

“*In situ*” Functionalized Polymers for siRNA Delivery

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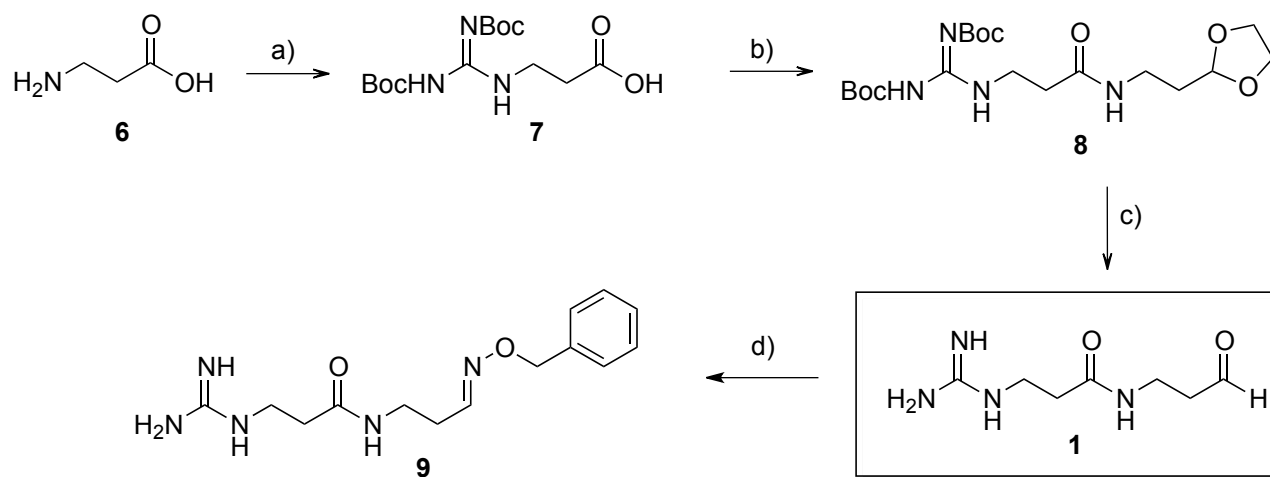
Materials and Methods

2-(((ethylthio)carbonothioyl)thio)-2-methylpropanoic acid (**CTA**)^{S1} was synthesised according to protocols described in the literature. 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS) was purchased from Sigma-Aldrich® and *p*-xylene-bis-pyridinium bromide (DPX) was purchased from Invitrogen™. Egg yolk L-α-

phosphatidylcholine (EYPC) was purchased from Avanti Polar Lipids, Inc. All other chemicals were purchased from Sigma-Aldrich®, Scharlau, Panreac Química SLU, Fisher Scientific® or Acros® and used without further purification. All solvents were HPLC grade, purchased from Sigma-Aldrich® or Fisher Scientific®, and used without further purification.

Nuclear Magnetic Resonance (NMR) spectra were recorded on either a Bruker Avance III 300 MHz, a Bruker Avance III 400 MHz spectrometer, a Varian Mercury 300 MHz or a Varian Inova 500 MHz spectrometer. Chemical shifts are reported in ppm (δ units) referenced to the following solvent signals: DMSO-*d*6 δ H 2.50, D₂O δ H 4.79 and CDCl₃, δ H 7.26. Electrospray ionization mass spectrometry (ESI-MS) for the characterization of new compounds was performed on a Finnigan MAT SSQ 7000 instrument or an ESI API 150EX and are reported as mass-per-charge ratio *m/z* (intensity in %, [assignment]). Accurate mass determinations (HR-MS) using ESI-MS were performed on a Sciex QSTAR Pulsar mass spectrometer. Infrared (IR) spectra were recorded on a Perkin Elmer Spectrum Two FT-IR spectrometer. Ultraviolet-visible (UV-vis) spectra were recorded on a Campsec M550 Double Beam Scanning UV-vis Spectrophotometer. Fluorescence measurements were performed with a FluoroMax-2 spectrofluorometer (Jobin-Yvon Spex) equipped with a stirrer and a temperature controller. Size Exclusion Chromatography (SEC) spectra were recorded on a Shimadzu Prominence LC-20A fitted with a Thermo Fisher Refractomax 521 Detector (**Boc-P1**) or a SPD20A UV-vis Detector (**P1**). **Boc-P1** was analyzed using 0.05 M LiBr in DMF at 60 °C as the eluent and a flow rate of 1 mL·min⁻¹. The instrument was fitted with a Polymer Labs PolarGel guard column (50 × 7.5 mm, 5 μ m) followed by two PLGel PL1110-6540 columns (300 × 7.5 mm, 5 μ m). Molecular weights were calculated based on a standard calibration method using polymethylmethacrylate. Activation of **P1** was analyzed using 100 mM acetic acid at pH 2.9 as the eluent and a flow rate of 1 mL·min⁻¹. The instrument was fitted with a Shodex Asaphipak GF-510 HQ column and a Shodex Asaphipak GF-310 HQ column (300 × 7.5 mm, 5 μ m). Vesicles were homogenized using a Mini-Extruder from Avanti Polar Lipids Inc. Activated polymer and Polyplex hydrodynamic diameter and ζ -potential were determined using a Malvern Zetasizer Nano ZS90. For cell experiments, the absorbance at 560 nm (cytotoxicity assays) and fluorescence (λ_{ex} 489nm; λ_{em} 509nm, transfection experiments) were measured using a microplate reader (Infinite F2000pro Tecan). Gels were resolved on an electrophoresis cell (Fisher Scientific UK), while an UV image station (Chem-genius, Syngene) was used to record and analyze gel images.

