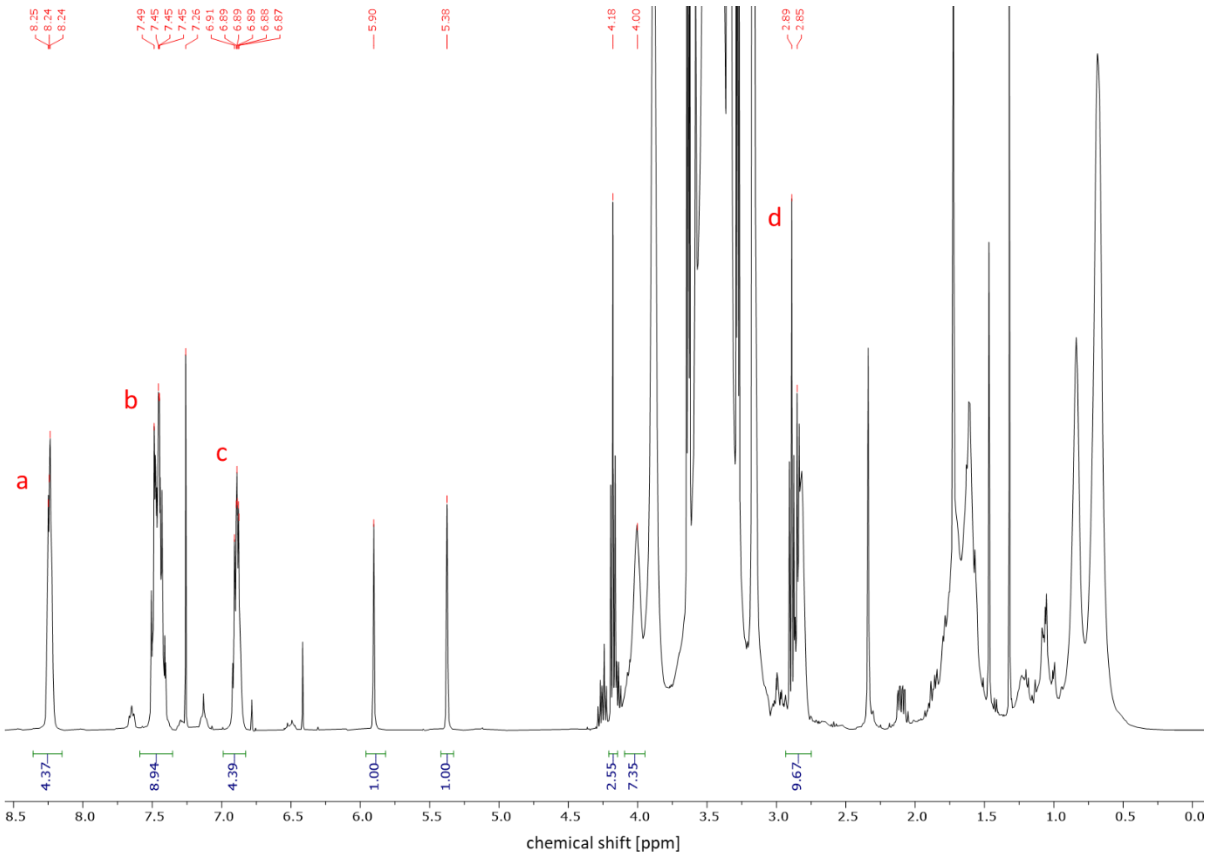


Figure S12. ^{1}H-NMR to determine PDSM monomere conversion.

For determining the conversion of the monomer, the superimposed NMR-signals of the monomer and the polymer species a, b, c, d were used. The respective contribution of the polymer signal to the superimposed signal was determined.

$$P = \frac{I(a) - 1.00 + I(b) - 2.00 + I(c) - 1.00 + I(d) - 2.00}{I(a) + I(b) + I(c) + I(d)}$$

$$= \frac{4.37 - 1.00 + 8.94 - 2.00 + 4.39 - 1.00 + 9.67 - 2.00}{4.37 + 8.94 + 4.39 + 9.67} = 0.7808$$

The reaction was quenched at 78% and, as described earlier, the product was isolated by precipitation against n-hexane and dried in vacuum overnight. 1153.5 mg (0.087 mmol, 92%

yield) of the product were obtained. The highly viscous, red product was then used as macro-CTA for the following polymerization.

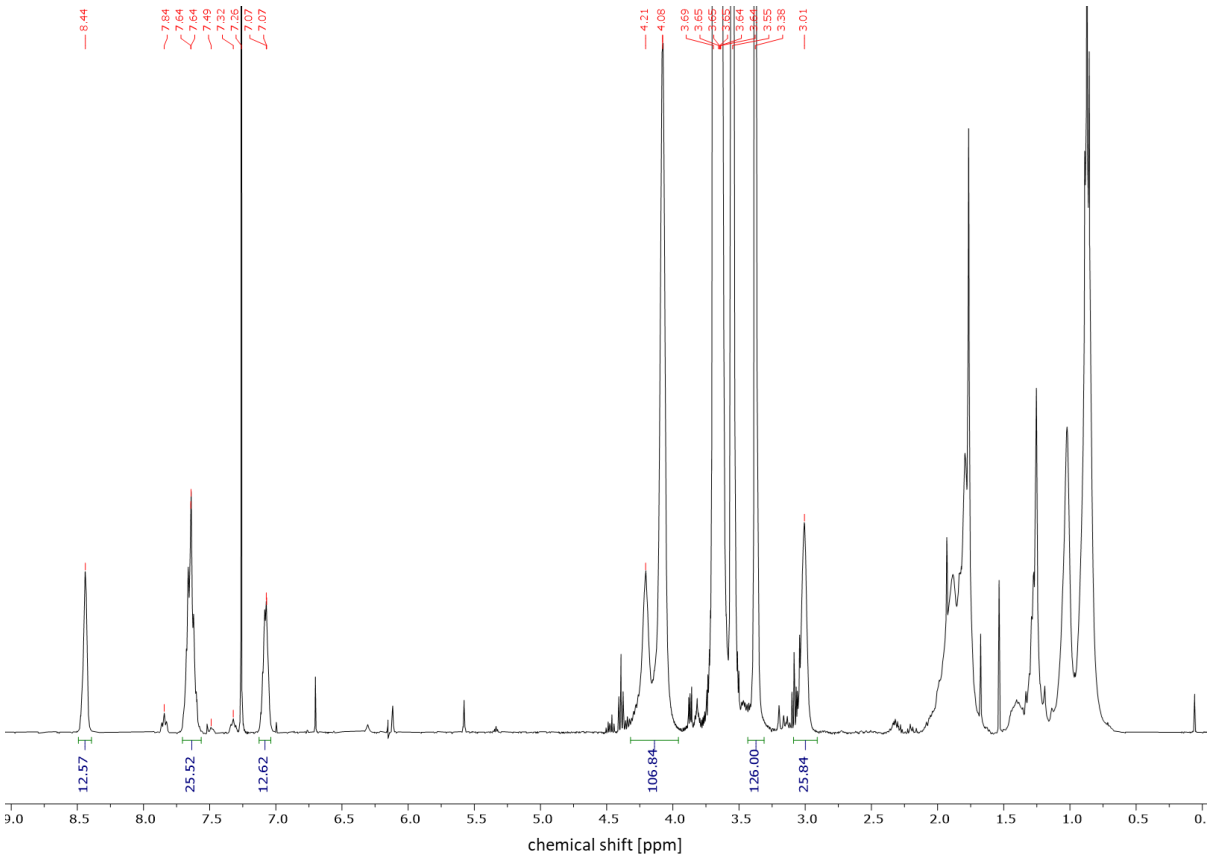


Figure S13. ^{1}H-NMR of azide-p(MEO$_3$MA)$_{42}$-*b*-p(PDSM)$_{12}$.

^{1}H-NMR (400 MHz; CDCl3): δ [ppm] = 8.44 (m, X_n(y)·1 H); 7.84 (m, 2 H); 7.64 (m, X_n(y)·2 H); 7.49 (m, 1 H); 7,32 (1 H); 7.07 (m, X_n(y)·1 H); 4.34-3.95 (m, (X_n(x) + X_n(y))·2 H), 3.69-3.64 (m, X_n(x)·8 H); 3.55 (m, X_n(x)·2 H); 3.38 (m, X_n(x)·3 H); 3.01 (m, X_n(y)·2 H).

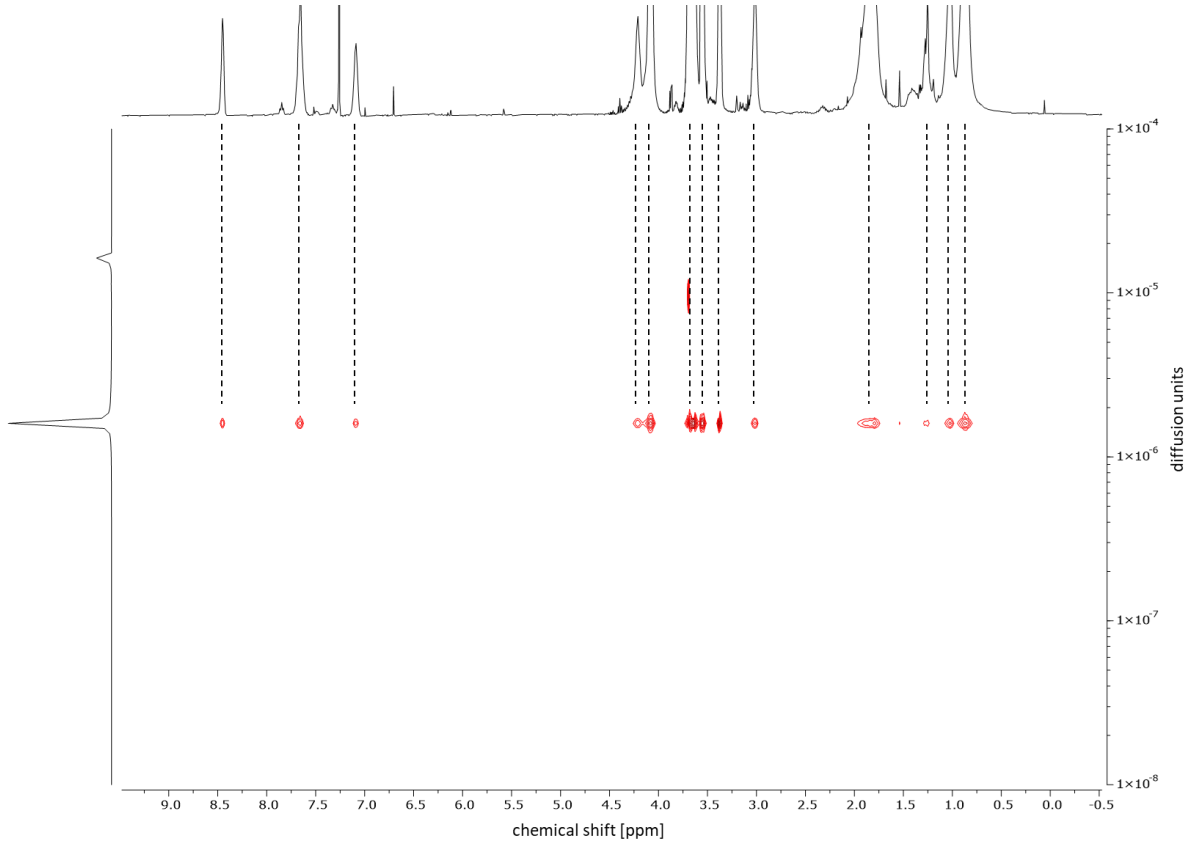


Figure S14. DOSY-NMR of azide-p(MEO_3MA)-*b*-p(PDSM)

