Supplementary Information

Making the negative positive – fluorination of indole as efficient strategy to improve guanidinium-containing gene carriers

Markus Kötzsche[‡ a], Jan Egger[‡ b], Andreas Dzierza [b], Liên Sabrina Reichel [a], Ivo Nischang [a,c,d,e], Anja Traeger [a,c], Dagmar Fischer [*b,f] and Kalina Peneva [*a,c]

a. Friedrich Schiller University Jena, Institute of Organic and Macromolecular Chemistry (IOMC), Humboldtstr. 10, 07743 Jena, Germany.

b. Friedrich-Alexander-Universität Erlangen-Nürnberg, Division of Pharmaceutical Technology and Biopharmacy, Cauerstr. 4, 91058 Erlangen, Germany.

c. Jena Center for Soft Matter, Philosophenweg 7, 07743 Jena, Germany.

d. Helmholtz Institute for Polymers in Energy Applications Jena (HIPOLE Jena), Lessingstr. 12-14, 07743, Jena, Germany

e. Helmholtz-Zentrum Berlin für Materialien und Energie GmbH (HZB), Hahn-Meitner-Platz 1, 14109 Berlin, Germany

f. FAU NeW - Research Center for New Bioactive Compounds, Nikolaus-Fiebiger-Str. 10, 91058 Erlangen, Germany

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1. Materials for chemical synthesis

Tryptamine (97%, Sigma Aldrich), 5-fluoro-tryptamine hydrochloride (99%, Sigma Aldrich), 6-fluoro-tryptamine hydrochloride (99%, Sigma Aldrich), 5,6-difluoro-tryptamine hydrochloride (95%, Sigma

Aldrich), *N*-succinimidyl methacrylate (98%, TCI) , 4-cyano-4-(phenylcarbonothioylthio) pentanoic acid (Sigma Aldrich), initiator 4,4'-azobis(4-cyanovaleric acid) (98%, Sigma Aldrich), trifluoroacetic acid (99%, TCI), 3-aminopropyl methacrylamide hydrochloride (98%, PolyScience), *N*,*N*'-di-Boc-1*H*-pyrazole-1-carboxamidine (97%, Carbolution), dry THF (99.5%, AcroSeal, FisherScientific), dry DMF (99.5%, AcroSeal, FisherScientific), triethylamine (99%, Sigma Aldrich), *N*-(2-hydroxypropyl) methacrylamide (98%, TCI) (HPMA)

2. Monomer synthesis

2.1 *N*-(2-(1*H*-indol-3-yl)ethyl)methacrylamide (IEMA)

1.466 g (9.15 mmol, 1 eq.) tryptamine was filled in a Schlenk flask, degassed and dissolved in 20 mL dry THF. 1.3 mL (9.33 mmol, 1 eq.) triethylamine and 1.664 g (9.09 mmol, 1 eq.) *N*-succinimidyl methacrylate in 7 mL dry THF were added. After 3 hours, the solvent was removed and the solid purified by column chromatography using n-hexane/ethyl acetate 1/2. The solvent was removed, the product washed with water and dried. 1.173 g (5.14 mmol, 57 %).

^{1}H-NMR (300 MHz, DMSO-d_6) δ = 10.81 (s, 1H), 8.05 (t, *J* = 5.8 Hz, 1H), 7.56 (dd, *J* = 7.7, 1.2 Hz, 1H), 7.34 (dt, *J* = 8.1, 1.0 Hz, 1H), 7.15 (d, *J* = 2.4 Hz, 1H), 7.07 (ddd, *J* = 8.1, 7.0, 1.3 Hz, 1H), 6.98 (ddd, *J* = 8.0, 7.0, 1.1 Hz, 1H), 5.68 – 5.63 (m, 1H), 5.32 (p, *J* = 1.6 Hz, 1H), 3.45 – 3.37 (m, 2H), 2.87 (dd, *J* = 8.6, 6.5 Hz, 2H), 1.86 (t, *J* = 1.2 Hz, 3H).

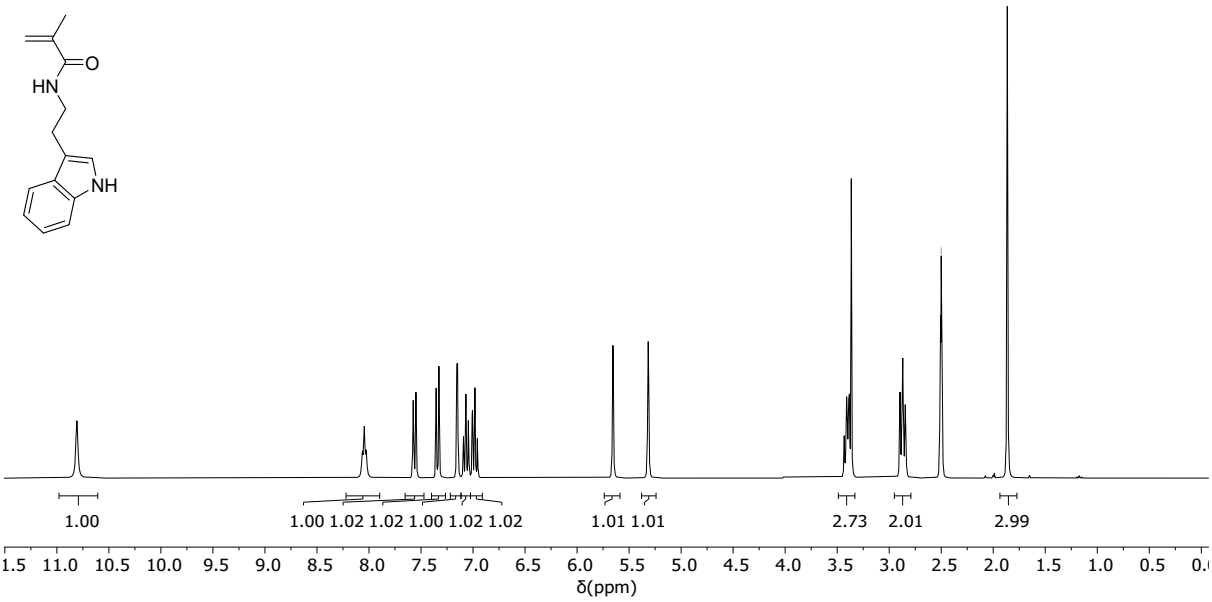

Figure S1. ^{1}H-NMR spectrum in DMSO-d_6 of *N*-(2-indoleethyl)methacrylamide.

2.2 *N*-(2-(6-fluoro-1*H*-indol-3-yl)ethyl)methacrylamide (6F-IEMA)

215 mg (1.17 mmol, 1 eq.) *N*-succinimidyl methacrylate were filled in a Schlenk flask, degassed and dissolved in 5 mL dry THF. 253 mg (1.18 mmol, 1 eq.) 6-fluoro-tryptamine hydrochloride in 5 mL dry THF and 0,32 mL (2.30 mmol, 2 eq.) triethylamine were added. After 6 hours, the solvent was removed, the remaining solid washed with water and purified by column chromatography using n-hexane/ethyl acetate 1/2. 186.4 mg (0.757 mmol, 65 %).

^{1}H-NMR: (400 MHz, DMSO-d_6) δ = 10.89 (s, 1H), 8.05 (t, *J* = 5.8 Hz, 1H), 7.53 (dd, *J* = 8.7, 5.5 Hz, 1H), 7.15 (d, *J* = 2.3 Hz, 1H), 7.10 (dd, *J* = 10.2, 2.4 Hz, 1H), 6.84 (ddd, *J* = 9.8, 8.6, 2.4 Hz, 1H), 5.63

(t, *J* = 1.3 Hz, 1H), 5.31 (q, *J* = 1.6 Hz, 1H), 3.37 (d, *J* = 1.2 Hz, 2H), 2.83 (t, *J* = 7.5 Hz, 2H), 1.85 (d, *J* = 1.3 Hz, 3H).

^{19}F-NMR: (376 MHz, DMSO-d_6) δ = -122.49 (td, *J* = 10.0, 5.4 Hz).

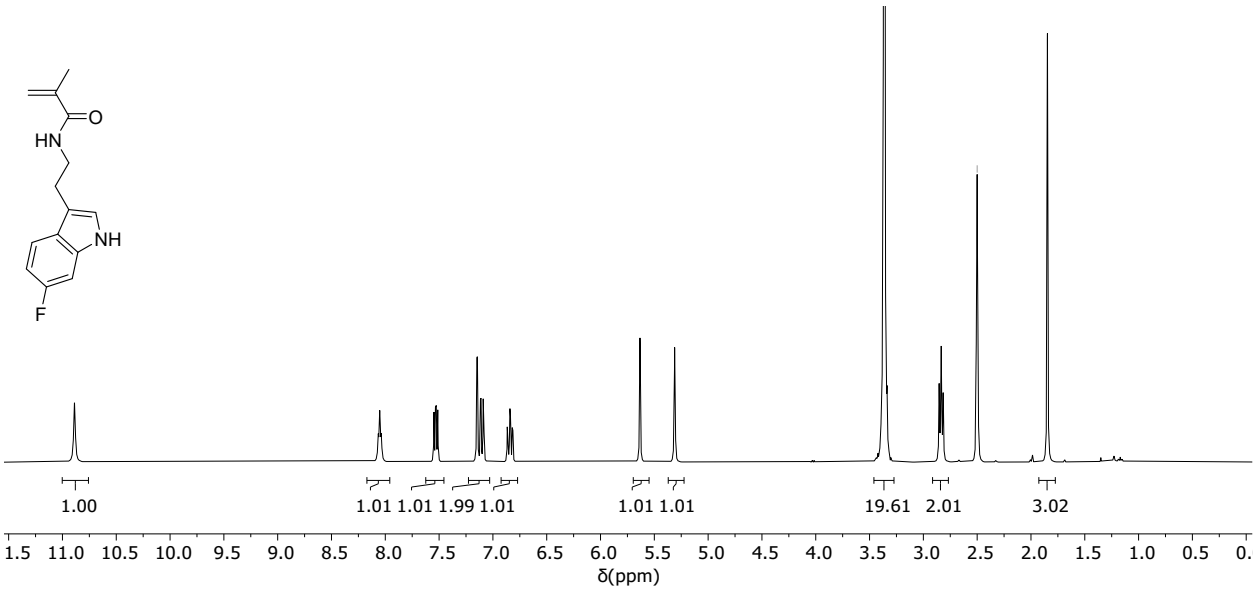

Figure S2. ^{1}H-NMR spectrum in DMSO-d$_6$ of *N*-(2-(6-fluoro-1*H*-indol-3-yl)ethyl)methacrylamide.