Synthesis of Guanidinium Aldehyde (1)



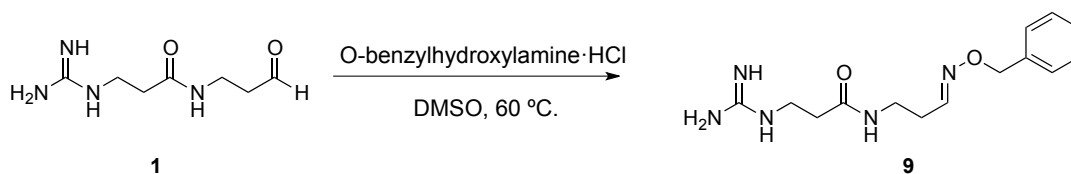
Scheme S1: Synthesis of aldehyde 1. a) *N,N'*-di-Boc-1*H*-pyrazole-1-carboxamide, DIPEA, CH₃CN/H₂O, 55 °C, 73%. b) 2-(1,3-dioxolan-2-yl)ethanamine, DCM, HBTU, DIPEA, rt, 80%. c) HCl 3M, 60 °C, 70%.

Compound 7. β -alanine (6) (750 mg, 8.42 mmoles) was dissolved in CH₃CN/H₂O (6:1, 35 mL) and treated with *N,N'*-di-Boc-1*H*-pyrazole-1-carboxamide (627.3 mg, 2.03 mmoles) and *N,N*-diisopropylethylamine (DIPEA) (868 μ l, 5.07 mmoles). The resulting solution was stirred for 2 h at 55 °C. The product was extracted with DCM (3x 10 mL) and the solution was washed with aqueous HCl (5%, 3 x 20 mL). The organic layers were combined, dried over Na₂SO₄ and concentrated under vacuum. The residue was purified by flash chromatography (gradient DCM/MeOH 98:2→90:10, *R_f*(90:10) = 0.70) to give 675 mg of compound 7 (73%). Spectroscopic data matched those reported in the literature.^{S2}

Compound 8. A solution of 7 (520 mg, 1.57 mmoles) in DCM (30 mL) was treated with TBTU (519.67 mg, 1.57 mmoles), 2-(1,3-dioxolan-2-yl)ethanamine (316 μ l, 2.83 mmoles) and DIPEA (1 mL, 6.28 mmoles, added dropwise). The reaction mixture was stirred at rt under Argon atmosphere for 1 hour. The reaction crude was washed with aqueous HCl (5%, 3 x 20 mL) and aqueous saturated NaHCO₃ (2 x 20 mL). The organic layer was dried with anhydrous Na₂SO₄, filtered and concentrated under vacuum. The residue was purified by flash chromatography (gradient DCM/MeOH 99:1→90:10, *R_f*(90:10) = 0.76) to give 542.6 mg of compound 8 (80%) (**Figure S19**). ¹H-NMR (300 MHz, CDCl₃) δ (ppm) 11.4 (s, 1H), 8.7 (t, ³*J_{H,H}* = 4.9 Hz, 1H), 6.66-6.57 (m, 1H), 4.8 (td, ³*J_{H,H}* = 4.3, 0.7 Hz, 1H), 3.95-3.91 (2H, m), 3.85-3.81 (2H, m), 3.70-3.59 (2H, m), 3.4-3.3 (2H, m), 2.41 (t, ³*J_{H,H}* = 6.2 Hz, 2H, m), 1.86-1.84 (2H, m), 1.49 (9H, s), 1.48 (9H, s). ¹³C-NMR (500 MHz, CDCl₃) δ (ppm) 170.79 (s), 163.40 (s), 156.25 (s), 152.84 (s), 103.89 (d), 83.09 (s), 79.28 (s), 64.89 (t), 36.05 (t), 35.40 (t), 34.71 (t), 32.80 (t), 28.30 (q), 28.05 (q). ESI-MS (H₂O/CH₃CN) *m/z* 431 (100, [M+H]⁺), 453 (20, [M+Na]⁺). IR (neat) ν_{\max} 3314 (m sh, N-H), 3124 (w, N-H), 2975 (m sh, C-O), 1721 (m sh, C=O), 1611 (s, N-H), 1409 (s, C-H), 1364 (s, C-H), 105 (s, C-O) cm⁻¹.

Compound 1. A solution of compound 8 (0.69 mmoles, 300 mg) in water was treated with an aqueous solution of HCl (3M, 10 mL). The reaction mixture was stirred at 60 °C for 1 hour. Then the solvent was evaporated under vacuum. The reaction crude was dissolved in H₂O and purified by RP-HPLC. The collected

fractions ($R_t = 4.0$ min,) were lyophilized and stored at -20 °C to give 90 mg of compound **1** (70%) (**Figure S20** and **S21**). RP-HPLC [Nucleosil 100-7 C18 H₂O (0.1% TFA)/CH₃CN (0.1% TFA) 100:0 (0→10 min), 100:0→75:35 (10→35 min), 0:100 (>35 min)]. Purity and characterization were confirmed by analytical RP-HPLC, ¹H-NMR and ESI-MS. ¹H-NMR (300 MHz, D₂O) δ (ppm) 9.53 (s, 1H), 5.00-4.77 (m, 1H), 3.40-3.25 (m, 2H), 3.24-3.04 (m, 2H), 2.65-2.60 (m, 2H), 2.52-2.29 (m, 2H), 1.94-1.73 (m, 1H), 1.70-1.60 (m, 2H). ESI-MS (H₂O/CH₃CN) m/z 187 (100, [M+H]⁺, 205 (30, [M+H₂O]⁺). HR-MS (MS): Calcd for C₇H₁₅N₄O₂: 187.1185; found: 187.1190. IR (neat) ν_{\max} 3312 (m b, N-H), 3121 (m b, N-H), 2976 (w sh, C-O), 1722 (m, C=O), 1614 (s, N-H), 1363 (s, C-H) 1057 (s, C-O) cm⁻¹. NMR analysis of **1** revealed a discrete mixture of isomers due to potential intramolecular cyclization and/or aldehyde oligomerization.^{S3} Treatment of **1** with benzylhydroxylamine afforded the corresponding oxime **9** as a pure single product.