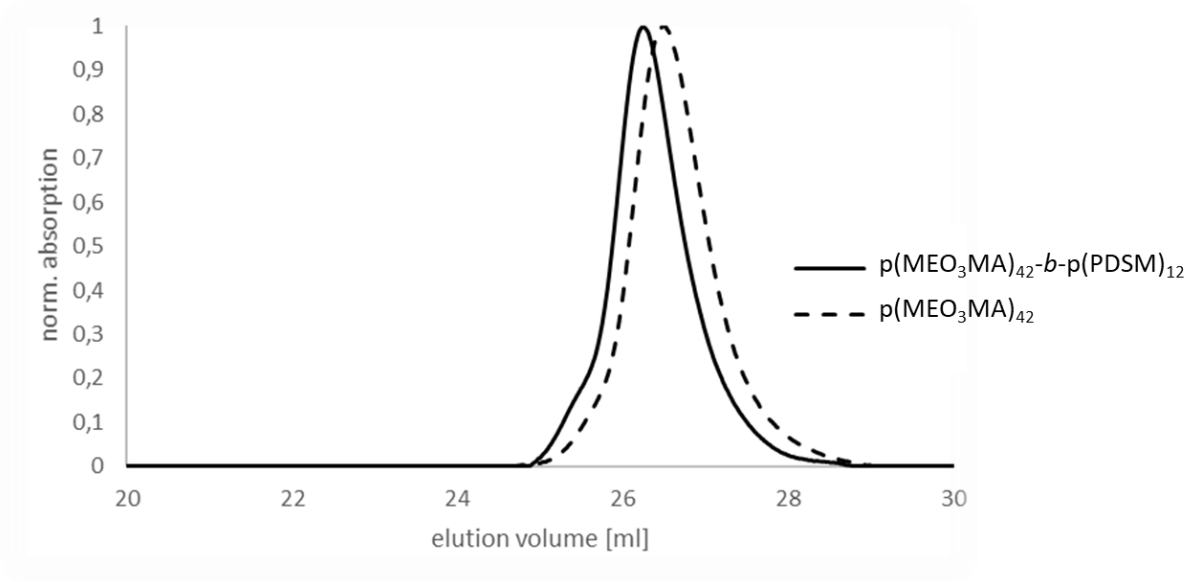


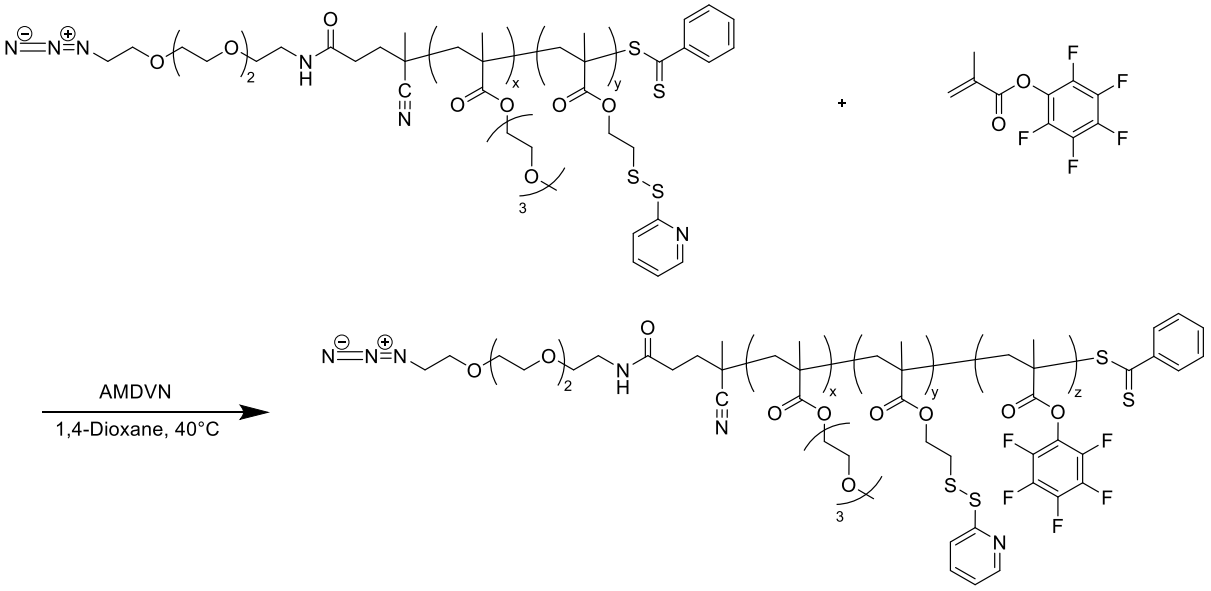
Figure S15. SEC of azide-p$(MEO_3MA)_{42}$-*b*-p$(PDSM)_{12}$ compared to the respective homopolymer.

Table S2. Characterization of p(MEO_3MA-*b*-PDSM) diblock-copolymers.

polymer	Composition	Mono: CTA: AMDVN	p [%][a]	M_N[b] [g/mol]	M_N[c] [g/mol]	M_W[d] [g/mol]	Đ[e]
P2-1	$P(MEO_3MA)_{42}$-*b*-$(PDSM)_{12}$	15:1:0.2	78	13300	9490	11300	1.19
P2-2	$P(MEO_3MA)_{42}$-*b*-$(PDSM)_{13}$	15:1:0.2	82	13370	8970	12090	1.18
P2-3	$P(MEO_3MA)_{52}$-*b*-$(PDSM)_{9}$	14:1:0.2	61	12410	10900	13120	1.20
P2-4	$P(MEO_3MA)_{52}$-*b*-$(PDSM)_{10}$	14:1:0.2	72	12650	11080	12990	1.17

[a]Conversion of the PDSM monomer. [b]Number average molecular weight, calculated from NMR data. [c]Number average molecular weight as determined by SEC. [d]Weight average molecular weight as determined by SEC. [e]Polydispersity as determined by SEC.

Synthesis of azide-poly((triethylenglycol-methylether methacrylate)-*block*-(pyridyldisulfidethyl methacrylate)-*block*-(pentafluorophenyl methacrylate) (azide-p(MEO_3MA-*b*-p(PDSM)-*b*-p(PFPMA)).



Scheme S7. Synthesis of azide-p(MEO3MA-*b*-p(PDSM)-*b*-p(PFPMA)

The azide-bearing triblock-copolymer was synthesized by dissolving 200.0 mg of the respective diblock-copolymer macro-CTA in 2.0 ml 1,4-dioxan. 349.0 mg (1.384 mmol) PFPMA was dissolved in 1.0 ml 1,4-dioxan and added to the reaction mixture. After the addition of 46.3 µl (0.003 mmol) of a 20.0 mg/ml stock solution of AMDVN in 1,4-dioxane, the polymerization was started by heating the reaction vessel to 40°C. The reaction was conducted over night. After 19 h a ^{1}H-NMR sample was taken to control the conversion.

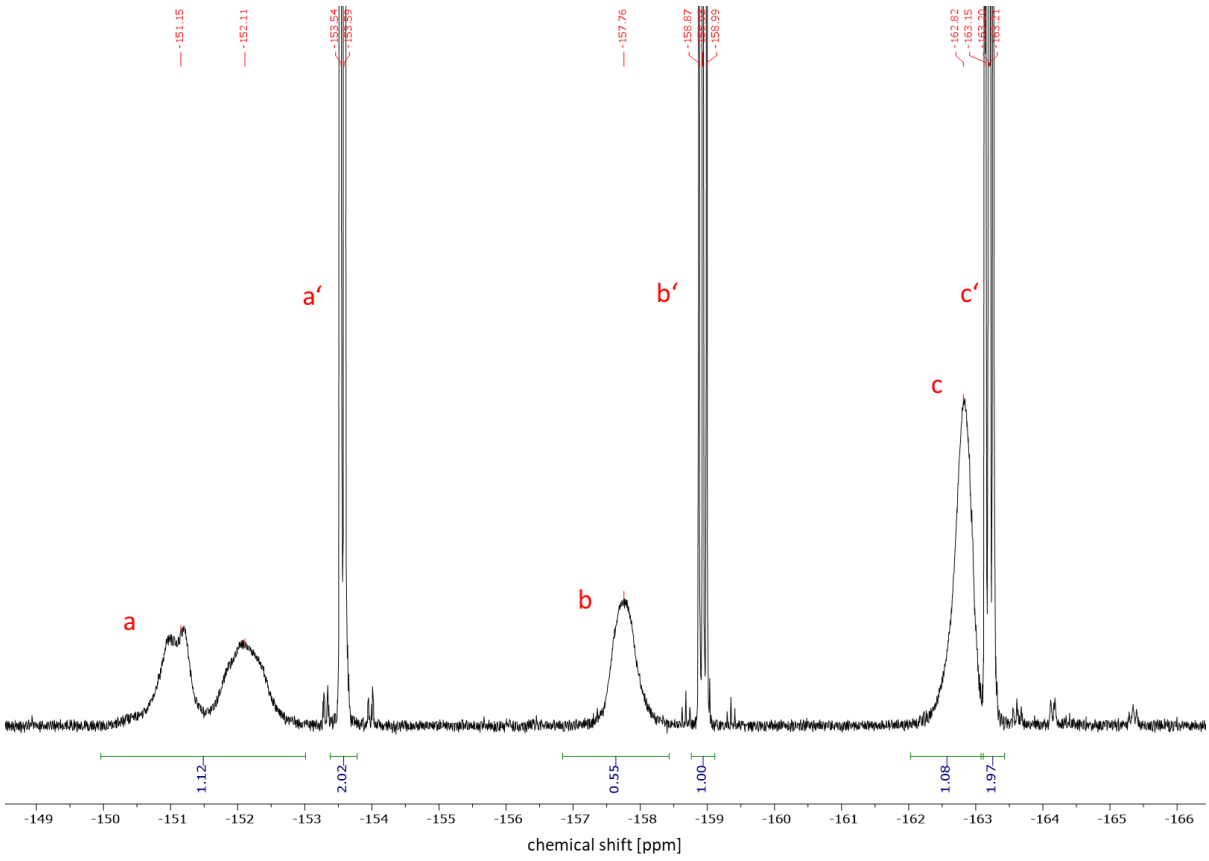


Figure S16. ^{19}F-NMR for determining PFPMA conversion.

To determine the monomer conversion, the intensities of the polymer signals was compared to the sum of the polymer and monomer signals:

$$P = \frac{I(a) + I(b) + I(c)}{I(a) + I(a') + I(b) + I(b') + I(c) + I(c')}$$

$$= \frac{1.12 + 0.55 + 1.08}{1.12 + 2.02 + 0.55 + 1.00 + 1.08 + 1.97} = 0.3553$$

At about 36% conversion the reaction was quenched by dipping the reaction vessel into liquid nitrogen. The light red, wax like product was isolated and purified by precipitation against n-hexane and dried overnight in vacuo. 271.0 mg (0.013 mmol, 87%) of the product were obtained.

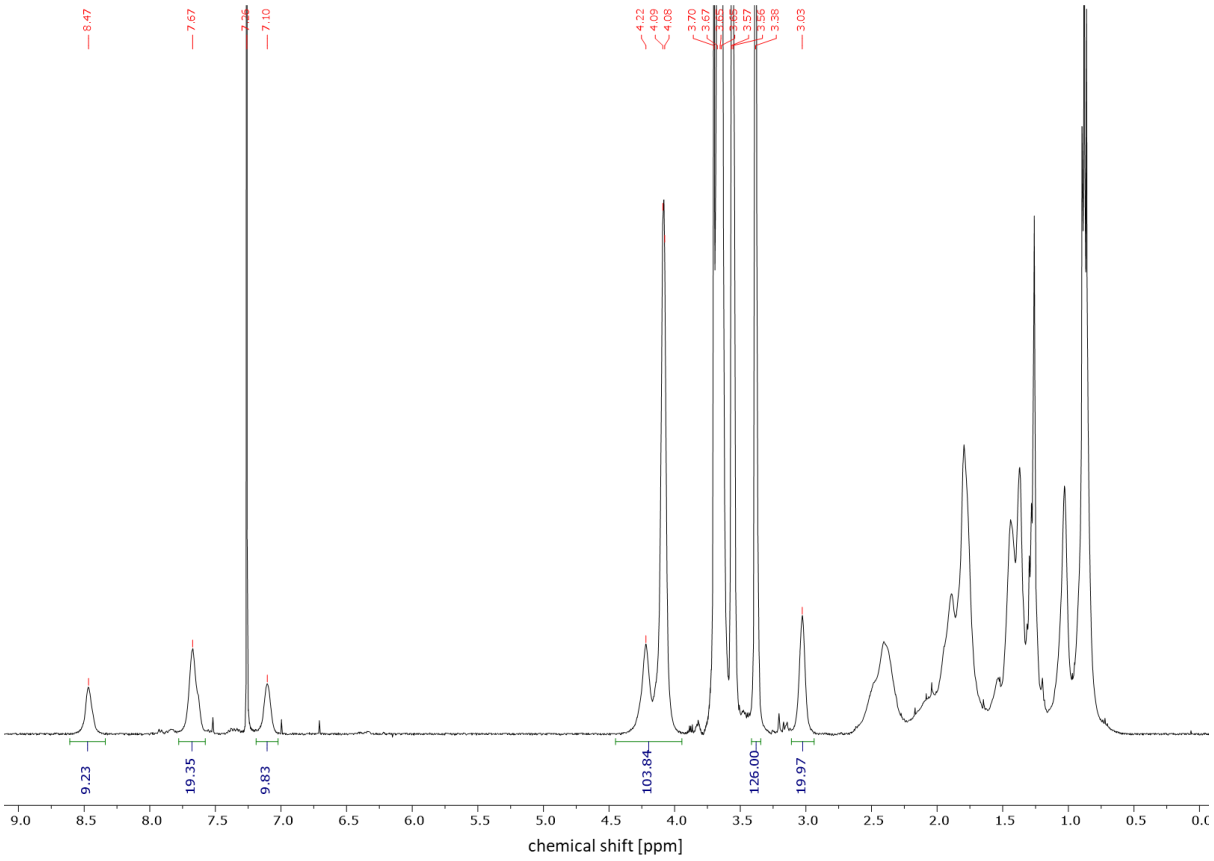


Figure S17. ^{1}H-NMR of azide-p(MEO$_3$MA)-*b*-p(PDSM)$_{12}$-p(PFPMA)$_{33}$

^{1}H-NMR (400 MHz; CDCl3): δ [ppm] = 8.47 (m, X_n(y)·1 H); 7.67 (m, X_n(y)·2 H); 7.10 (m, X_n(y)·1 H); 4.34-3.95 (m, (X_n(x) + X_n(y))·2 H); 3.70-3.65 (m, X_n(x)·8 H); 3.56 (m, X_n(x)·2 H); 3.38 (m, X_n(x)·3 H); 3.03 (m, X_n(y)·2 H).