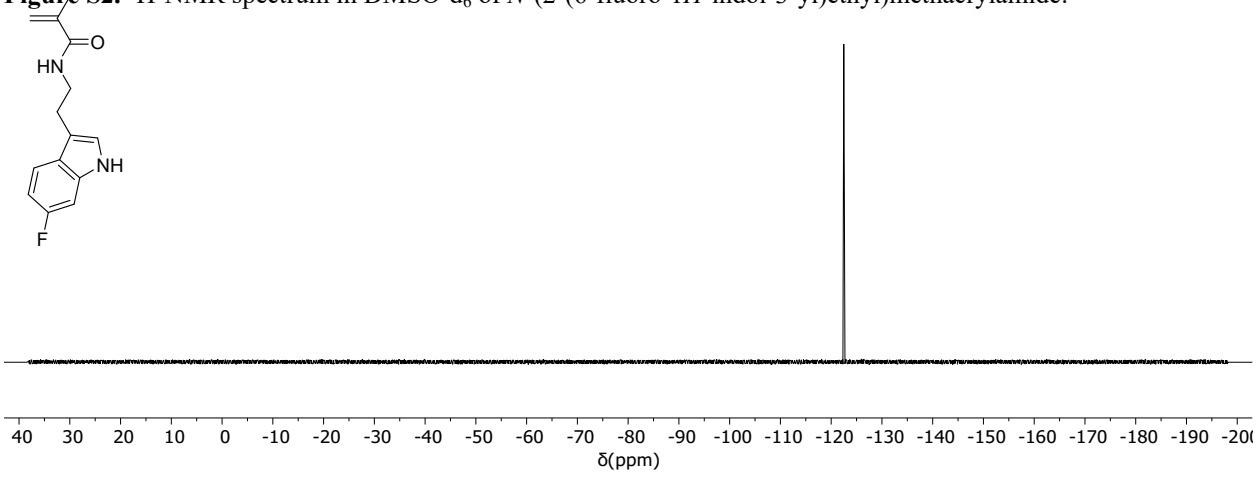

Figure S3. ^{19}F-NMR spectrum in DMSO-d$_6$ of *N*-(2-(6-fluoro-1*H*-indol-3-yl)ethyl)methacrylamide.

***N*-(2-(5-fluoro-1*H*-indol-3-yl)ethyl)methacrylamide (5F-IEMA)**

247 mg (1.15 mmol, 1 eq.) 5-fluoro-tryptamine hydrochloride were filled in a Schlenk flask, degassed and dissolved in 5 mL dry THF. 0,32 mL (2.30 mmol, 2 eq.) triethylamine and 218 mg (1.19 mmol, 1 eq.) *N*-succinimidyl methacrylate in 5 mL dry THF were added. After 6 hours, the solvent was removed, the remaining solid washed with water and purified by column chromatography using n-hexane/ethyl acetate 1/2. 120 mg (0.487 mmol, 42 %).

^{1}H-NMR: (300 MHz, DMSO-d_6) δ = 10.90 (s, 1H), 8.03 (t, J = 5.8 Hz, 1H), 7.36 – 7.26 (m, 2H), 7.22 (d, J = 2.4 Hz, 1H), 6.90 (td, J = 9.2, 2.6 Hz, 1H), 5.63 (dt, J = 1.8, 0.9 Hz, 1H), 5.31 (p, J = 1.6 Hz, 1H), 3.37 (m, 2H), 2.82 (dd, J = 8.4, 6.5 Hz, 2H), 1.85 (dd, J = 1.6, 0.9 Hz, 3H).

^{19}F-NMR: (282 MHz, DMSO-d_6) δ = -125.72 (td, J = 9.8, 4.6 Hz).

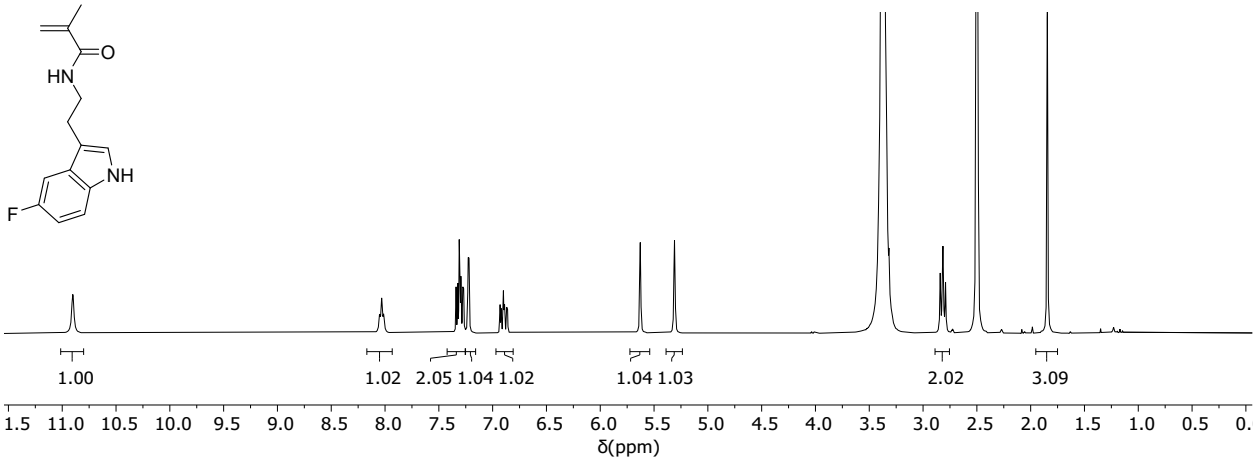

Figure S4. ^{1}H-NMR spectrum in DMSO-d_6 of *N*-(2-(5-fluoro-1*H*-indol-3-yl)ethyl)methacrylamide.

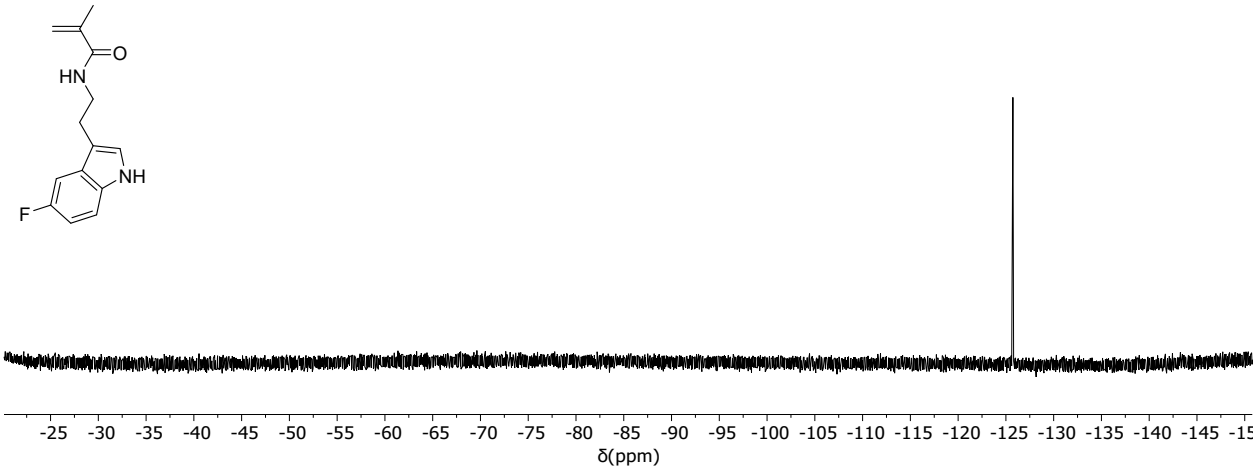

Figure S5. ^{19}F-NMR spectrum in DMSO-d_6 of *N*-(2-(5-fluoro-1*H*-indol-3-yl)ethyl)methacrylamide.

2.3 *N*-(2-(5,6-difluoro-1*H*-indol-3-yl)ethyl)methacrylamide (5,6F-IEMA)

100 mg (0.430 mmol, 1 eq.) 5,6-difluoro-tryptamine hydrochloride were filled in a Schlenk flask, degassed and dissolved in 2.5 mL dry DMF. 0,12 mL (0.860 mmol, 2 eq.) triethylamine and 87 mg (0.473 mmol, 1.1 eq.) *N*-succinimidyl methacrylate were added. After 4.5 hours, the reaction mixture was diluted with 50 mL water and the precipitate separated by centrifugation (8000 rpm for 12 min). The obtained solid was dried and purified by column chromatography using n-hexane/ethyl acetate 1/1. 88 mg (0.333 mmol, 77 %).

^{1}H-NMR (300 MHz, DMSO-d_6) δ = 10.97 (s, 1H), 8.02 (t, J = 5.8 Hz, 1H), 7.51 (dd, J = 11.5, 8.0 Hz, 1H), 7.32 (dd, J = 11.4, 7.0 Hz, 1H), 7.22 (d, J = 2.3 Hz, 1H), 5.65 – 5.59 (m, 1H), 5.31 (p, J = 1.6 Hz, 1H), 3.33 (m, 2H), 2.81 (t, J = 7.4 Hz, 2H), 1.87 – 1.81 (m, 3H).

^{19}F-NMR (282 MHz, DMSO-d_6) δ = -146.08 (ddd, J = 22.1, 11.7, 8.3 Hz), -149.52 (ddd, J = 22.3, 11.4, 6.9 Hz).

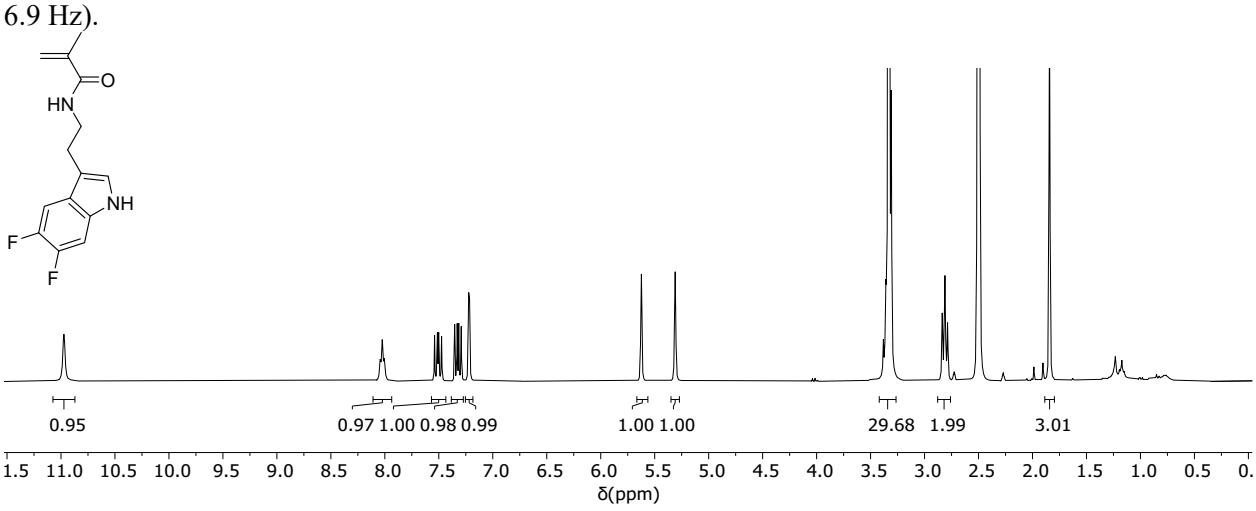

Figure S6. ^{1}H-NMR spectrum in DMSO-d$_6$ of *N*-(2-(5,6-difluoro-1*H*-indol-3-yl)ethyl)methacrylamide.