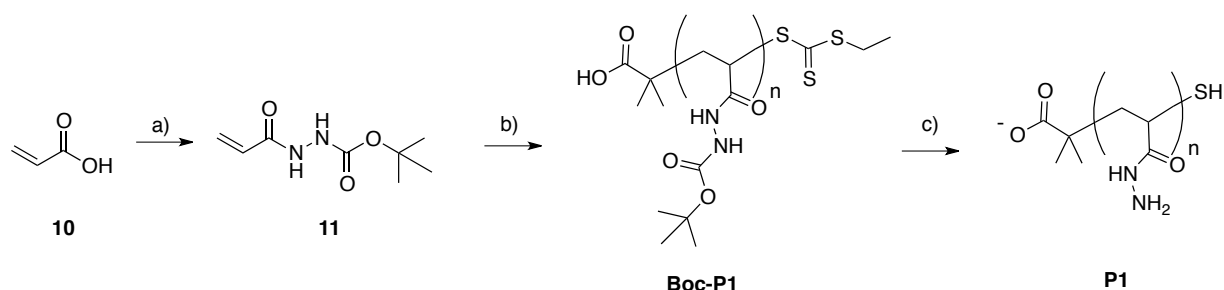


Scheme S2. To determine the purity of the isomer mixture present in **1**, this mixture was reacted with benzyl hydroxylamine to afford oxime **9** as a pure single product. This result suggested that all isomers of compound **4** reacted, under the coupling conditions, to afford the desired oxime **5**.

Compound 9. A solution of compound **1** (100 mM) in aqueous acetic acid (AcOH_{aq}) (100 mM, pH = 4.5) was mixed with 1.2 equivalents of O-benzylhydroxylamine (100 mM) in DMSO. The mixture was stirred at 60 °C for 2 h. The crude was purified by RP-HPLC (The collected fractions ($R_t = 18.8$ min) were lyophilized and stored at -20 °C to give 9.0 mg of compound **9** (80%) (**Figures S22, S23** and **S24**). RP-HPLC [Nucleosil 100-7 C18 H₂O (0.1% TFA)/CH₃CN (0.1% TFA) 100:0 (0→5 min), 100:0→35:75 (5→35 min), 0:100 (>35 min)]. The presence and purity of the O-alkyloxime **9** was checked by analytical RP-HPLC, NMR and ESI-MS. ¹H-NMR (500 MHz, D₂O) δ (ppm) 7.5 (t, ³ $J_{H,H} = 6.4$ Hz, 1H, Isomer *E*), 7.43-7.35 (5H, m), 6.85 (t, ³ $J_{H,H} = 5.7$ Hz; 1H, Isomer *Z*), 5.10 (s, 2H, Isomer *Z*), 5.05 (s, 2H, Isomer *E*), 3.40-3.30 (m, 4H), 2.59 (dd, ³ $J_{H,H} = 12.3, 6.4$ Hz, 1H), 2.40-2.30 (m, 3H). ¹³C-NMR (500 MHz, D₂O) δ (ppm) 173.5 (s), 152.2 (d) Isomer *E*, 152.0 (d) Isomer *Z*, 137.2 (s), 137.0 (s), 128.6 (d), 128.3 (d), 128.2 (d), 128.1 (d), 128.0 (d), 75.39 (t) Isomer *Z*, 75.0 (t) Isomer *E*, 37.4 (t), 36.0 (t) Isomer *Z*, 35.9 (t) Isomer *E*, 34.7 (t) Isomer *E*, 34.6 (t) Isomer *Z*, 29.2 (t), 25.7 (t). ESI-MS (H₂O/CH₃CN) m/z 292 (100, [M+H]⁺). HR-MS (ESI): Calcd for C₁₄H₂₂N₅O₂: 292.1771; found: 283.1768. IR (neat) ν_{\max} 3369 (m b, N-H), 3246 (m b, N-H), 3010 (w, C-H), 1671 (s, C=O), 1491 (m, C=C), 1382 (m, N-O), 1347 (s, C-H), 1120 (s) cm⁻¹.

Synthesis of Activated Polymers

• Synthesis of Poly(acryloyl hydrazide) P1



Scheme S3: a) *tert*-butyl carbazate, EDC, H₂O/THF (2:1), 25 °C, 50%. b) CTA, ACVA, DMSO, 70 °C, 73%. c) *i.* TFA, 25 °C; *ii.* H₂O, NaHCO₃, 25 °C, 92%.

Compound 11. Acrylic acid (**10**) (3.81 mL, 54.95 mmol) and *tert*-butyl carbazate (8.89 g, 65.95 mmol) were dissolved in a H₂O/THF mixture (2:1, 180 mL) at rt. *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) (11.75 g, 61.29 mmol) was added in portions to the solution over 15 minutes and left stirring for 3 h. The crude reaction was extracted with EtOAc (3 x 75 mL) and the organic layer was washed with 0.1 M HCl (3 x 75 mL), H₂O (50 mL) and brine (2 x 50 mL). The organic phase was dried with anhydrous Na₂SO₄ and the solvent was removed under reduced pressure to afford the crude product as a white solid. The crude product was purified by recrystallization from EtOAc (70 °C to rt) to afford a 5.05 g of a white crystalline powder identified as **11** (50%) (**Figure S25**). $R_f = 0.87$ (100% EtOAc). ¹H-NMR (300 MHz, DMSO-*d*₆) δ (ppm) 9.79 (s, 1H), 8.84 (s, 1H), 6.17-6.20 (m, 2H), 5.69 (dd, ³ $J_{H,H} = 7.8, 4.5$ Hz, 1H), 1.40 (s, 9H). ¹³C-NMR (100 MHz, DMSO-*d*₆) δ (ppm) 164.3 (s), 155.3 (s), 129.4 (d), 126.2 (t), 79.2 (s), 28.1 (q). IR (neat) ν_{\max} 3311 (m sh, N-H), 3221 (m sh, N-H), 2981 (w sh, C-H), 1715 (s sh, C=O), 1668 (s sh, C=O) cm⁻¹.