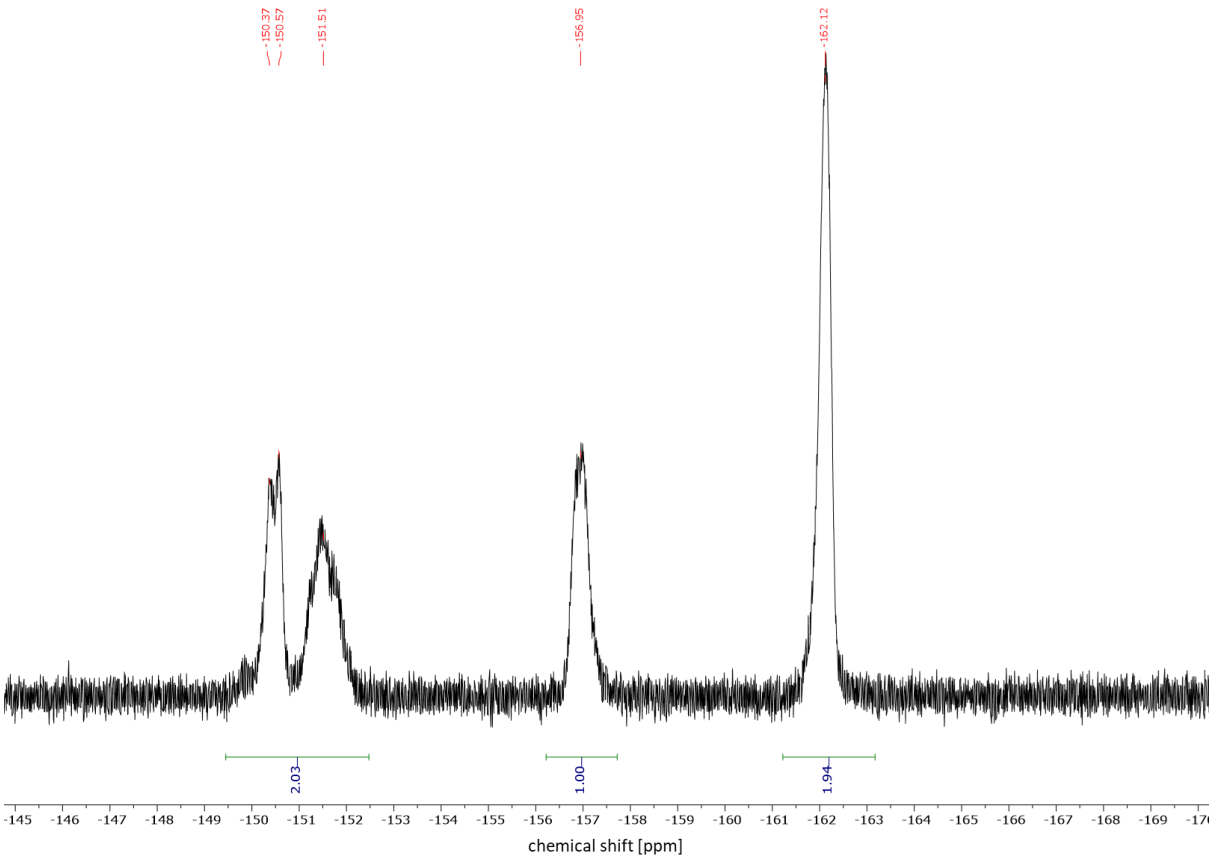


Figure S18. ^{19}F-NMR of azide-p(MEO$_3$MA)-*b*-p(PDSM)$_{12}$-p(PFPMA)$_{33}$.

^{19}F-NMR (400 MHz; CDCl3): δ [ppm] = -149.96-(-152.31) (m, X_n(z)·2 F); -156.95 (m, X_n(z)·1 F); -162.12 (m, X_n(z)·2 F).

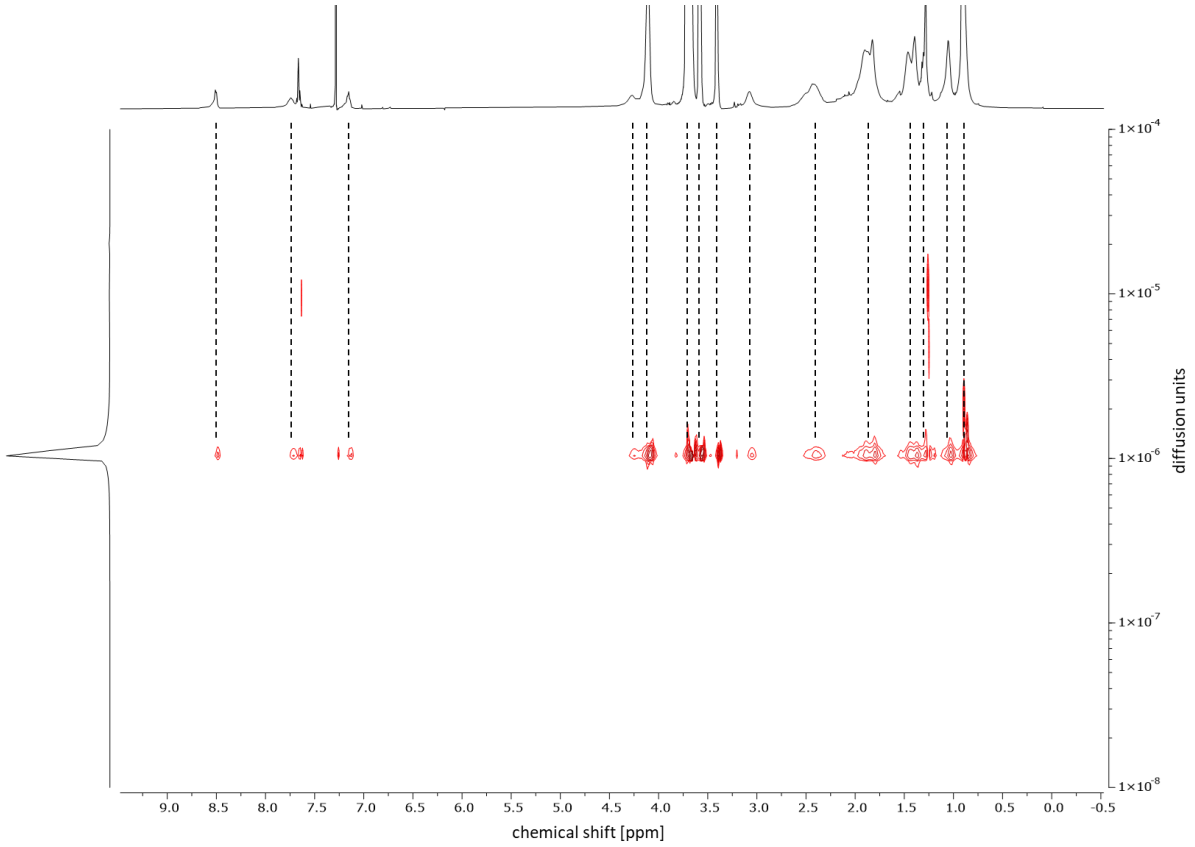


Figure S19. DOSY-NMR of azide-p(MEO_3MA)-*b*-p(PDSM)$_{12}$-p(PFPMA)$_{33}$.

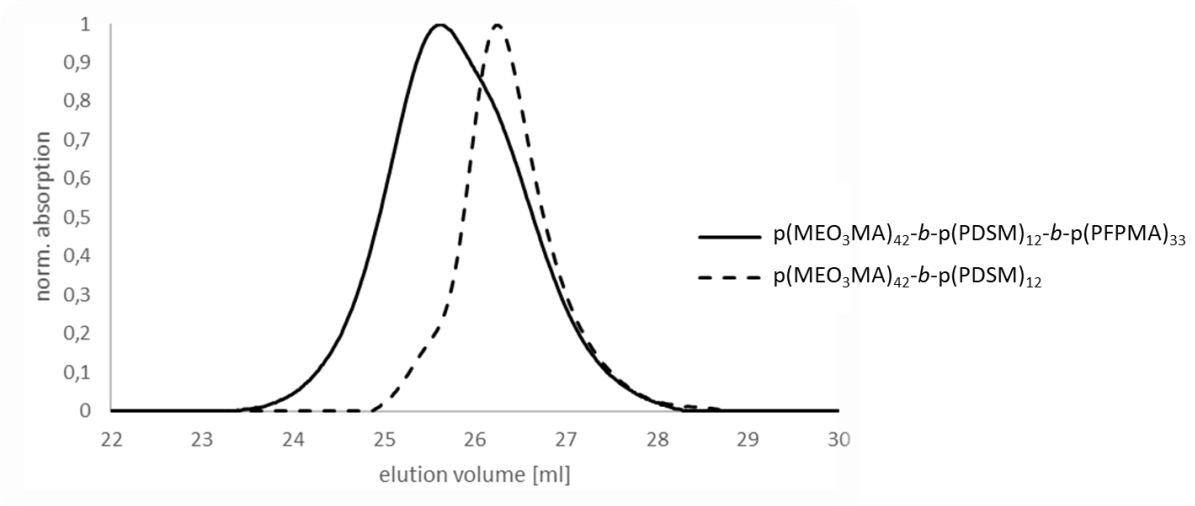


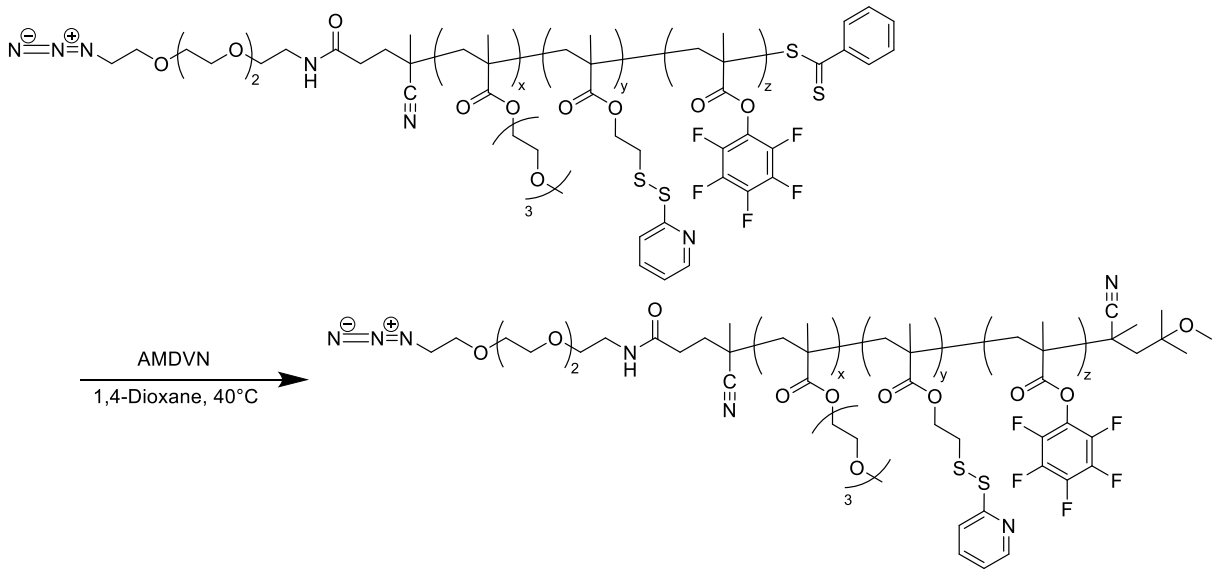
Figure S20. SEC of azide-p(MEO_3MA)$_{42}$-*b*-p(PDSM)$_{12}$-*b*-p(PFPMA)$_{33}$.