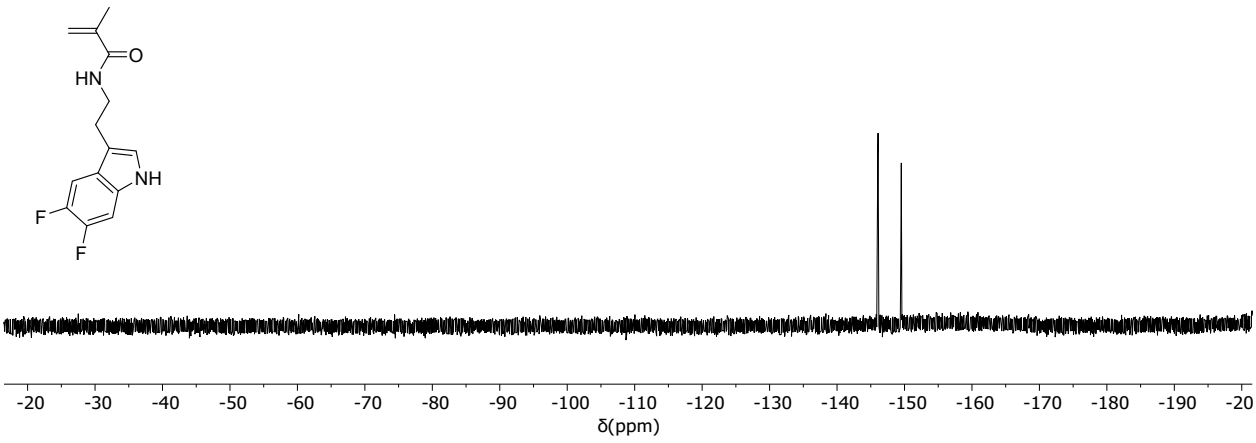

Figure S7. ^{19}F-NMR spectrum in DMSO-d$_6$ of *N*-(2-(5,6-difluoro-1*H*-indol-3-yl)ethyl)methacrylamide.

2.4 3-Guanidinopropyl methacrylamide (GPMA)

10 g (56.0 mmol, 1.2 eq) 3-aminopropyl methacrylamide hydrochloride were dissolved in 60 mL acetonitrile, 6 mL water and 24 mL (172 mmol, 3.75 eq) triethylamine. 14.33 g (46.2 mmol, 1 eq) *N*,*N*'-di-Boc-1*H*-pyrazole-1-carboxamidine was dissolved in 60 mL acetonitrile and added to the solution. After 1 day at room temperature, the product precipitated and the suspension was diluted with 200 mL water. The precipitate was filtered, washed with water and freeze-dried to obtain 16.36 g (42.6 mmol, 92%) Boc-protected 3-guanidinopropyl methacrylamide.

^{1}H-NMR (300 MHz, Chloroform-*d*) δ = 11.53 (s, 1H), 8.47 (t, *J* = 6.7 Hz, 1H), 7.35 (t, *J* = 7.2 Hz, 1H), 5.84 – 5.77 (m, 1H), 5.31 (p, *J* = 1.5 Hz, 1H), 3.49 (q, *J* = 6.3 Hz, 2H), 3.33 (q, *J* = 6.2 Hz, 2H), 2.00 (t, *J* = 1.3 Hz, 3H), 1.75 – 1.65 (m, 2H), 1.50 (s, 9H), 1.46 (s, 9H).

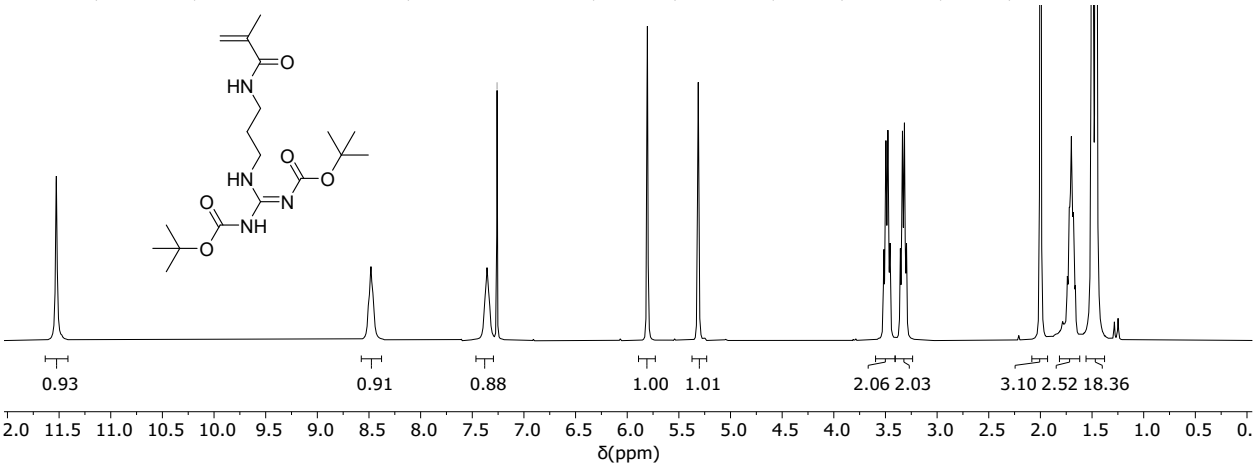

Figure S8. ^{1}H-NMR spectrum in CDCl3 of *N*,*N*'-di-Boc-3-guanidinopropyl methacrylamide.

7.90 g (20.5 mmol, 1 eq) *N*,*N*'-di-Boc-3-guanidinopropyl methacrylamide was dissolved in 80 mL dichlormethane and 20 mL (261 mmol, 13 eq) trifluoroacetic acid in 20 mL dichloromethan were added. The reaction was stirred over night and the solvent removed. 9.37 g of 3-guanidinopropyl methacrylamide trifluoroacetate were obtained.

^{1}H-NMR (300 MHz, Deuterium Oxide) δ = 5.66 (p, *J* = 0.9 Hz, 1H), 5.42 (dq, *J* = 2.5, 1.5 Hz, 1H), 3.31 (t, *J* = 6.8 Hz, 2H), 3.20 (t, *J* = 6.8 Hz, 2H), 1.90 (dd, *J* = 1.6, 1.0 Hz, 3H), 1.81 (p, *J* = 6.8 Hz, 2H).

^{19}F-NMR (282 MHz, Deuterium Oxide) δ = -75.66.

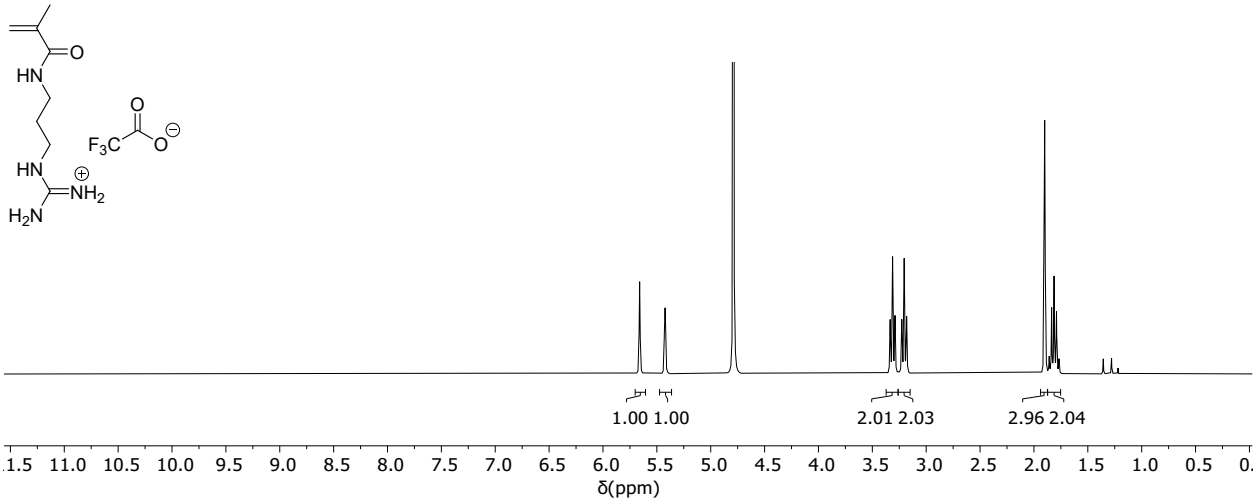

Figure S9. ^{1}H-NMR spectrum in D_2O of 3-guanidinopropyl methacrylamide trifluoroacetate.

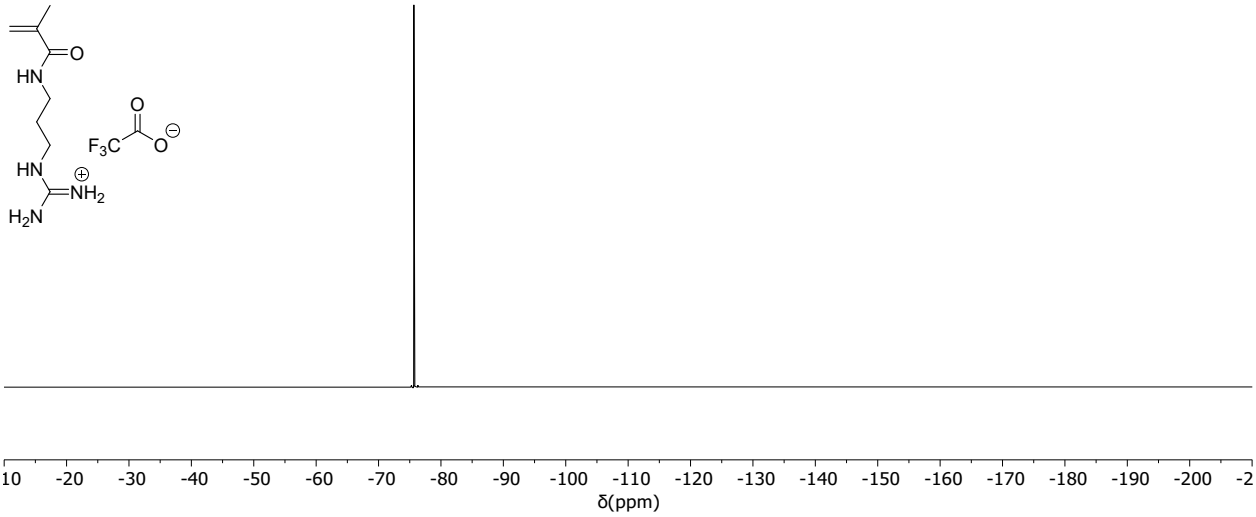

Figure S10. ^{19}F-NMR spectrum in D_2O of 3-guanidinopropyl methacrylamide trifluoroacetate.