Boc-P1. A solution of 4,4'-azobis(4-cyanovaleric acid) (ACVA) (18.4 mg, 0.064 mmol) in DMSO (1.5 mL) and a solution of CTA (72.3 mg, 0.322 mmol) in DMSO (1.5 mL) were added sequentially to a solution of *tert*-butyl-2-acryloylhydrazine-1-carboxylate (**11**) (3.00 g, 16.095 mmol) in DMSO (14.88 mL). A 50 μ L aliquot of this solution was taken at this stage to aid in the calculation of conversion. The reaction mixture was then sealed and degassed with Argon for 30 min. The degassed solution was left to react at 70 °C for 7 h. The reaction was stopped by allowing it to cool down to room temperature and by exposing it to air. A 50 μ L aliquot of this solution was taken at this stage to aid in the calculation of conversion. The polymer was purified by dialysis against water. The water was removed by lyophilisation and by drying in a desiccator with P₂O₅ to afford 2.2 g of **Boc-P1** (**Figure S26**) as an off-white powder (73% yield). UV (DMSO) λ_{\max} 300 nm. ¹H-NMR (300 MHz, DMSO-*d*₆) δ (ppm) 9.22 (1H, br), 8.60 (1H, br), 2.03 (1H, br), 1.41 (11H, br). Conversion 80%. M_n (DMF GPC) 10270, D_M (DMF GPC) 1.39. DP (UV-Vis) 45.

– Calculation of Conversion

50 μ L aliquots of the reaction mixture were taken before and after the polymerization. These aliquots were mixed with 50 μ L of a 0.8 M stock solution of syringic acid (used as internal standard) and 650 μ L of DMSO-*d*₆.

Monomer conversion was calculated by $^1\text{H-NMR}$ spectra by comparing the integration of the vinyl proton signals from the monomers (5.69 and 6.19 ppm) to the integration from the aromatic groups in syringic acid (7.20 ppm).

– Calculation of Degree of Polymerization (DP) Using UV

DP in **Boc-P1** was calculated by measuring the absorbance at 300 nm (λ_{max} for **Boc-P1**), 305 nm and 310 nm (λ_{max} for **CTA**) and comparing against calibration curves using **CTA** (**Figure S1** and **Table S1**). This way, the amount ($\text{mg}\cdot\text{mL}^{-1}$) of trithiocarbamate in **Boc-P1** was estimated,[†] and the ratio between monomer units and end-groups calculated.

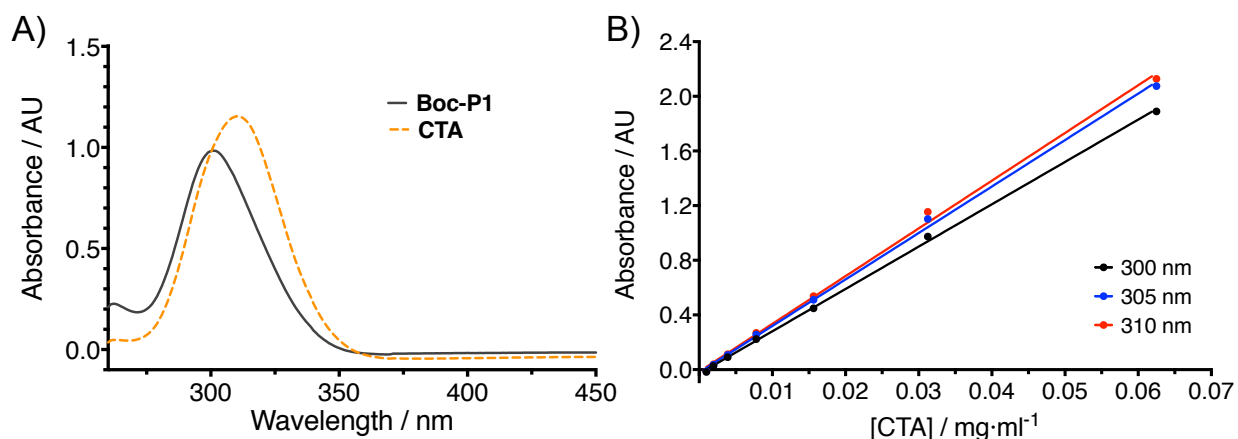


Figure S1: A) UV-vis of **Boc-P1** ($1.3 \text{ mg}\cdot\text{mL}^{-1}$) and **CTA** ($0.03 \text{ mg}\cdot\text{mL}^{-1}$) solutions in DMSO. B) Calibration curves showing the linear relationship between absorbance and [CTA].

Poly(acryloyl hydrazide) P1. Trifluoroacetic acid (TFA) (15 mL) was added dropwise to poly(*tert*-butyl-2-acryloylhydrazine-1-carboxylate) (**Boc-P1**) (1.5 g) and the yellow solution was stirred at rt for 2 h. Excess of TFA was removed by blowing a steady stream of Argon and the resulting oil was diluted in water (15 mL). The **P1**·TFA salt formed was neutralised by adding NaHCO_3 until no foaming was observed. The colourless solution was allowed to stir overnight. The crude polymer was purified by dialysis against water. The water was removed by lyophilisation and by drying in a desiccator with P_2O_5 to afford 650 mg of **P1** (**Figure S27**) as a white powder (92%). $^1\text{H-NMR}$ (300 MHz, D_2O) δ (ppm) 1.59-2.08 (br m, (3·DP)H), 1.01 (s, 3H), 0.95 (s, 3H). $^{13}\text{C-NMR}$ (100 MHz, D_2O) δ (ppm) 174.9 (s), 40.2-40.5 (d), 34.4-35.7 (d). DP ($^1\text{H-NMR}$) 40. IR (neat) ν_{max} 3254 (w br, N-H), 1609 (m br, C=O), 1428 (s sh) cm^{-1} .

– Calculation of DP using $^1\text{H-NMR}$

DP in **P1** was calculated from the $^1\text{H-NMR}$ spectra by comparing the integration of the methyl substituents in the end-group (0.95 and 1.01 ppm, 6 H) to the integration from the aliphatic region in the polymer backbone (1.59-2.08 ppm) (**Figure S2** and **Table S1**).

[†] DP of polymerization calculated this way is approximate. Absorption (*i.e.* molar extinction coefficient, λ_{max}) properties for **CTA** and **P1** are not necessarily the same. Similarly, not all polymer chains will incorporate a trithiocarbamate as an end-group. However, the calculated value is within error of those calculated by $^1\text{H-NMR}$ (**Table S1**).