Table S3. Characterization of p(MEO_3MA-*b*-PDSM-*b*-PFPMA) triblock-copolymers

polymer	Composition	Mono: CTA: AMDVN	p [%][a]	M_N[b] [g/mol]	M_N[c] [g/mol]	M_W[d] [g/mol]	Đ[e]
P3-1	P$(MEO_3MA)_{42}$-*b*-P$(PDSM)_{12}$-*b*-P$(PFPMA)_{33}$	92:1:0.2	36	21620	14890	18840	1.27
P3-2	P$(MEO_3MA)_{42}$-*b*-P$(PDSM)_{12}$-*b*-P$(PFPMA)_{35}$	92:1:0.2	38	22120	15350	19860	1.29
P3-3	P$(MEO_3MA)_{42}$-*b*-P$(PDSM)_{12}$-*b*-P$(PFPMA)_{33}$	92:1:0.2	36	21620	13440	17710	1.32
P3-4	P$(MEO_3MA)_{42}$-*b*-P$(PDSM)_{12}$-*b*-P$(PFPMA)_{28}$	92:1:0.2	31	20360	14800	19750	1.33
P3-5	P$(MEO_3MA)_{42}$-*b*-P$(PDSM)_{13}$-*b*-P$(PFPMA)_{49}$	116:1:0.2	42	25650	15930	21600	1.36
P3-6	P$(MEO_3MA)_{42}$-*b*-P$(PDSM)_{13}$-*b*-P$(PFPMA)_{50}$	116:1:0.2	44	25910	16040	20930	1.31
P3-7	P$(MEO_3MA)_{42}$-*b*-P$(PDSM)_{13}$-*b*-P$(PFPMA)_{88}$	203:1:0.2	43	35490	19800	26760	1.35
P3-8	P$(MEO_3MA)_{42}$-*b*-P$(PDSM)_{13}$-*b*-P$(PFPMA)_{101}$	240:1:0.2	42	38770	23420	32300	1.38

[a]Conversion of the PFPMA monomer. [b]Number average molecular weight, calculated from NMR data. [c]Number average molecular weight as determined by SEC. [d]Weight average molecular weight as determined by SEC. [e]Polydispersity as determined by SEC.

Deactivation of the CTA end group.



Scheme S8. Deactivation of the CTA end group

To remove the end group, 256.6 mg (0.012 mmol) of the triblock-copolymer were dissolved in 1.5 ml 1,4-dioxane under argon atmosphere. 55.1 mg (0.179 mmol; 15 equivalents) of AMDVN in 1.0 ml 1,4-dioxane were added. The reaction mixture was stirred vigorously at 40°C oil bath temperature and the reaction vessel was flushed with a continuous flow of nitrogen for 14 h. During the process, the light red solution turned colorless. 227.2 mg (0.011 mmol, 92% yield) of the colorless, wax like product could be isolated by precipitation in n-hexane and subsequent lyophilization. The absence of the reactive end group was confirmed by UV-Vis spectroscopy.

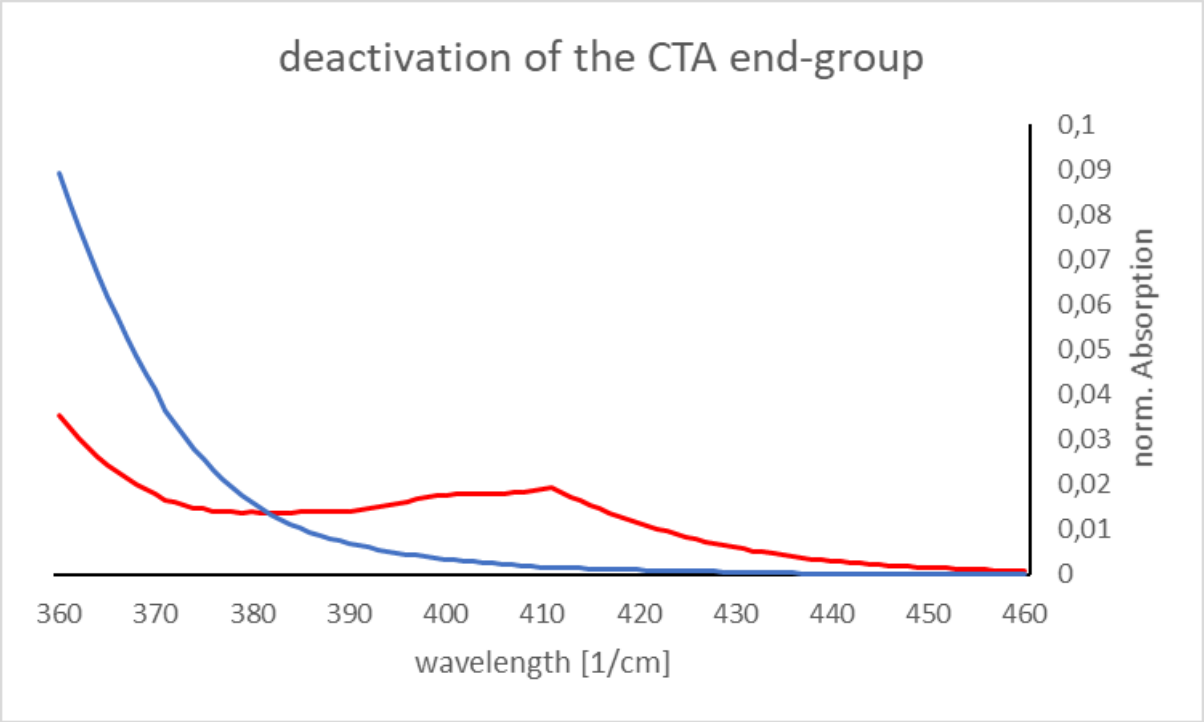


Figure S21. UV-Vis spectrum as reaction control of the deactivation reaction. Red curve: UV-Vis absorption signal before deactivation. Blue curve: UV-Vis absorption signal after the deactivation reaction.

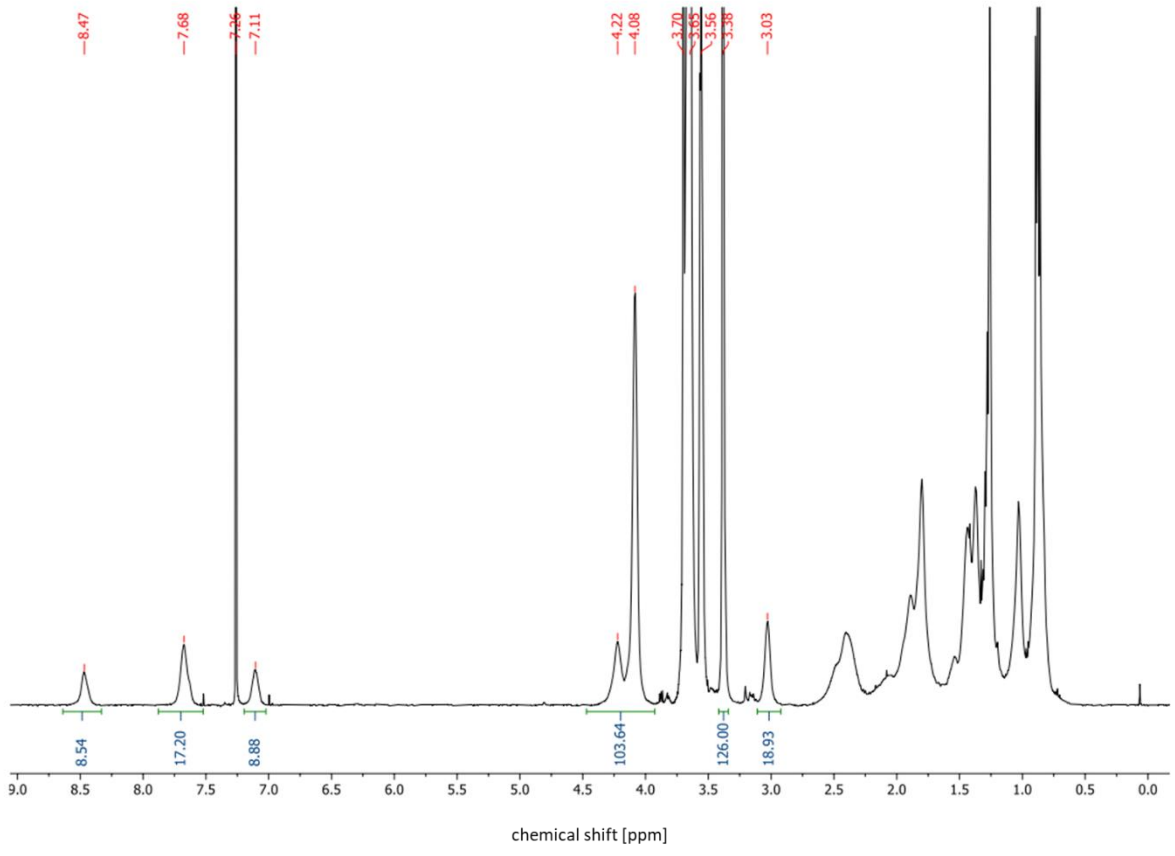


Figure S22. ^{1}H-NMR of the deactivated azide-p(MEO_3MA)$_{42}$-*b*-p(PDSM)$_{12}$-*b*-p(PFPMA)$_{33}$.

^{1}H-NMR (400 MHz; CDCl3): δ [ppm] = 8.47 (m, X_n(y)·2 H); 7.68 (m, X_n(y)·1 H); 7.11 (m, X_n(y)·2 H); 4.34-3.95 (m, (X_n(y) + X_n(y))·2 H); 3.70-3.65 (m, X_n(x)·8 H); 3.56 (m, X_n(x)·2 H); 3.38 (m, X_n(x)·3 H); 3.03 (m, X_n(y)·2 H).

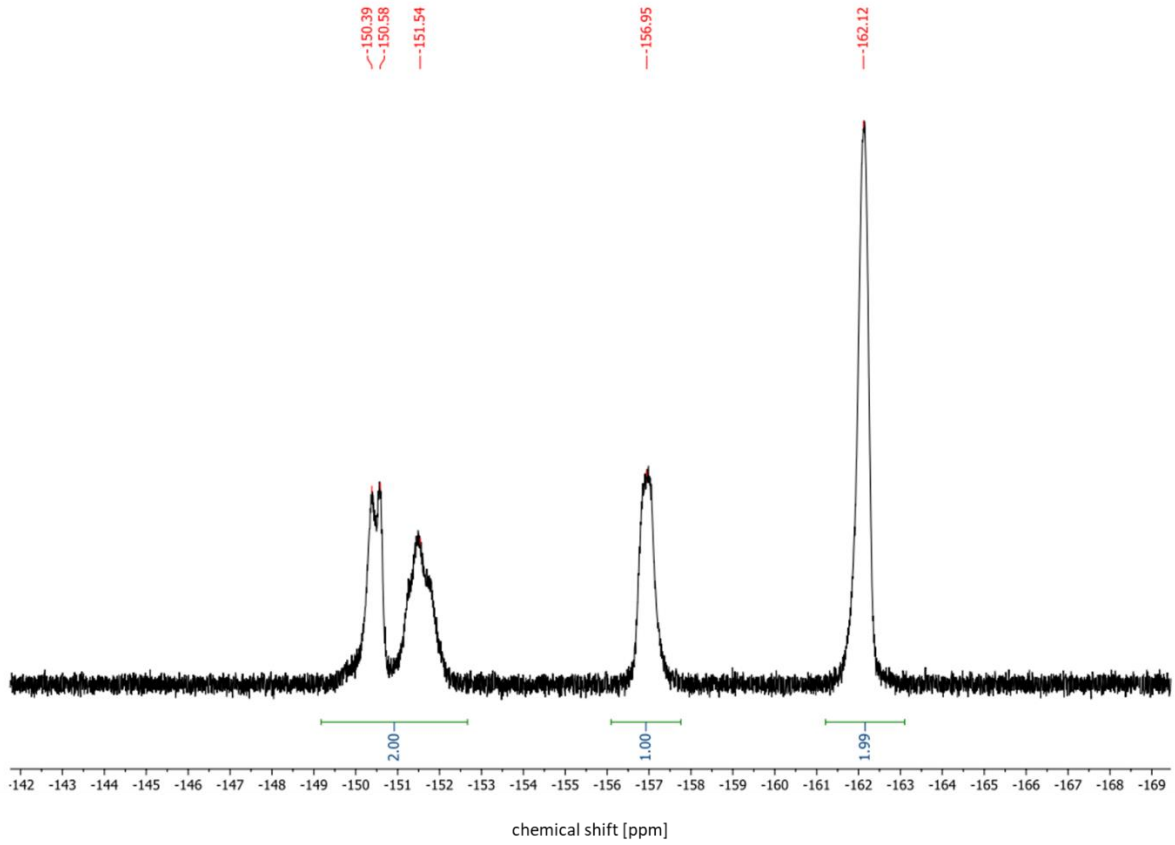


Figure S23. ^{19}F-NMR of of the deactivated azide-p(MEO$_3$MA)$_{42}$-*b*-p(PDSM)$_{12}$-*b*-p(PFPMA)$_{33}$.