2.5 Contact angle measurement

2 wt% solutions of the indole monomers were prepared in methanol and used for spin coating with a Laurell WS-650 at 1500 rpm for 2 minutes on silicon wafers. The contact angle of water was measured with a Lauda LSA 100.

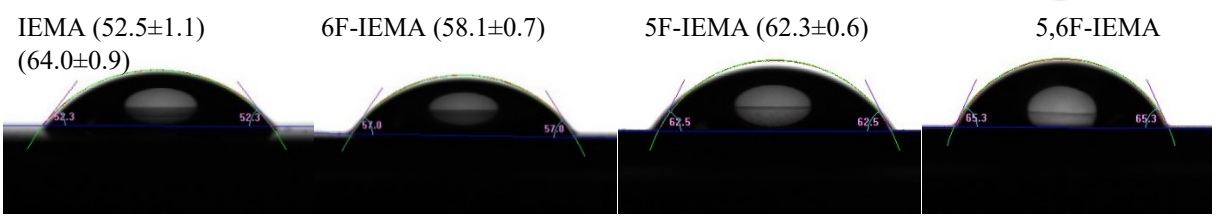

Figure S11. Contact angle measurement of water on IEMA derivate films on silicon wafer.

3. Polymer synthesis

3.1 Synthesis

Each polymer was prepared according to the described procedure with the amounts listed in Table *S1*. Polymer 5,6-di-F-IEMA was additionally dialysed against methanol/water 1/1 first for 1 day to remove remaining *N*-(2-(5,6-difluoro-1*H*-indol-3-yl)ethyl)methacrylamide. 5F-IEMA6 and 6F-IEMA6 were terminated with 56 mg (0.2 mmol, 100 eq) initiator in 56 µL DMF for 30 minutes to remove the chain-transfer agent.

Table S1. Amounts used in an aqueous RAFT polymerisation at 80 °C for 24 hours.

Polymer	GPMA	HPMA	IEMA analogue	CTA	I	Acetate buffer
IEMA6	449.4 mg 1.090 mmol 450 eq	41.4 mg 0.289 mmol 120 eq	17.9 mg 0.078 mmol 30 eq	2.71 mg 9.70 µmol 4 eq	0.68 mg 2.42 µmol 1 eq	1.38 mL
IEMA10	244.0 mg 0.592 mmol 410 eq	28.0 mg 0.196 mmol 135 eq	17.1 mg 0.075 mmol 50 eq	1.61 mg 5.76 µmol 4 eq	0.41 mg 1.45 µmol 1 eq	1.61 mL
IEMA20	2003 mg 4.86 mmol 360 eq	278.8 mg 1.947 mmol 140 eq	318.6 mg 1.396 mmol 100 eq	15.06 mg 53.9 µmol 4 eq	3.78 mg 13.5 µmol 1 eq	6.8 mL
5F-IEMA6	500.1 mg 1.213 mmol 450 eq	46.6 mg 0.325 mmol 120 eq	20.3 mg 0.082 mmol 30 eq	3.00 mg 10.7 µmol 4 eq	0.75 mg 2.68 µmol 1 eq	1.54 mL
5F-IEMA10	250.4 mg 0.549 mmol 390 eq	29.0 mg 0.203 mmol 140 eq	19.8 mg 0.0804 mmol 60 eq	1.57 mg 5.62 µmol 4 eq	0.39 mg 4.11 µmol 1 eq	0.76 mL
5F-IEMA20	198.8 mg 0.436 mmol 330 eq	28.2 mg 0.197 mmol 150 eq	39.4 mg 0.160 mmol 120 eq	1.49 mg 5.32 µmol 4 eq	0.37 mg 1.33 µmol 1 eq	0.64 mL
6F-IEMA6	498.4 mg 1.209 mmol 450 eq	46.9 mg 0.328 mmol 120 eq	20.3 mg 0.082 mmol 30 eq	3.00 mg 10.7 µmol 4 eq	0.75 mg 2.68 µmol 1 eq	1.54 mL
5,6-di-F-IEMA	299.6 mg 0.657 mmol 450 eq	24.7 mg 0.172 mmol 120 eq	11.6 mg 0.0439 mmol 30 eq	1.63 mg 5.85 µmol 4 eq	0.41 mg 1.46 µmol 1 eq	0.83 mL

3.2 NMR spectra

The compositions of the statistical copolymers were calculated from the ^{1}H-NMR integrals. The signal at 7.2 ppm in combination with the signal at 3.4 ppm was used to determine the equivalents of IEMA. The signal at 3.9 ppm was used to determine the equivalents of HPMA and the remaining signals were used to determine the equivalents of GPMA. The mol% were then calculated from the equivalents. The calculation for IEMA6 is given as example.

Table S2. Calculation for the composition of the terpolymer IEMA6.

ppm	7.2	3.9	3.0	1.75	1
F-IEMA-proton	3/4/5	0	4	2	3
HPMA-proton	0	1	2	2	6
GPMA-proton	0	0	4	4	3
Integrals	1.36	1.00	15.00	13.94	16.45
-1 HPMA protons	1.36	0	13	11.94	10.45
-0.272 IEMA protons	0	0	11.91	11.40	9.63
-2.98 GPMA protons	0	0	0	-0.52	0.70

1/(1+0.272+2.98) = 23.5%, 0.272/(1+0.272+2.98) = 6.4%, 2.98/(1+0.272+2.98) = 70.1%

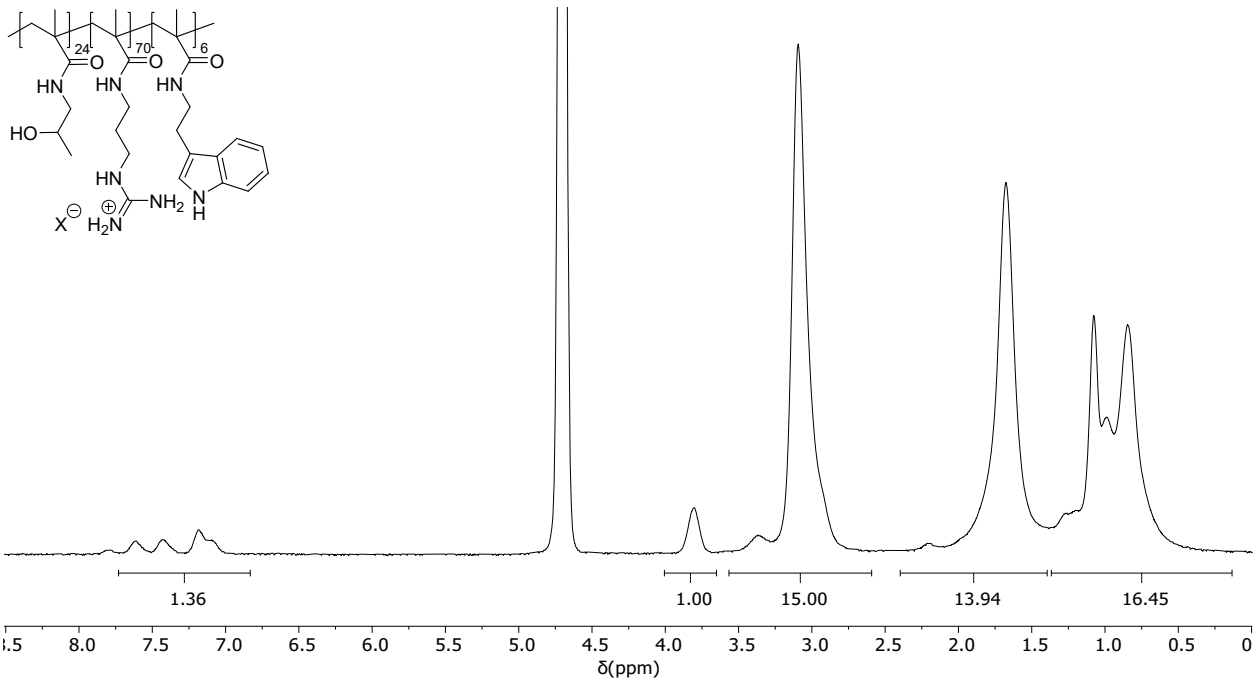

Figure S12. ^{1}H-NMR spectrum in D_2O of the polymer IEMA6.

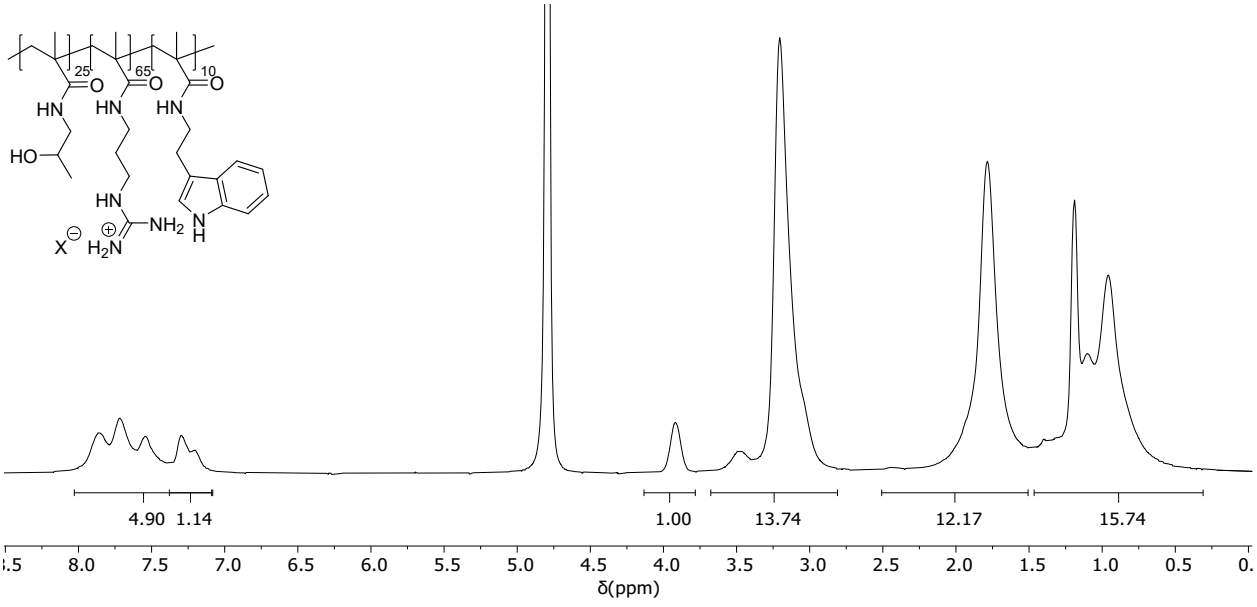

Figure S13. ^{1}H-NMR spectrum in D_2O of the polymer IEMA10.