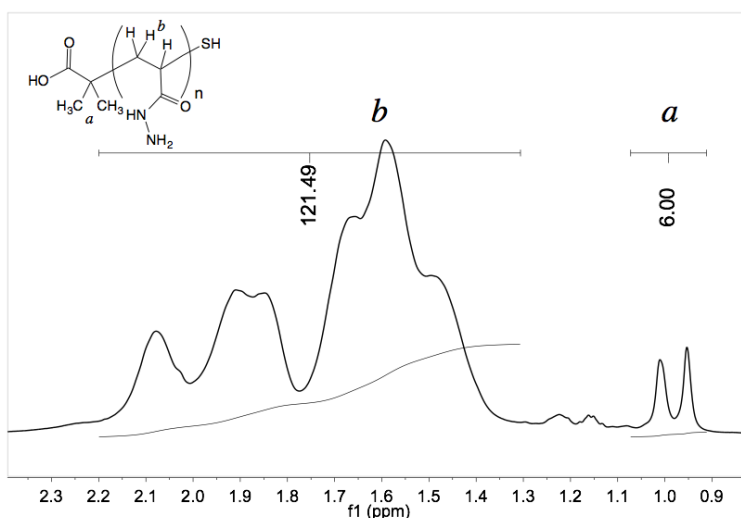


Figure S2: $^1\text{H-NMR}$ spectra of **P1** showing the relevant region where the methyl substituents (a) and the aliphatic hydrogens (b) can be identified.

Table S1

	$[\text{M}] / [\text{CTA}]^{\text{a}}$	DP (conversion) ^b	DP (UV-vis) ^c	DP ($^1\text{H-NMR}$) ^d
Boc-P1	50:1	40	45	-
P1	-	-	-	40

^a Calculated from the initial monomer and **CTA** concentrations used for the polymerization.

^b Calculated from the initial monomer and **CTA** ratio and the conversion of monomer from $^1\text{H-NMR}$.

^c Calculated by UV-vis against a **CTA** standard as the ratio of monomer to trithiocarbamate in **Boc-P1** (**Figure S1**).

^d Calculated by $^1\text{H-NMR}$, using the methyl groups as an internal standard, as the ratio between monomer and end-group (**Figure S2**).

• Conjugation of Poly(acryloyl hydrazide) with Aldehydes

Poly(acryloyl hydrazide) (**P1**) in aqueous acetic acid (AcOH_{aq} , 100 mM, pH 3.0) was reacted with 6 equivalents of a solution of different molar fractions of **1** and hydrophobic aldehyde in DMSO. For instance, in a model experiment with pure guanidinium aldehyde ($\chi_1 = 1$), **P1** (25 μl , 35 mM in AcOH_{aq}) was reacted with **1** (25 μl , 200 mM in DMSO) to afford a final concentration of activated polymer of 50 mM. In a typical experiment with a mixture of aldehydes ($\chi_1 = 0.85$, $\chi_2 = 0.15$), 25 μl of a solution of **P1** (35 mM in AcOH_{aq} pH 3.0) was mixed with a solution of 25 μl composed by 3.8 μl of a solution of hydrophobic aldehyde (200 mM in DMSO) and 21.2 μl of a solution of **1** (200 mM in DMSO) to give a final concentration of polymer of 50 mM (**Table S2**). This mixture was shaken at 60 $^\circ\text{C}$ for 2 h. Activated polymers were used without further purification in the transport vesicle experiments.

– Calculation of loading using $^1\text{H-NMR}$

Loading in **P1-4-imidazolecarboxaldehyde** was calculated from the $^1\text{H-NMR}$ spectra by comparing the integration of the residual aldehyde (9.7 ppm, XH) against the overall number of protons in that region (3H, 1H from aldehyde or hydrazone, 2H from 4-imidazolecarboxaldehyde ring). Signal at 6.0 ppm corresponds to the cyclic acetal and has been included in the overall integration.

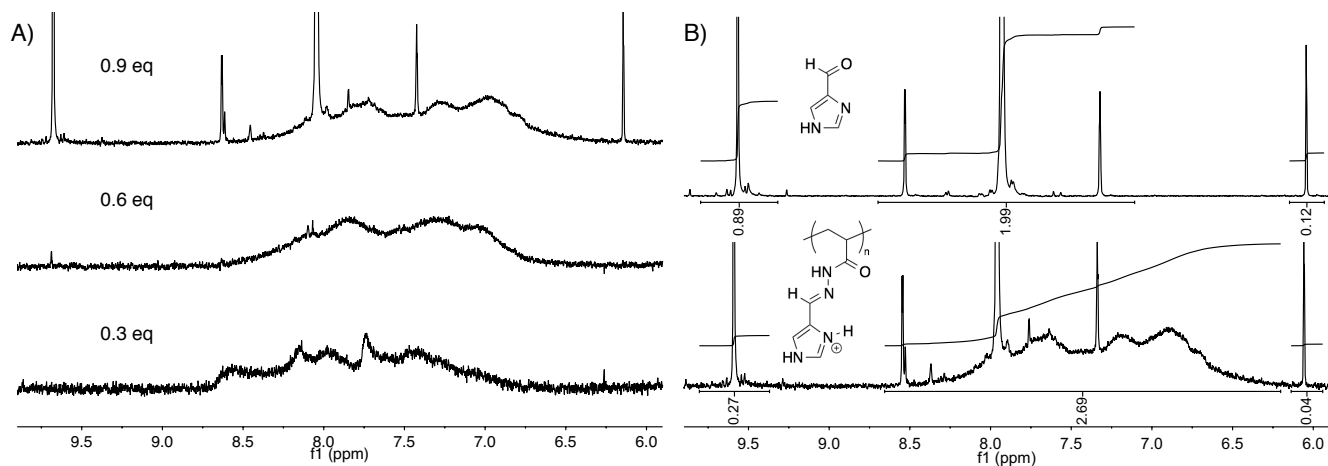


Figure S3: A) ¹H NMR spectra of **P1** (1.3 mg/mL) incubated (< 1h) with increasing amounts of 4-imidazolecarboxaldehyde. From bottom: 0.3, 0.6 and 0.9 eq. of 4-imidazolecarboxaldehyde. B) Integration of residual aldehyde (9.7 ppm) against the overall number of protons (3H) in this region.