^{19}F-NMR (400 MHz; CDCl3): δ [ppm] = -149.58-(-152.47) (m, X_n(z)·2 F); -156.95 (m, X_n(z)·1 F); -162.12 (m, X_n(z)·2 F).

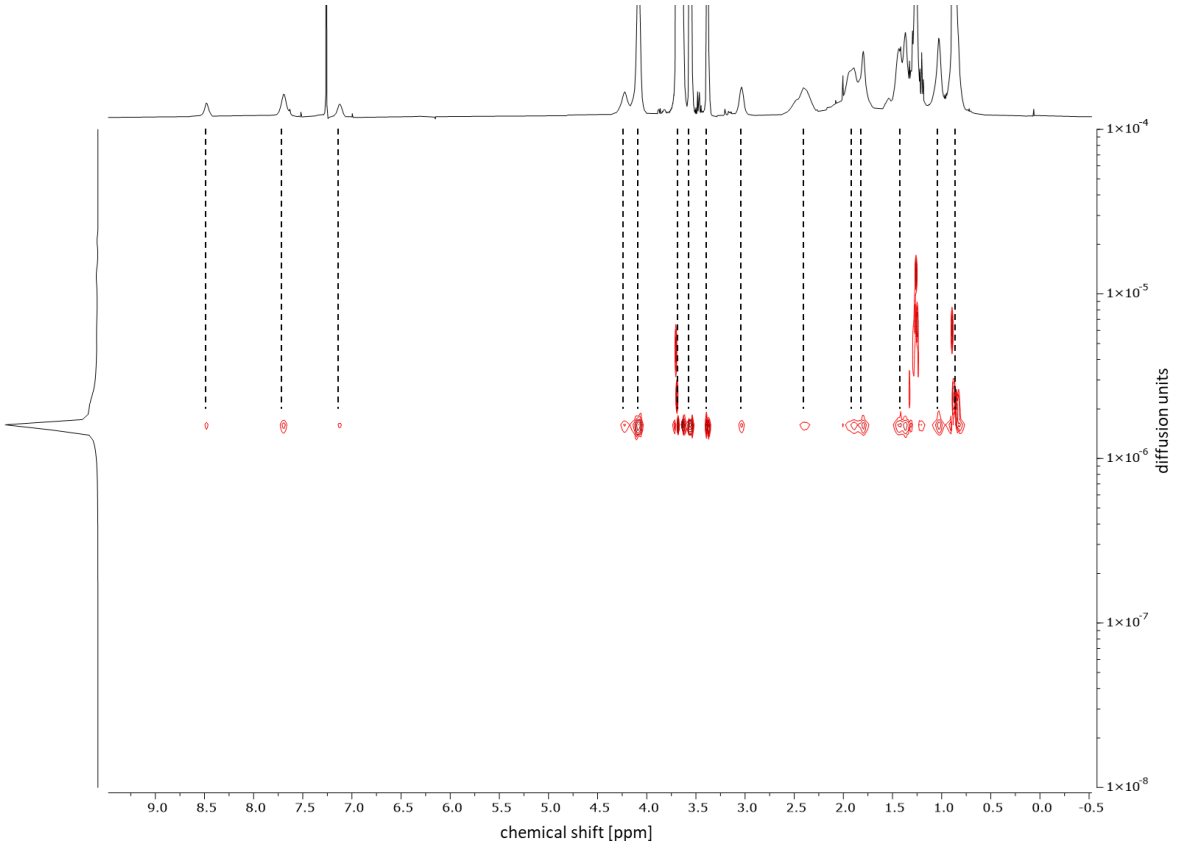


Figure S24. DOSY-NMR of the deactivated azide-p(MEO_3MA)$_{42}$-*b*-p(PDSM)$_{12}$-*b*-p(PFPMA)$_{33}$.

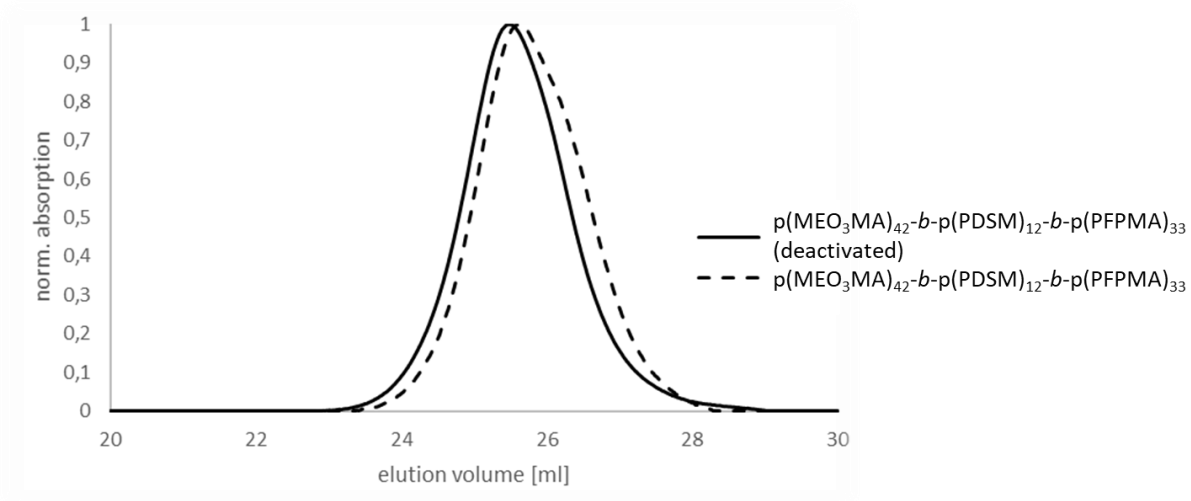
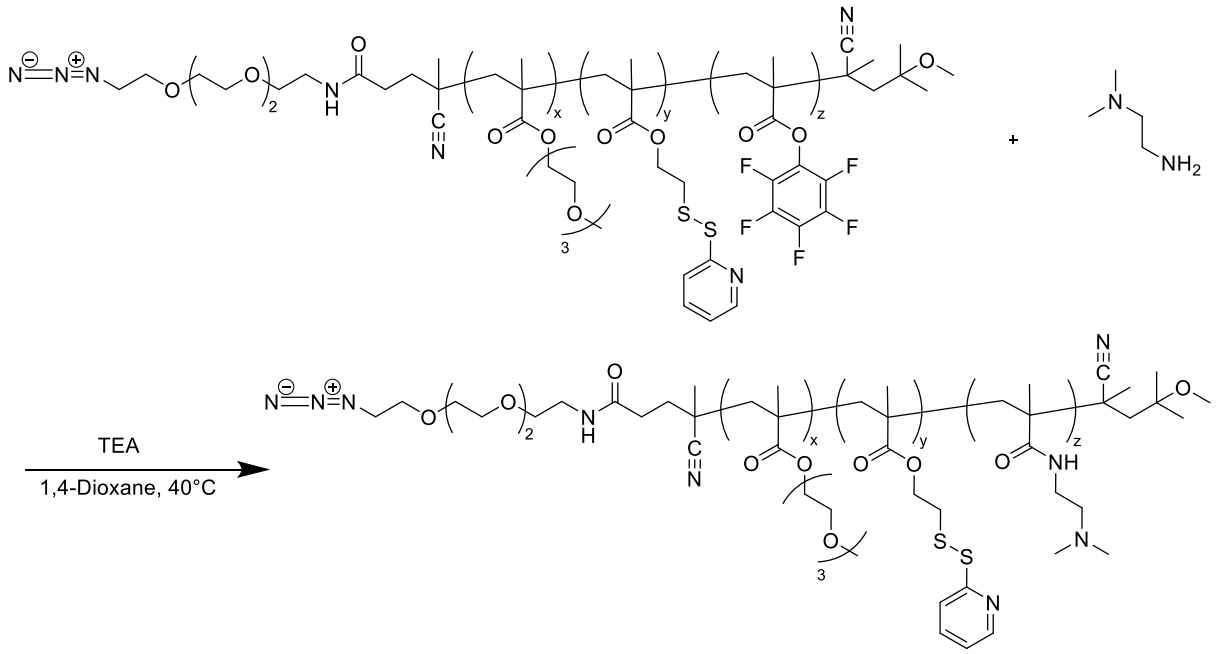


Figure S25. SEC of the deactivated azide-p(MEO_3MA)$_{42}$-*b*-p(PDSM)$_{12}$-*b*-p(PFPMA)$_{33}$.

Amidation of the PFPMA chain units.



Scheme S9. Amidation of the PFPMA side chain units with DMEDA

200.0 mg (9.256 µmol) of the deactivated triblock-copolymer were dissolved in 2.0 ml 1,4-dioxane in a 25 ml Schlenk tube under argon atmosphere. Subsequently, 52.6 mg (0.520 mmol) triethylamine (TEA) in 250 µl 1,4-dioxane and 38.4 mg (0.436 mmol) N,N-dimethyl ethylendiamine (DMEDA) in 250 µl 1,4-dioxane were added to the reaction solution. The reaction vessel was heated to 40°C. After 14 h 16.3 mg (0.185 mmol) DMEDA in 250 µl 1,4-dioxane and 26.1 mg TEA in 250 µl 1,4-dioxane were added to the solution. After further 22 h a ^{1}H-NMR sample was taken to confirm full conversion.

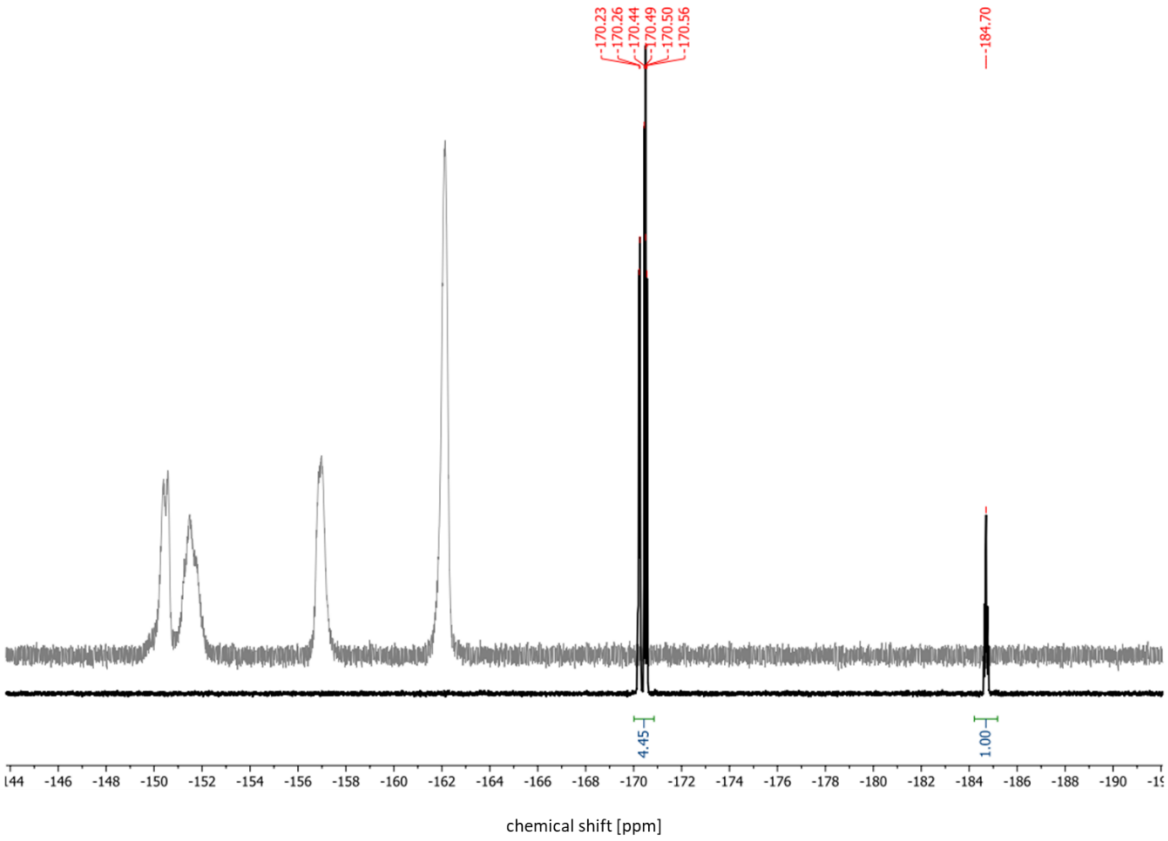


Figure S26. ^{19}F-NMR confirming full conversion of the PFPMA side chains. Black curve: signals of free pentafluorophenolate. Grey curve: NMR-signals of the PFPMA side chain before modification.