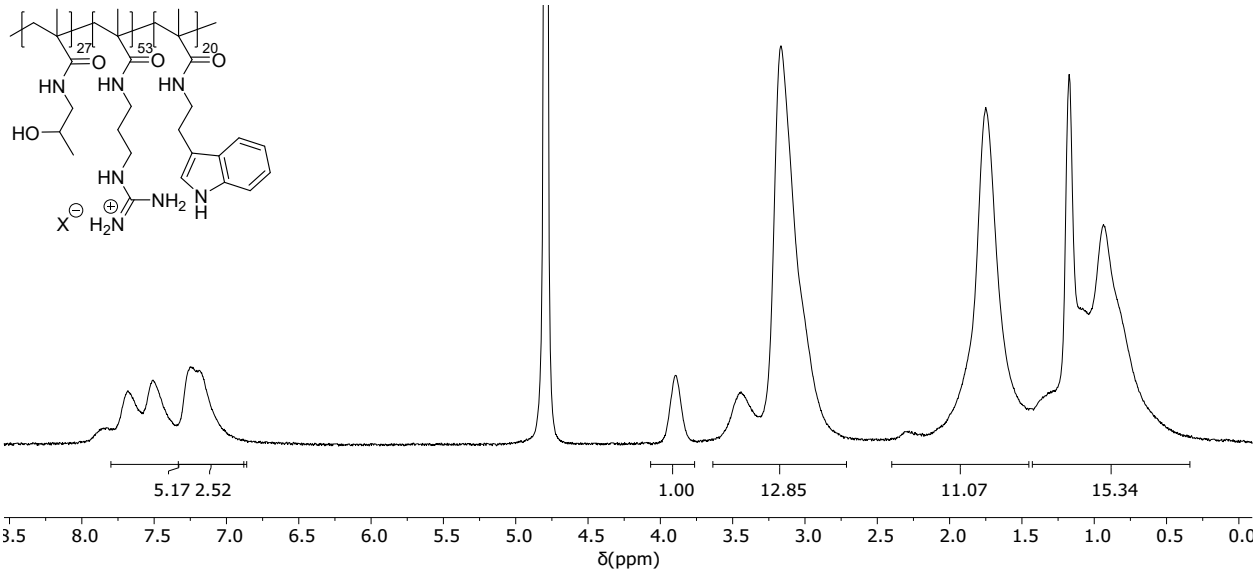

Figure S14. ^{1}H-NMR spectrum in D_2O of the polymer IEMA20.

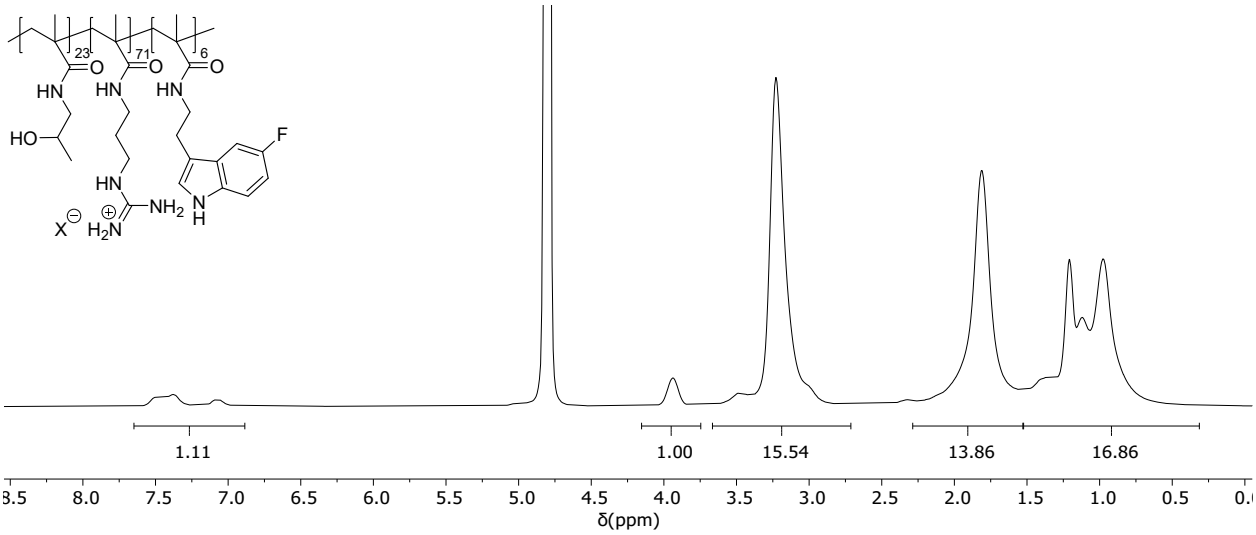

Figure S15. ^{1}H-NMR spectrum in D_2O of the polymer 5F-IEMA6.

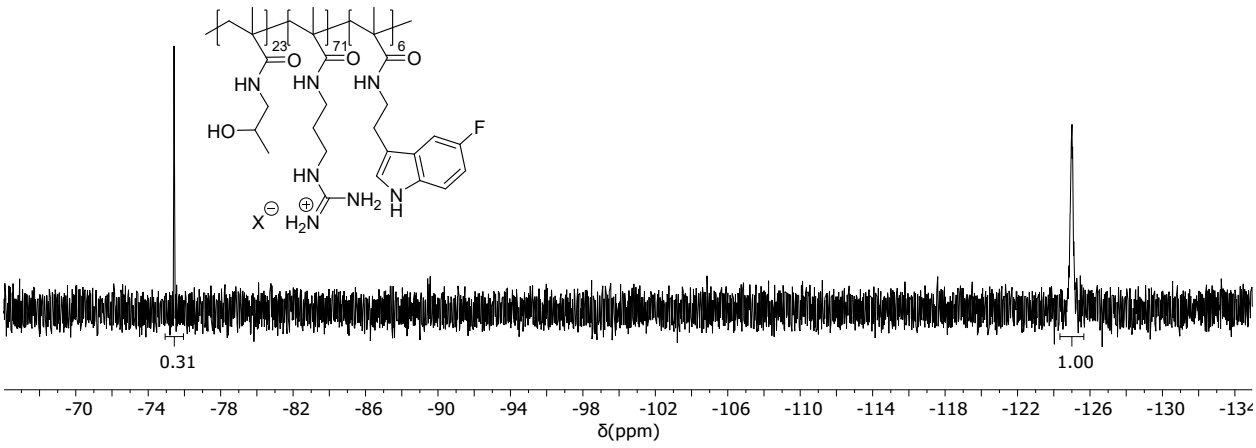

Figure S16. ^{19}F-NMR spectrum in D_2O of the polymer 5F-IEMA6.

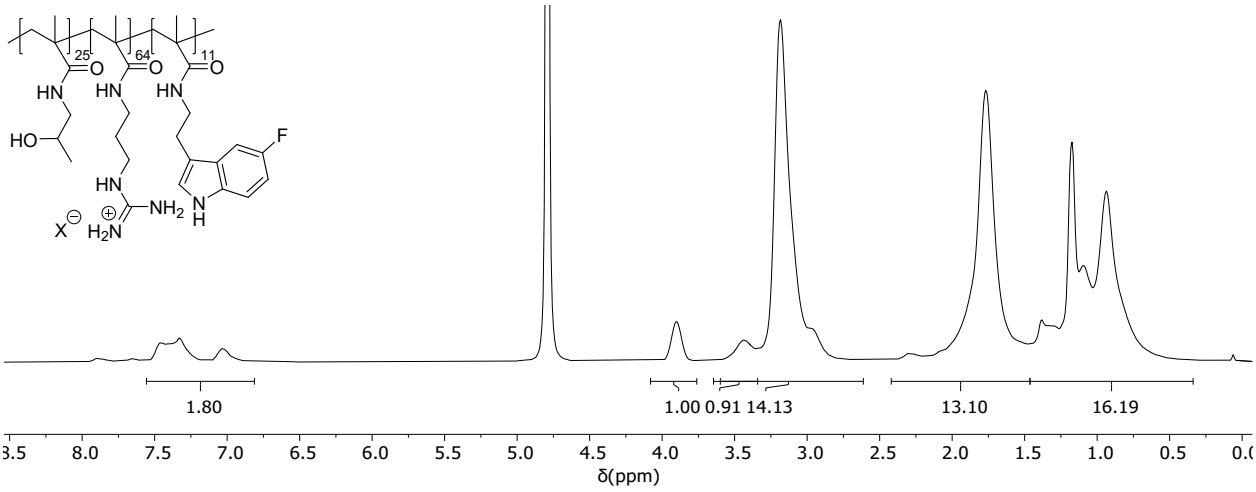

Figure S17. ^{1}H-NMR spectrum in D_2O of the polymer 5F-IEMA10.

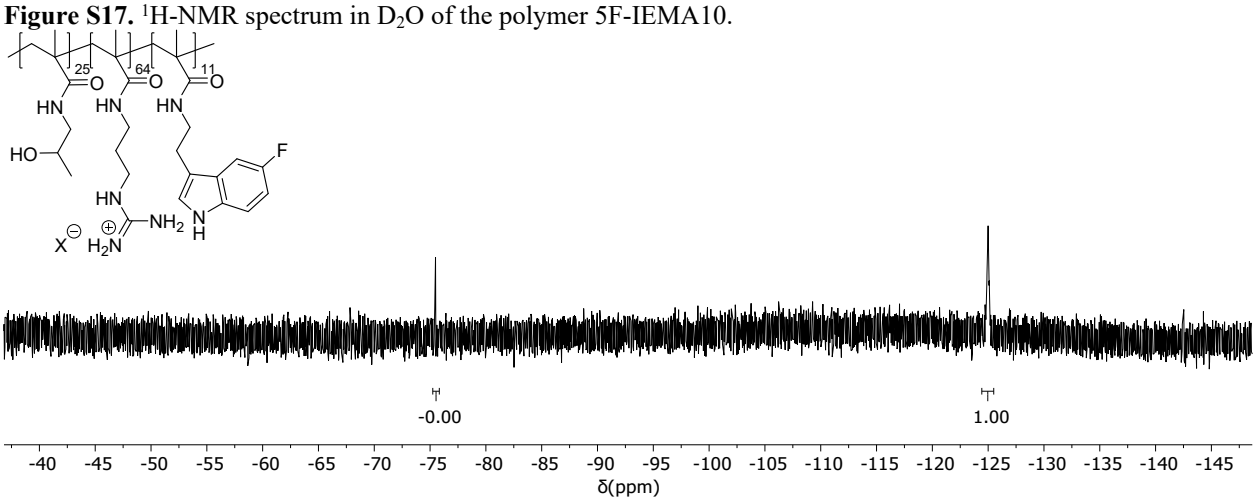

Figure S18. ^{19}F-NMR spectrum in D_2O of the polymer 5F-IEMA10.