– Dynamic light scattering of **P1(1)₈₅(2)₁₅**

P1(1)₈₅(2)₁₅ was prepared accordingly to the general procedure for the conjugation of poly(acryloyl hydrazide) with aldehyde aldehydes. The final concentration in the reaction vessel was 17 mM. From this stock solution 20 μl were diluted with 1980 μl of MilliQ water to afford a final concentration of 17 μM of activated polymer. For DNA conjugates, 20 μl of 17 mM activated polymer stock solution was diluted with 1960 μl of MilliQ water and 20 μl of 2 μM DNA from Herring sperm were added and the mixture was gently mixed by pipetting and immediately transferred to the cuvette for DLS experiment.

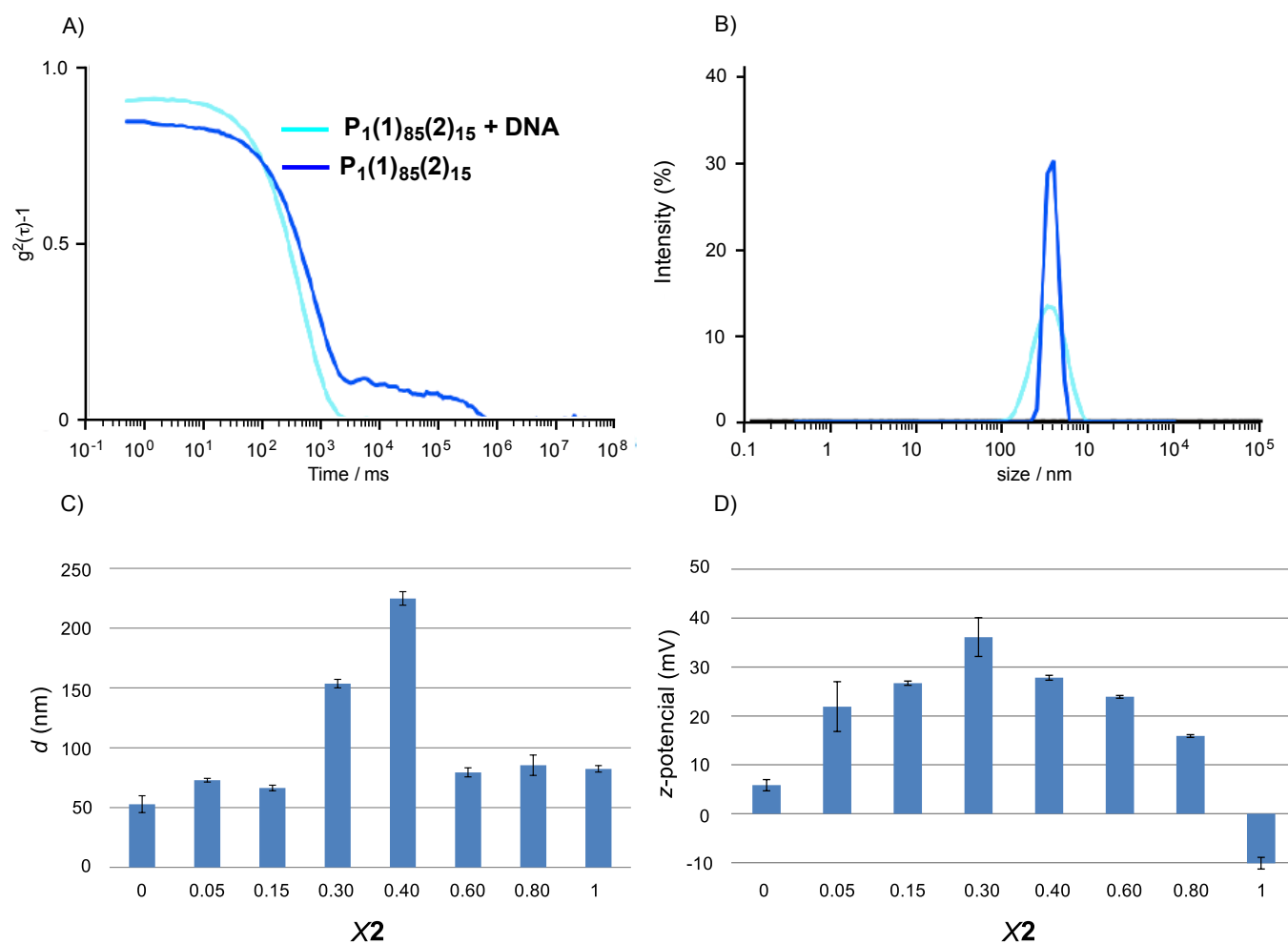


Figure S4: Representative dynamic light scattering data for $P_1(1)_{85}(2)_{15}$ in the absence and presence of dsDNA (Herring DNA). A) Autocorrelation function (ACF) curves and B) intensity distributions measured in milliQ water. [activated polymer] = 17 μM . C) Diameter and D) ζ -potential for representative activated polymer $P_1(1)_{85}(2)_{15}$ = 3 μM at increasing molar fractions of isovaleraldehyde ($X_2 = 0-1$).

Evaluation of Transport Across Model Membranes: Vesicle Experiments

• Preparation of Large Unilamellar Vesicles (LUV)

A thin lipid film was prepared by evaporating a solution of EYPC (25 mg) in MeOH/ CHCl_3 (1:1, 1 mL) on a rotary evaporator (at rt) and then in vacuo overnight. The resulting film was hydrated with 1.0 mL buffer (5 mM HPTS, 16.5 mM DPX, 10 mM Tris, 72 mM NaCl, pH 7.4) for more than 30 min, subjected to freeze-thaw cycles (5 x) and extrusions (15 x) through a polycarbonate membrane (pore size 100 nm). Extravesicular components were removed by gel filtration (Sephadex G-50) with 10 mM Tris, 107 mM NaCl, pH 7.4. Final conditions: ~5 mM EYPC; inside: 5 mM HPTS, 16.5 mM DPX, 10 mM Tris, 72 mM NaCl, pH 7.4; outside: 10 mM Tris, 107 mM NaCl, pH 7.4.