132.4 mg (7.179 µmol, 78% yield) of the colorless, wax like product were obtained after repeated precipitation in n-hexane and after lyophilization.

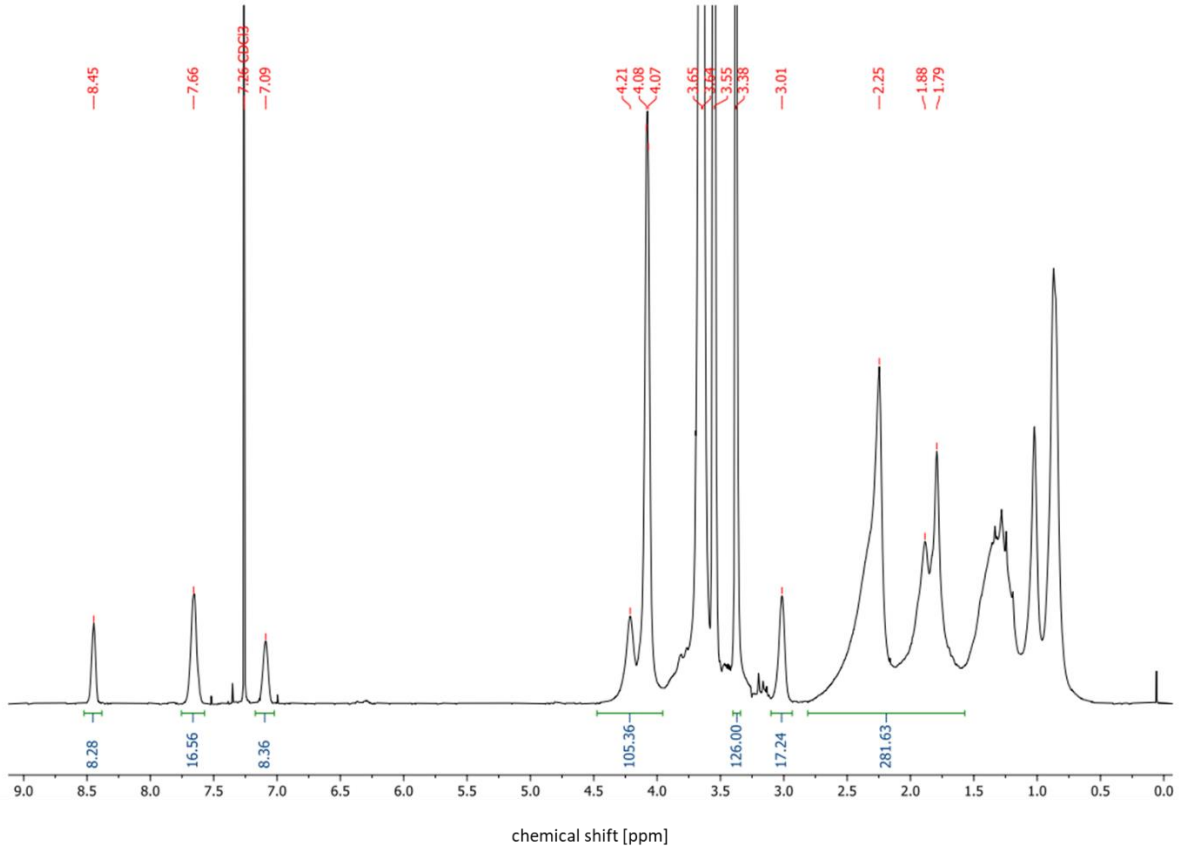


Figure S27. ^{1}H-NMR of azide-p(MEO$_3$MA)$_{42}$-*b*-p(PDSM)$_{12}$-*b*-p(DMEDAMA)$_{33}$.

^{1}H-NMR (400 MHz; CDCl3): δ [ppm] = 8.45 (m, X_n(y)·2 H); 7.66 (m, X_n(y)·1 H); 7.09 (m, X_n(y)·2 H); 4.34-3.95 (m, (X_n(y) + X_n(y))·2 H); 3.70-3.64 (m, X_n(x)·8 H); 3.55 (m, X_n(x)·2 H); 3.38 (m, X_n(x)·3 H); 3.01 (m, X_n(y)·2 H); 2.80-1.60 (m, X_n(z)·8 H).

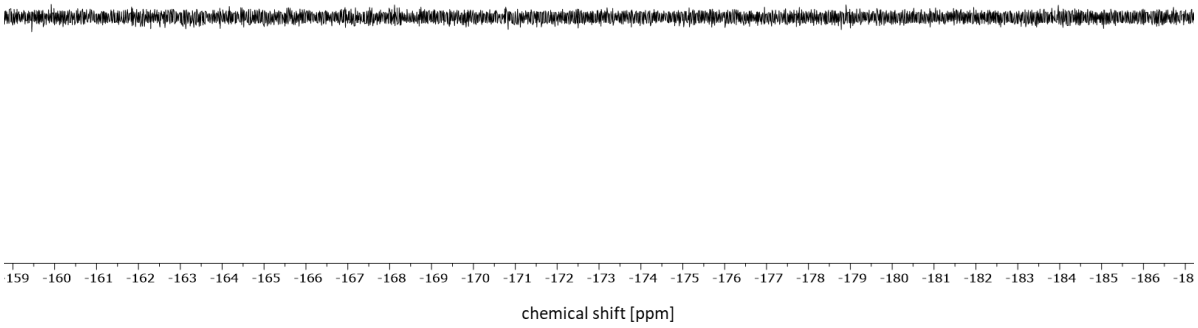


Figure S28. ^{19}F-NMR confirming absence of PFPMA side chains in azide-p(MEO_3MA)$_{42}$-*b*-p(PDSM)$_{12}$-*b*-p(DMEDAMA)$_{33}$.

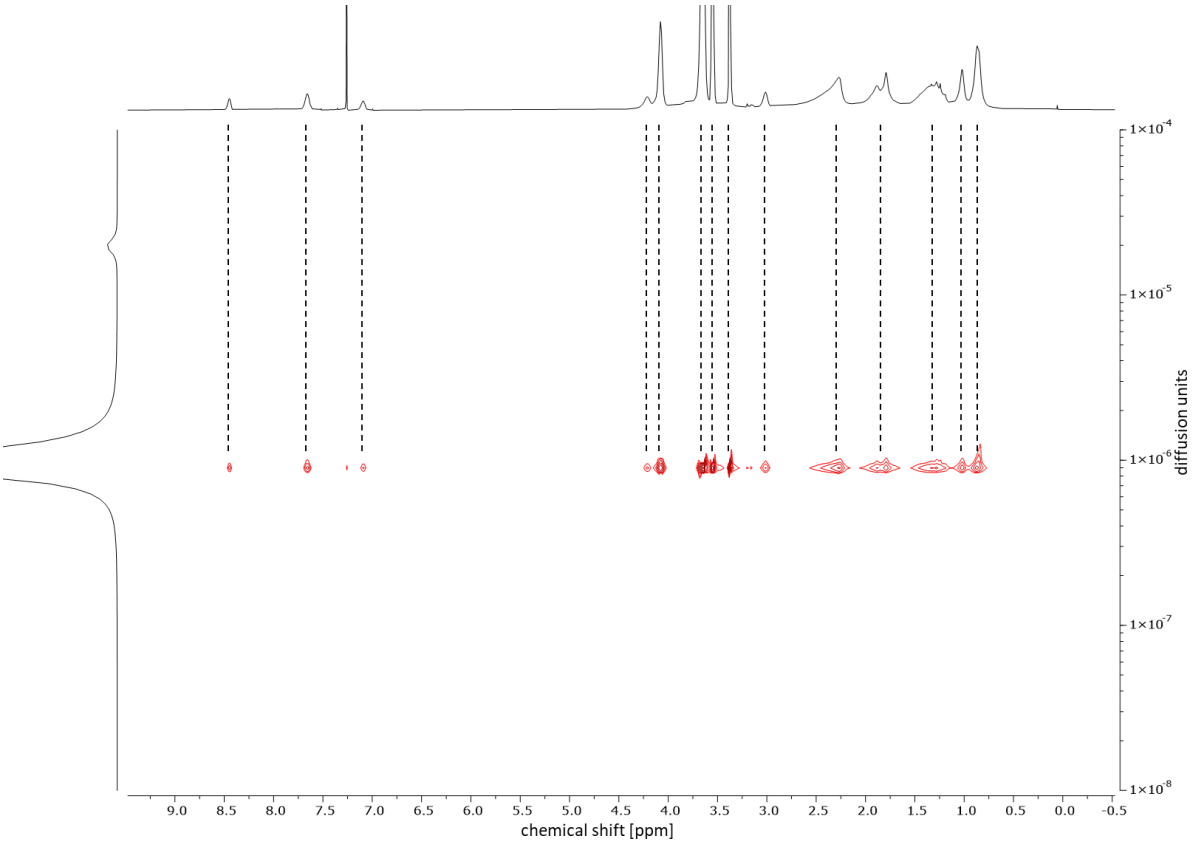


Figure S29. DOSY-NMR of azide-p(MEO_3MA)$_{42}$-*b*-p(PDSM)$_{12}$-*b*-p(DMEDAMA)$_{33}$.

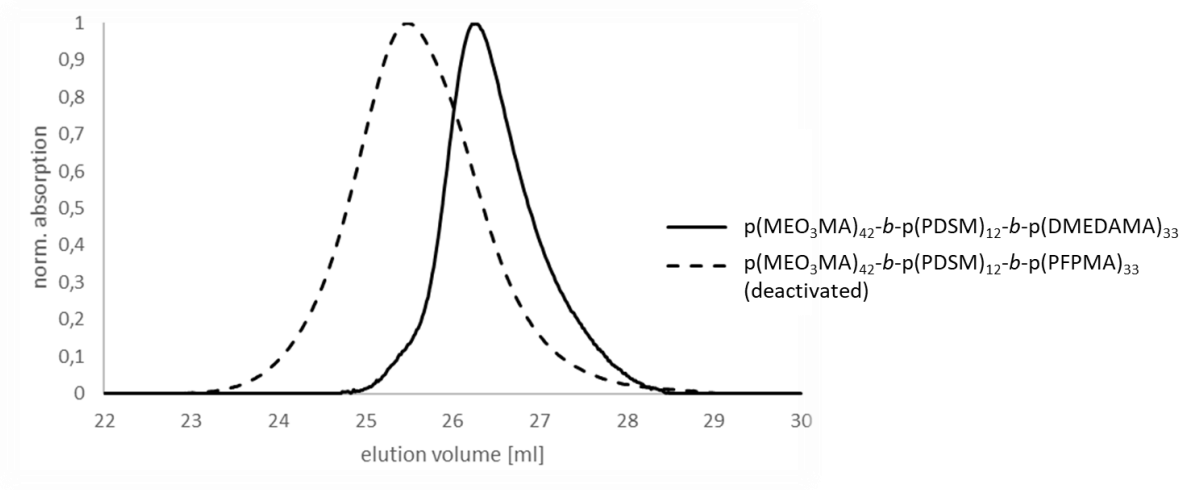


Figure S30. SEC of azide-p(MEO_3MA)$_{42}$-*b*-p(PDSM)$_{12}$-*b*-p(DMEDAMA)$_{33}$.