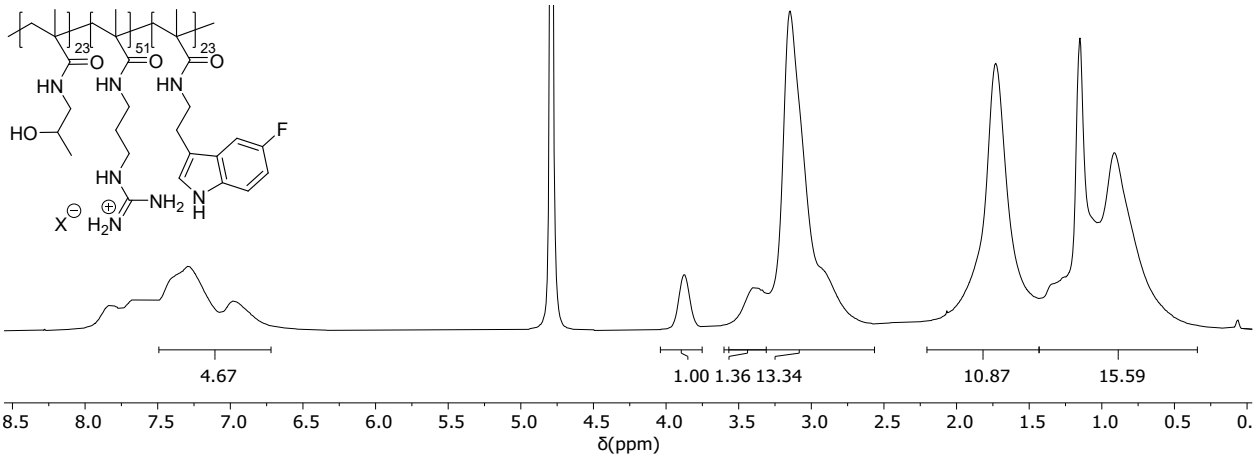

Figure S19. ^{1}H-NMR spectrum in D_2O of the polymer 5F-IEMA20.

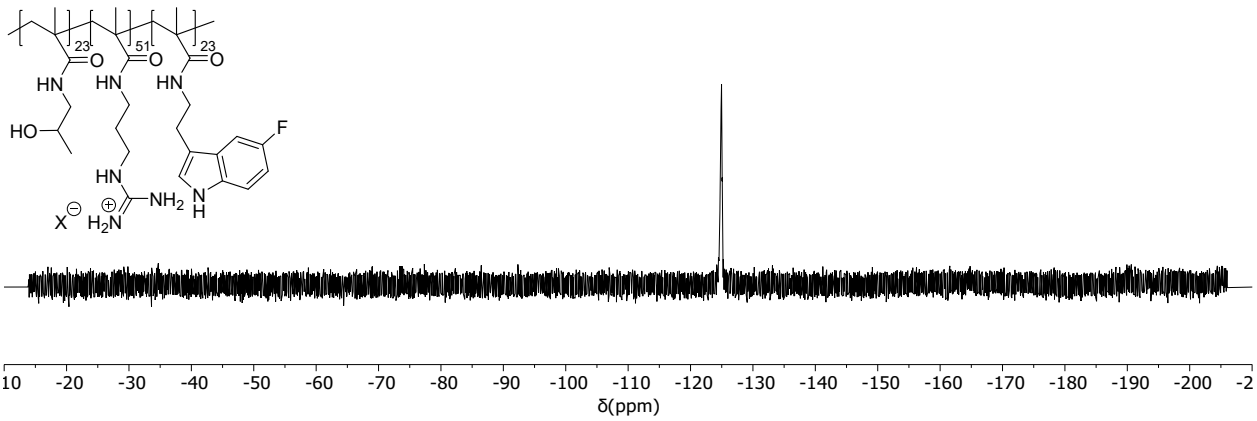

Figure S20. ^{19}F-NMR spectrum in D_2O of the polymer 5F-IEMA20.

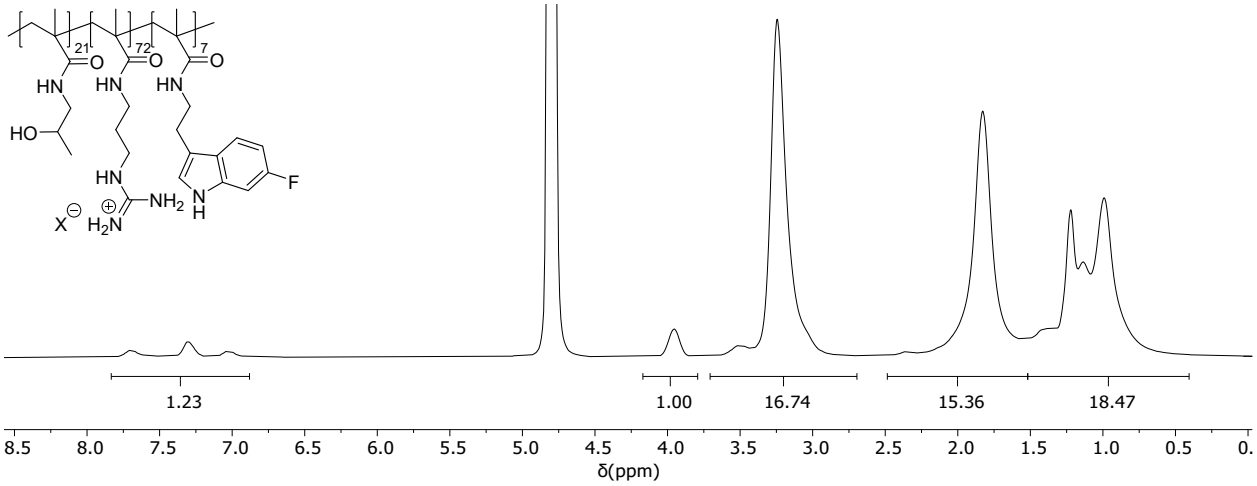

Figure S21. ^{1}H-NMR spectrum in D_2O of the polymer 6F-IEMA6.

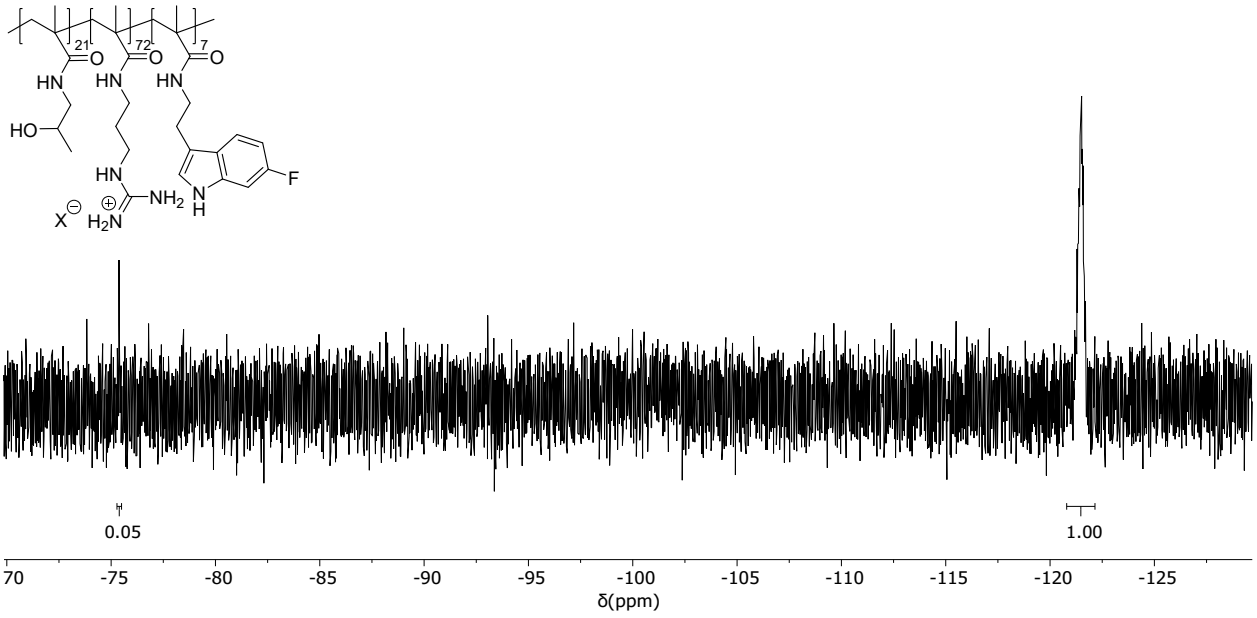

Figure S22. ^{19}F-NMR spectrum in D_2O of the polymer 6F-IEMA6.

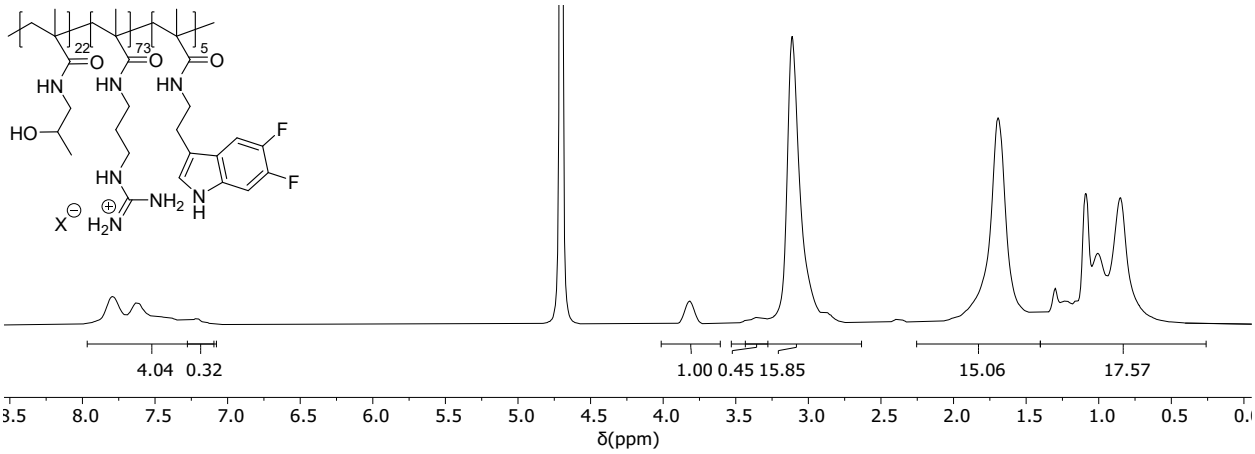

Figure S23. ^{1}H-NMR spectrum in D_2O of the polymer 5,6-di-F-IEMA.