• **Evaluation of Transport of Nucleic Acids across EYPC-LUV**

EYPC-LUV stock solutions (5 μ l) were diluted with buffer (10 mM Tris, 107 mM NaCl, pH 7.4), placed in a thermostated fluorescence cuvette (25 $^{\circ}$ C) and gently stirred (total volume \sim 2000 μ l; final lipid concentration \sim 13 μ M). HPTS efflux was monitored at λ 511 nm (λ_{ex} 413 nm) as a function of time after addition of activated polymer (20 μ l in DMSO/AcOH buffer, $t = 25$ s), nucleic acid (NA, 20 μ l of 2 μ M stock solution in buffer, $t = 50$ s) and aqueous triton X-100 (1.2%, 40 μ l, 370 μ M final concentration, $t = 225$ s). Total experiment time = 250 s. Fluorescence intensities were normalized to fractional emission intensity $I(t)$ using Equation S1.

$$\text{Equation S1: } I(t) = (I_t - I_0)/(I_{\infty} - I_0)$$

where $I_0 = I_t$ at NA addition, $I_{\infty} = I_t$ at saturation after lysis. Effective concentration for activated polymer or NA - EC_{50} - and Hill coefficient - n - were determined by plotting the fractional activity $Y (= I(t)$ at saturation just before lysis, $t = 200$ s) as a function of activated polymer or NA concentration $[Analyte]$ and fitting them to the Hill equation (Equation S2).

$$\text{Equation S2: } Y = Y_0 + (Y_{max} - Y_0) / \left\{ 1 + \left(\frac{EC_{50}}{[Analyte]} \right)^n \right\}$$

where Y_0 is Y without NA (or activated polymer), Y_{max} is Y with an excess of activated polymer (or NA) at saturation, EC_{50} is the concentration of NA (or activated polymer) required to reach 50% activity and n is the Hill coefficient (**Figure S5** and **Table S2**).

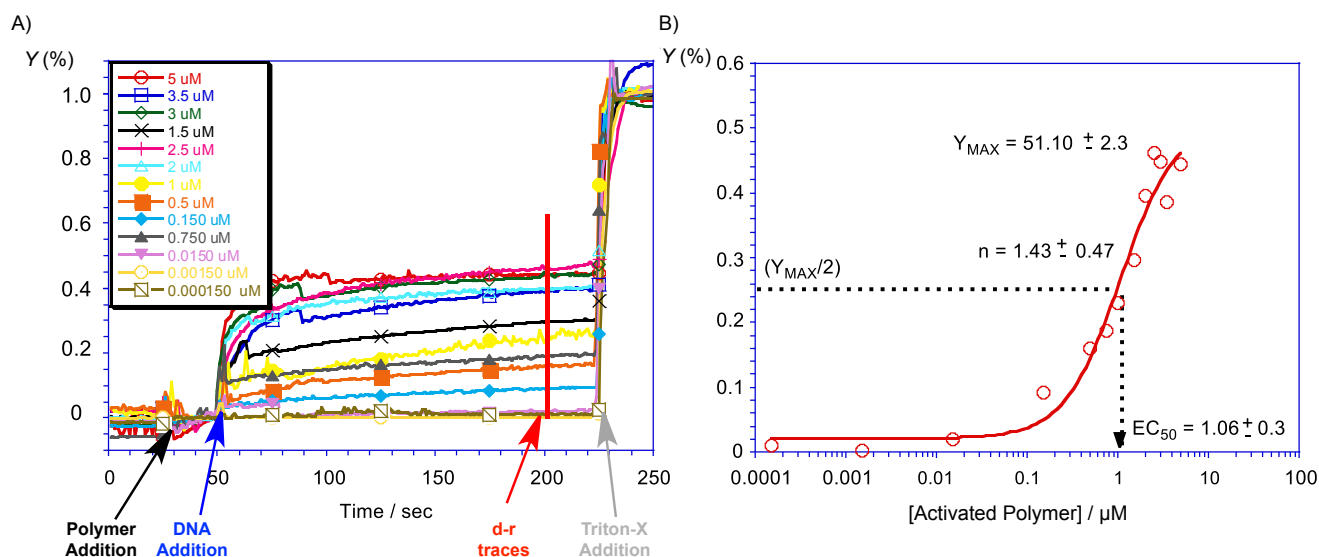


Figure S5: A) Changes to fractional emission intensity $I(t)$ for EYPC-LUVs \Rightarrow HPTS/DPX after the addition of activated polymer (17 μ M, χ_1 : 0.85 and χ_5 : 0.15) at $t = 25$ sec, Herring DNA (5 μ M-0.15 nM final concentrations) at $t = 50$ sec and Triton-X (370 μ M final concentration) at $t = 225$ sec. B) Dose response curve obtained from the plot of fractional activity vs activated polymer concentration and fitting to the Hill equation (Equation S2) $Y_{MAX} = 51.10 \pm 2.3\%$, $EC_{50} = 0.33 \pm 0.3 \mu$ M, $n = 1.43 \pm 0.47$.