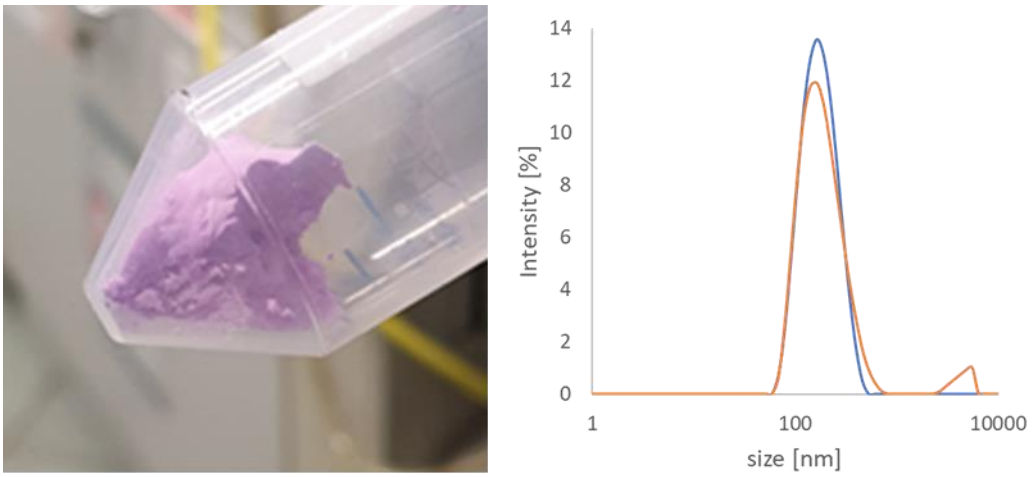


Figure S31. Azide-p(MEO_3MA)-*b*-p(PDSM)-*b*-p(DMEDAMA) after modification with TexasRed cadaverine (sulforhodamine 101 cadaverine). Polyplexes were synthesized in 10 mM NaCl solution. Orange: DLS of a pGL3-Basic polyplex (P5-1, N/P = 3) with 100% DMEDA functionalization. Blue: DLS of a pGL3-Basic polyplex (P5-1, N/P = 3) with 1 % Texas Red cadaverine (sulforhodamine 101 cadaverine) and 99% DMEDA functionalization.

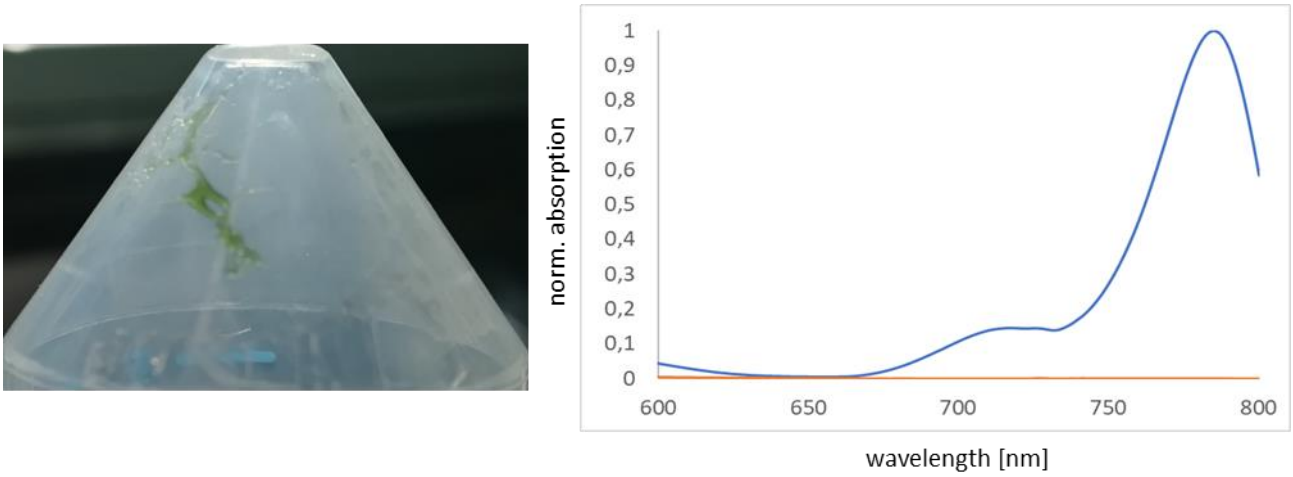
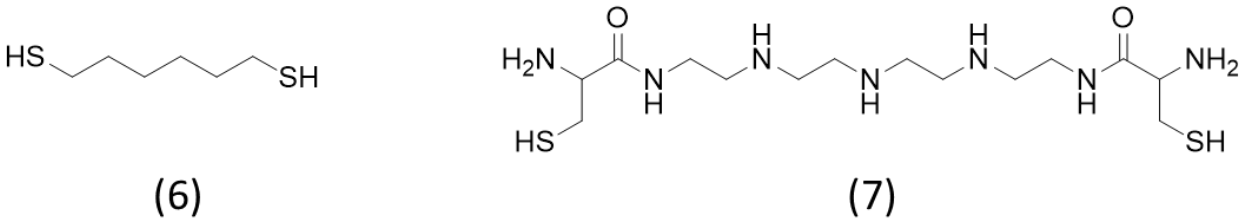


Figure S32. UV-VIS spectroscopy: modification of azide-p(MEO_3MA)-*b*-p(PDSM)-*b*-p(DMEDAMA) with the IR-dye CW800-DBCO. Orange: UV-VIS signal of the polymer before modification; Blue: UV-VIS signal of the polymer after modification, dialysis and lyophilization.

Polyplex formation



(6) (7)

Scheme S10. Structure of crosslinking agents: 1,6-hexanedithiol (6) and C-TEPA-C (7)

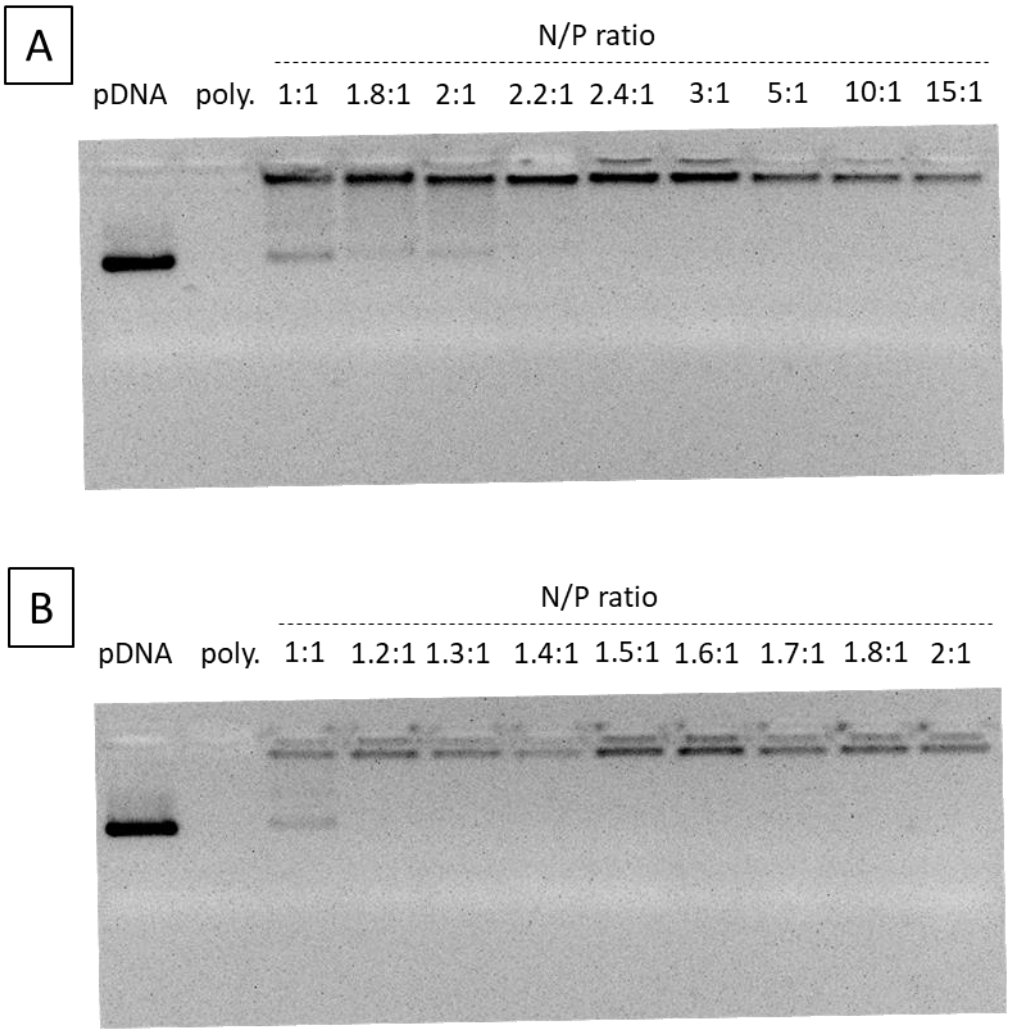


Figure S33. Agarose gel electrophoresis of azide-p(MEO_3MA)$_{42}$-*b*-p(PDSM)$_{12}$-*b*-p(DMEDAMA)$_{33}$ (P5-1) polyplexes with pGL3-BASIC pDNA. (A) Non-crosslinked polyplexes. (B) Polyplexes crosslinked with 1,6-hexanedithiol.

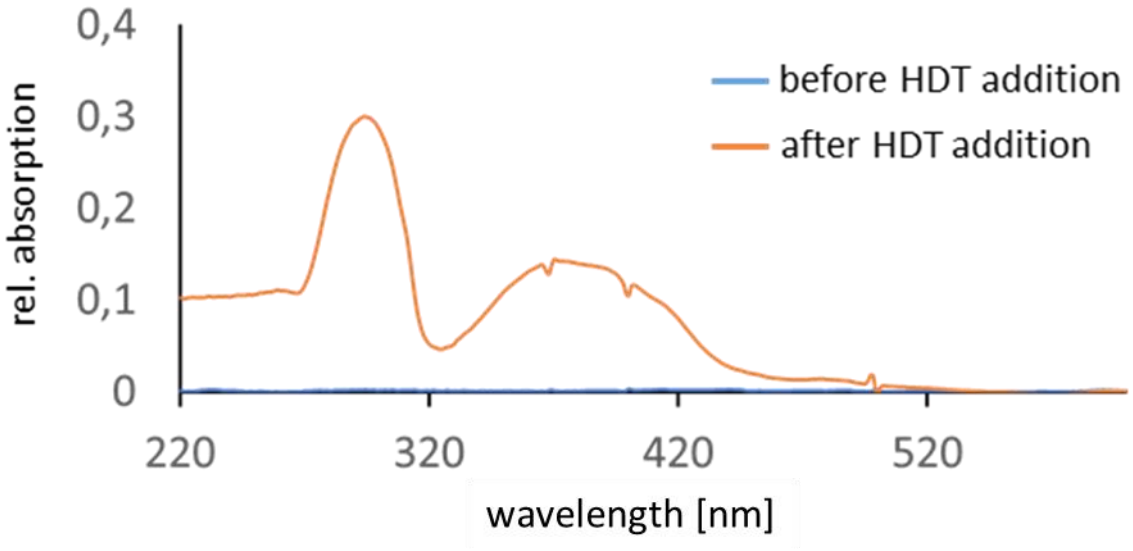


Figure S34. UV-VIS spectroscopy of azide-p(MEO_3MA)$_{42}$-*b*-p(PDSM)$_{12}$-*b*-p(DMEDAMA)$_{33}$ (P5-1) before and after reaction with 1,6-hexanedithiol (HDT).