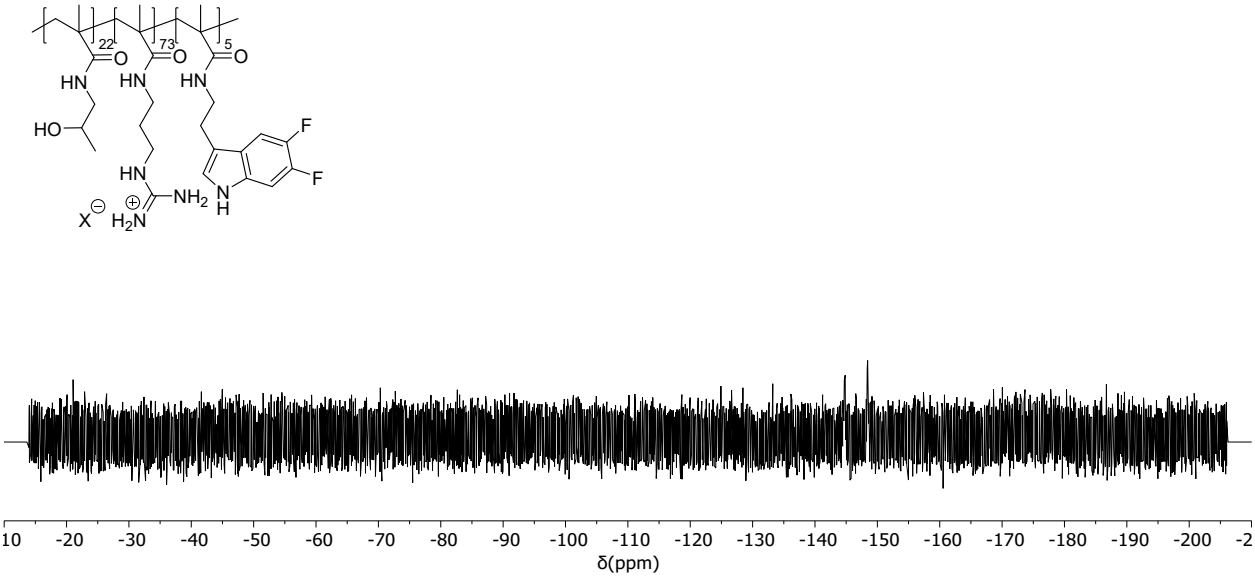

Figure S24. ^{19}F-NMR spectrum in D_2O of the polymer 5,6-di-F-IEMA.

3.3 Polymerization Kinetics and Monomer Conversion Analysis

A monomer feed consisting of 20 mol% HPMA, 65 mol% GPMA, and 15 mol% 6F-IEMA was used. Samples were collected at 0, 1, 2, 4, 6, and 8 hours, and the reaction mixture was diluted with deuterated DMSO and D_2O. Monomer composition was determined using the proton signals of the double bond at 5.5 ppm, while conversion was calculated from the integral at 1 ppm. Due to signal overlap, the spectra from 4 and 8 hours could not be used for analysis. The ^{1}H-NMR spectrum after 6 hours is shown as an example, revealing a conversion of 60%. During this time, the proportion of GPMA increased slightly from 64% to 68%, while HPMA and 6F-IEMA decreased from 20% to 18% and from 15% to 13%, respectively, suggesting a slower incorporation of GPMA compared to HPMA and 6F-IEMA. Nevertheless, the resulting copolymers can be regarded as statistical copolymers.

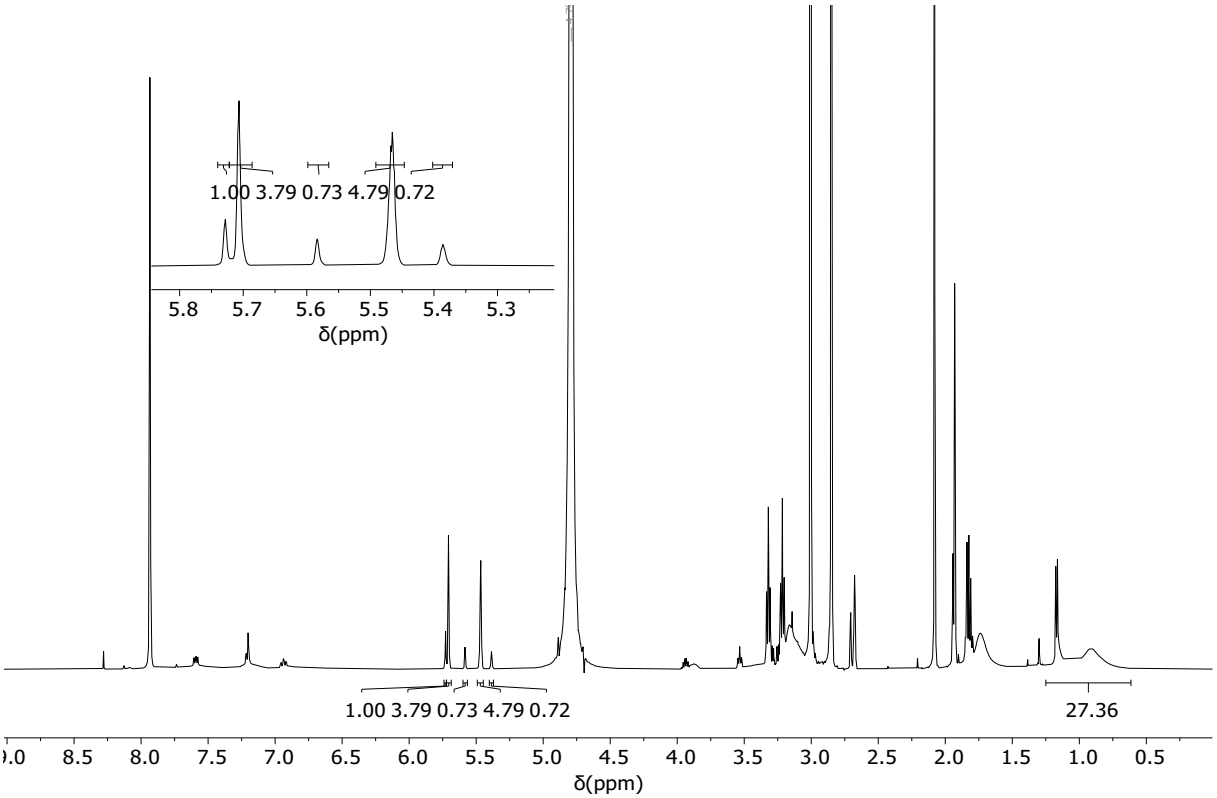

Figure S25. ^{1}H-NMR spectrum of the kinetic reaction mixture after 6 hours.

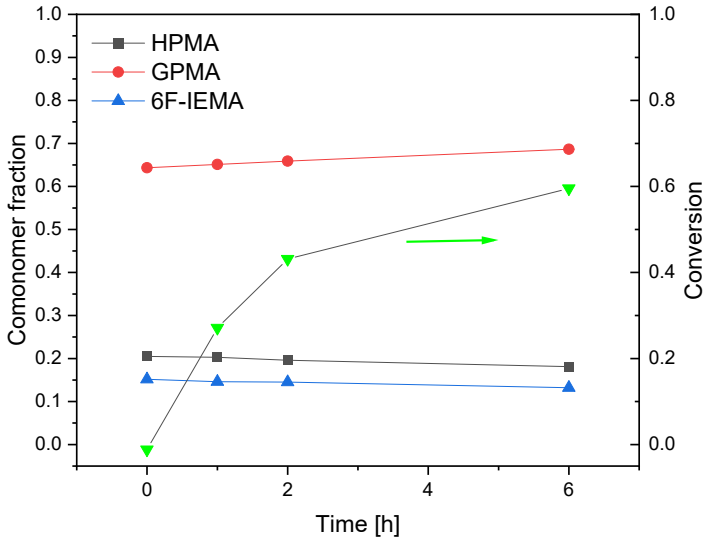

Figure S26. Monomer proportion and conversion for the kinetic with 20 mol% HPMA, 65 mol% GPMA and 15 mol% 6F-IEMA as monomer feed at 80°C.

3.4 Size exclusion chromatography

Size exclusion chromatography was performed in dimethylacetamide and 0.21% lithium chloride as the eluent at 40 °C using PSS GRAM guard/1000/30 Å columns at a flow rate of 1 mL/min. A G1362A refractive indes detector and a G1315D UV detector (290 nm) were used and the calibration was done with poly (methyl methacrylate) (PMMA) standards.

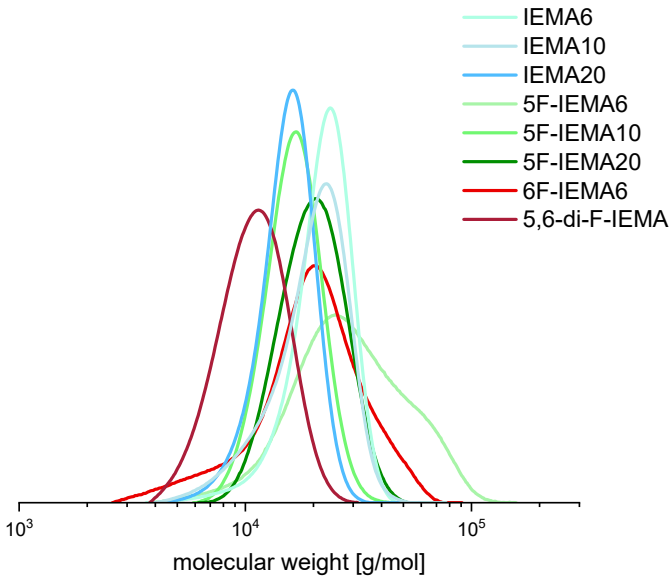

Figure S27. SEC curves of the terpolymers in dimethylacetamide with PMMA calibration.

4. Liquid chromatography

Liquid chromatographic analysis of the polymers was enabled by a Vanquish Core HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) with a diode array detector (DAD) and a charged aerosol detector (CAD). The UHPLC chromatographic system was equipped with a monolithic Chromolith® High Resolution RP-18 end capped (100 × 4.6 mm) column from Merck KGaA (Darmstadt, Germany) as the stationary phase. The polymers were dissolved at a concentration of 1 mg/mL in a 10 mM aqueous triethyl ammonium acetate buffer (TEAA) adjusted to a pH of 7. In all the measurements, the injection volume was 10 µL. The samples were placed in the autosampler tempered at 20 °C. Prior to the analyses, all samples were filtered through a 0.45 µm pore size regenerated cellulose (RC) filter (Carl Roth GmbH + Co. KG, Karlsruhe, Germany).

The column was operated with a binary mobile phase composition. The utilized flow rate was 1 mL/min, consisting of aqueous 10 mM TEAA buffer (pH=7) and CH_3CN by linear gradient elution programming. Initially, the mobile phase composition of 5 / 95 (%, v / v) CH_3CN / aqueous TEAA buffer was kept constant for 2 min. Afterward, the CH_3CN content was linearly increased from 5% to 95% within 23 min, followed by an isocratic hold at 95% CH_3CN for 10 min before decreasing back to 5% ACN within 3 min. Here, the column was reconditioned at injection conditions for another 5 min. The total runtime per sample was 43 min. The column oven temperature was set to 40 °C. For the CAD, data were collected at 10 Hz acquisition frequency with the nebulizer tempered at 50 °C. The DAD trace was followed at 280 nm (polymer absorbance maximum) at 5 Hz acquisition frequency. Chromatographic data were extracted from the Thermo Scientific™ Dionex™ Chromeleon™ 7 Chromatography Data System.

Figure S26 shows elution traces of an example polymer (IEMA 20) via the CAD elution trace. Both UV and CAD detector showed similar elution times. As typical for polymers (low diffusivity, slow mass transfer as well as dispersity) the elution signals appear broad in gradient elution liquid chromatography. To rationalize the data, elution times at the peak maximum were correlated to the respective polymers in Figure S27.

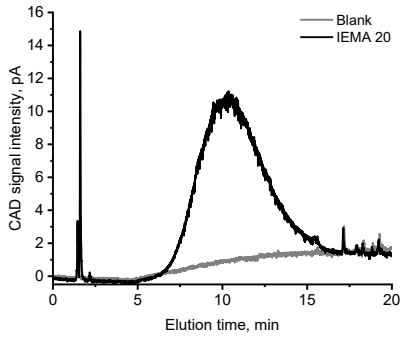

Figure S28. Elution trace of IEMA 20 recorded by CAD.

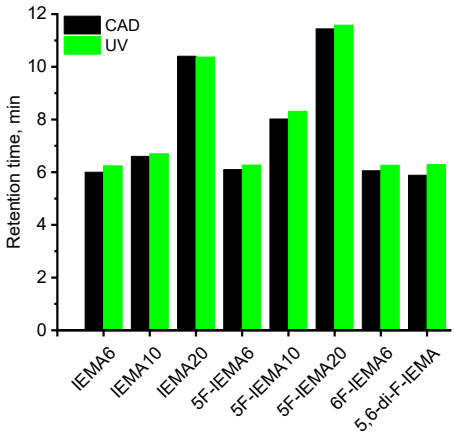

Figure S29. Elution time of polymers from the CAD and DAD at 280 nm.

5. DNA binding studies

5.1 Fluorescent dye exclusion assay (AccuBlue®)

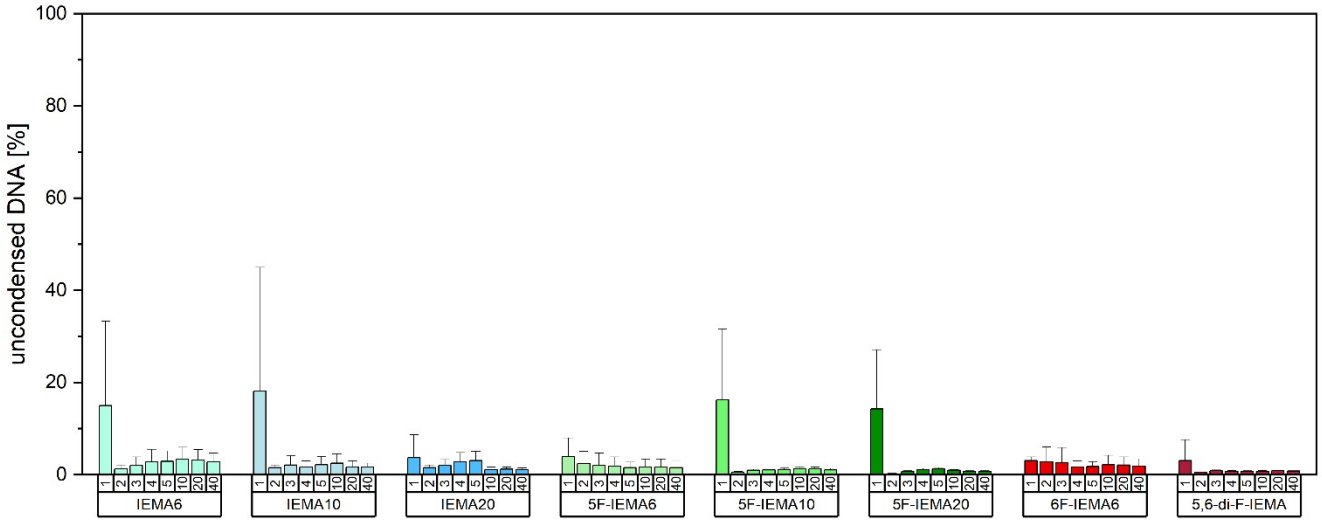

Figure S30. DNA binding efficiency of the terpolymers determined with the AccuBlue® High Sensitivity dsDNA Quantification Kit, measured in triplicates at different N/P ratios (n = 3, mean ± SD).

5.2 Horizontal gel electrophoresis

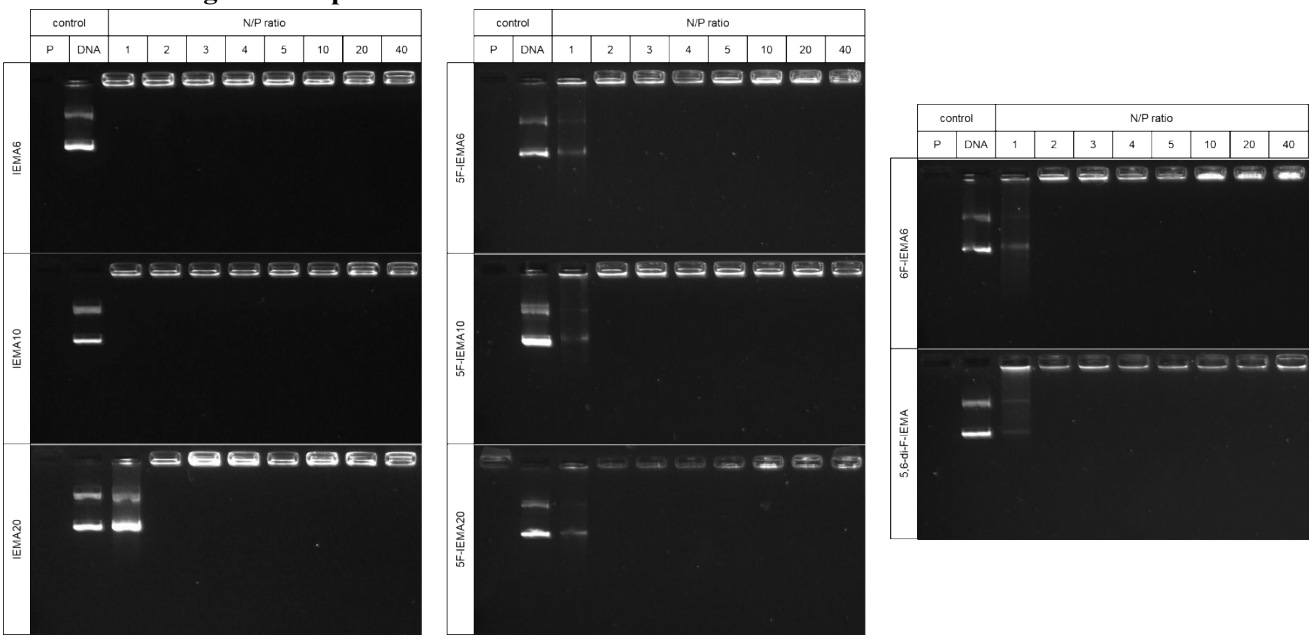

Figure S31. DNA binding efficiency of the polymers determined with agarose gel electrophoresis at different N/P ratios 1 to 40. Free polymer (P) and free pBR322 plasmid DNA were used as controls. Gels were captured under UV transillumination (312 nm) after electrophoretic separation at 80 V for 60 min.

6. CytoTox-ONE™ Assay

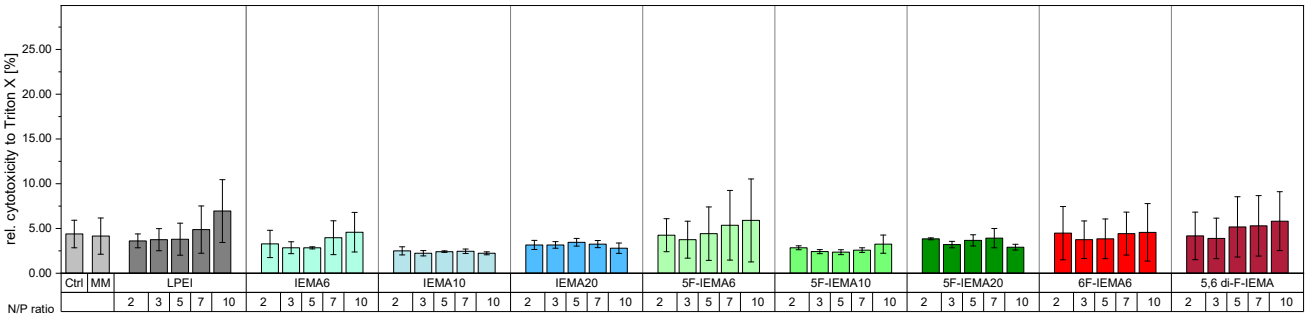

Figure S32. N/P ratio dependent membrane integrity test (CytoTox-ONE™) of HEK293T cells conducted for 24 h in combination with the transfection experiments, in comparison to LPEI, polyplexes with pCMV pDNA (Ctrl) and pure mEGFP-N1 pDNA without polymer (MM); values represent mean +/- SD of 3 experiments.