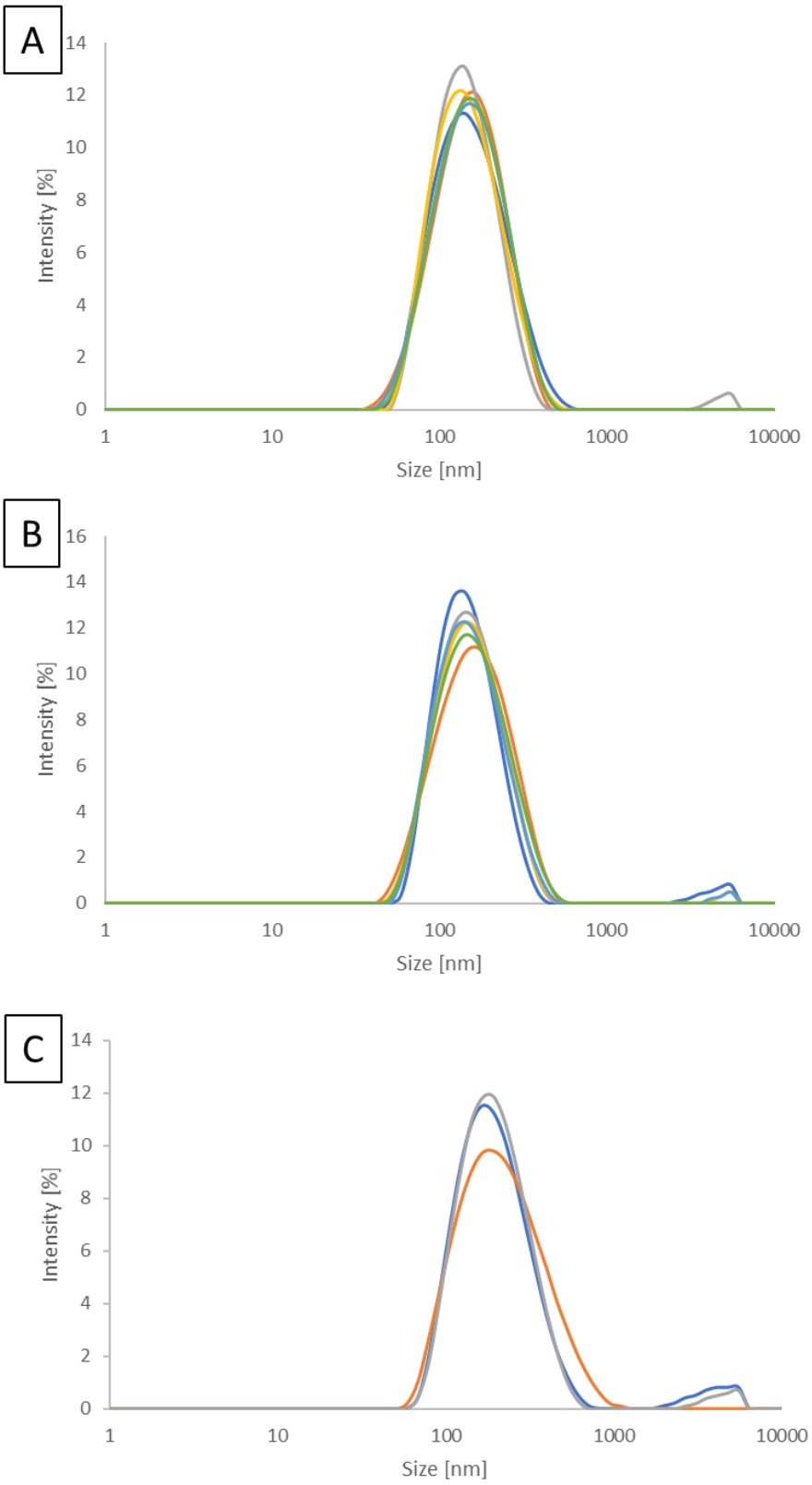


Figure S35. Dynamic light scattering measurements of polyplexes (N/P = 3) based on azide-p(MEO_3MA)$_{42}$-*b*-p(PDSM)$_{12}$-*b*-p(DMEDAMA)$_{33}$ (P5-1) and pGL3-BASIC pDNA (A + B) and pCMV-Luc pDNA (C). (A) measurement ensemble: non-crosslinked polyplex. (B) measurement ensemble: polyplex crosslinked with 1,6-hexandithiol. (C) measurement ensemble: non-crosslinked polyplex.

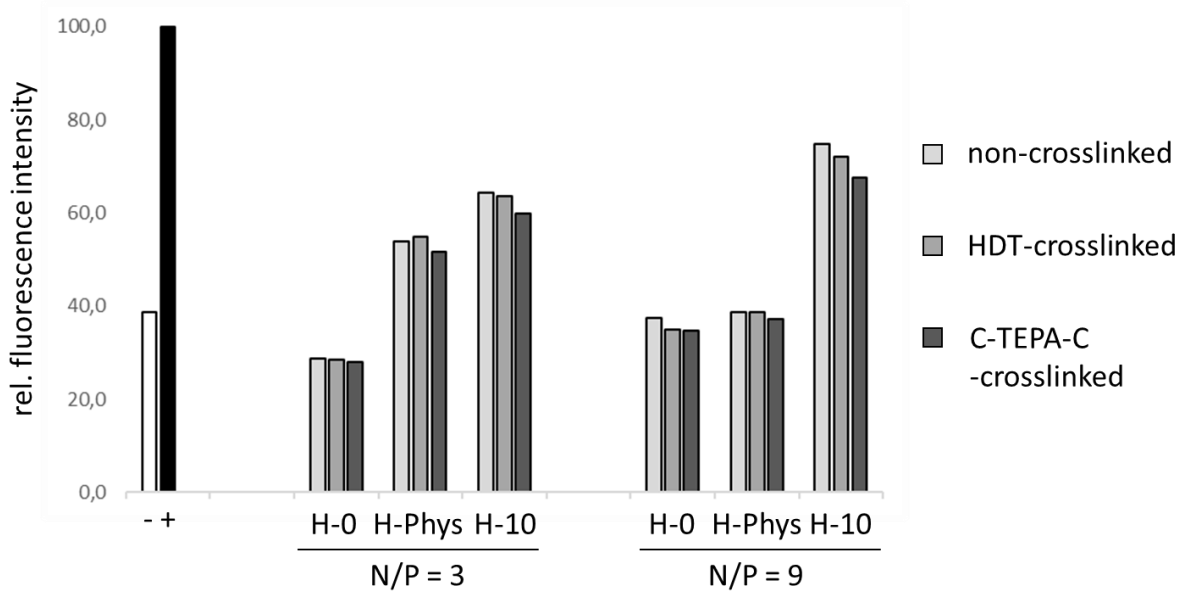


Figure S36. Heparin challenge of P5-1. Fluorescence spectroscopy with ethidium bromide as intercalative dye. Different levels of heparin were applied to challenge non-crosslinked and crosslinked P5-1 polyplexes of different N/P-ratios. Batches were treated 30 minutes prior to the measurement with 0 µg/ml heparin (H-0), 1,65 µg/ml heparin (H-Phys) and 10 µg/ml (H-10). As negative control (-) the equivalent amount of ethidium bromide was dissolved in PBS. As positive control (+) the equivalent amount of DNA was incubated with ethidium bromide.

Table S4: Results of the DLS (hydrodynamic diameter) and Zeta potential measurements

	Non crosslinked polyplexes[a]		**Crosslinked polyplexes[b]**	
Polymer	mean hydrodynamic diameter [nm]	mean PDI[c]	mean hydrodynamic diameter [nm]	mean PDI
P5-1[c]	230.8	0.200	237.6	0.212
P5-2[c]	187.2	0.151	213.3	0.230
P5-3[c]	256.1	0.217	224.4	0.201
P5-4[c]	258.1	0.179	231.4	0.206
Polymer	mean Zeta potential [mV]		mean Zeta potential [mV]	
P5-1[c]	-2		+1	
P5-2[c]	-2		+3	
P5-3[c]	±0		±0	
P5-4[c]	+1		+1	

[a]Polyplexes were synthesized with an N/P-Ratio of 3:1. [b]Crosslinked polyplexes were prepared with an N/P-Ratio of 3:1, with 1,6-hexanedithiol as crosslinking agent and a calculated crosslinking density of 100%. [c]As determined in 1x PBS buffer.

As Zeta Potentials were measured in PBS buffer, which has a extremely high conductivity, due to high salt concentrations. Thus, only 1 run was performed per measurement to prevent electrode corrosion. Because of that, no values for Zeta potential deviation could be acquired.

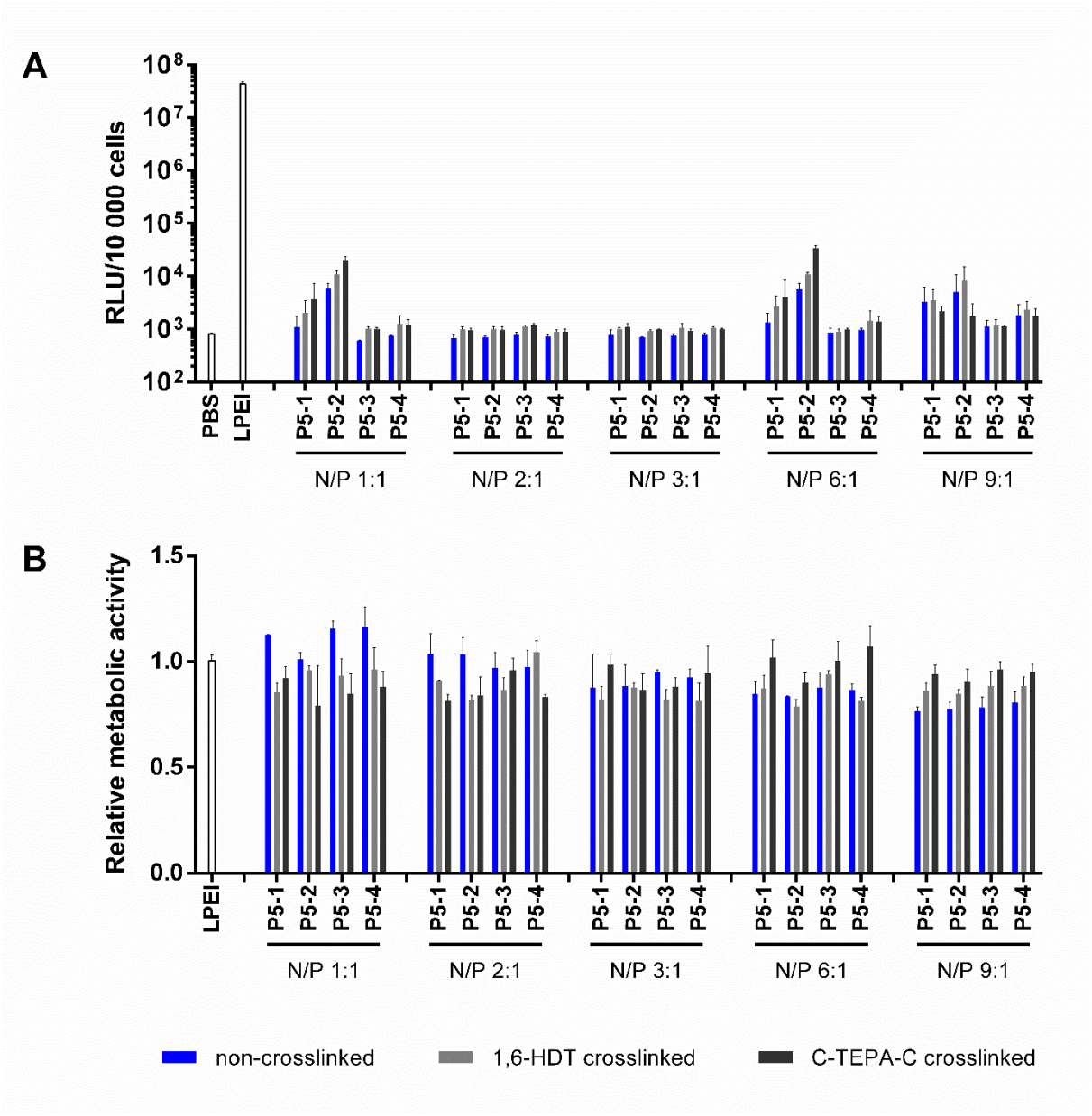


Figure S37. *In vitro* evaluation in N2a cells of polyplexes prepared from polymers P5-1 to P5-4 and pCMV-Luc at different N/P ratios. Non crosslinked polyplexes were compared with polyplexes crosslinked with 1,6-hexanedithiol (1,6-HDT) or C-TEPA-C, respectively (for both crosslinkers: crosslinking density = 100%). PBS served as negative control, LPEI polyplexes (N/P 6) as positive control. Experiments were performed in triplicate. (A) Luciferase gene transfer assay. (B) MTT assay. Metabolic activity is displayed relatively to PBS-treated control cells.