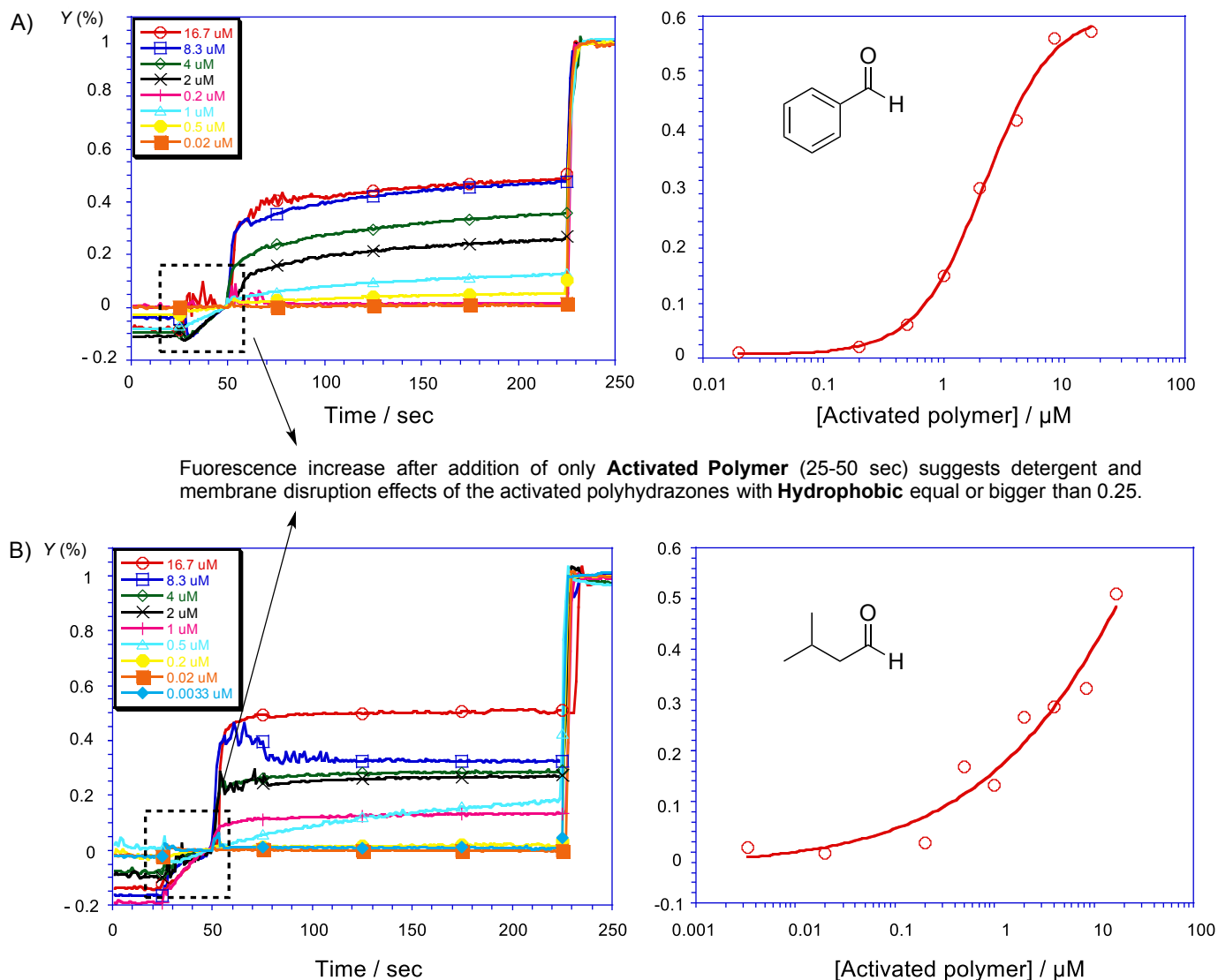


Figure S6: Changes to fractional emission intensity $I(t)$ and dose-response curve for the transport of Herring DNA (2 μM) in EYPC-LUVs with HPTS/DPX in the presence of activated polymer (17 μM , χ_1 : 0.75 and $\chi_{\text{Hydrophobic}}$: 0.25). A) $\chi_{\text{Hydrophobic}}$ = Benzaldehyde and in B) $\chi_{\text{Hydrophobic}}$ = isovaleraldehyde (2). The increase in fluorescence observed immediately after the addition of activated polymer depicts membrane disruption profiles when using molar fractions of 0.25 of the hydrophobic aldehydes.

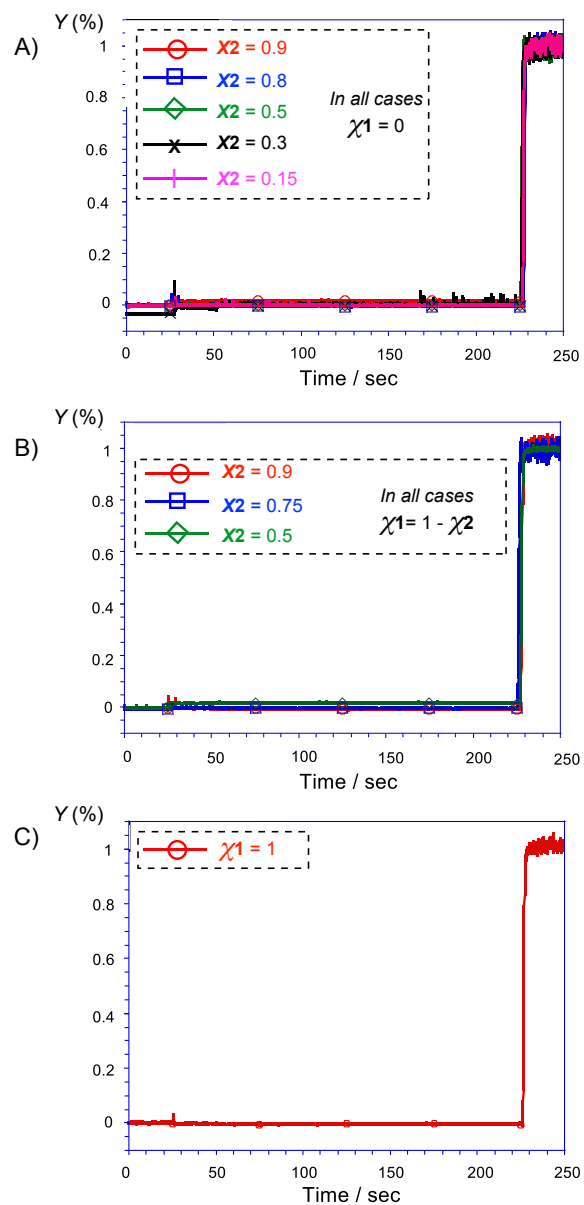
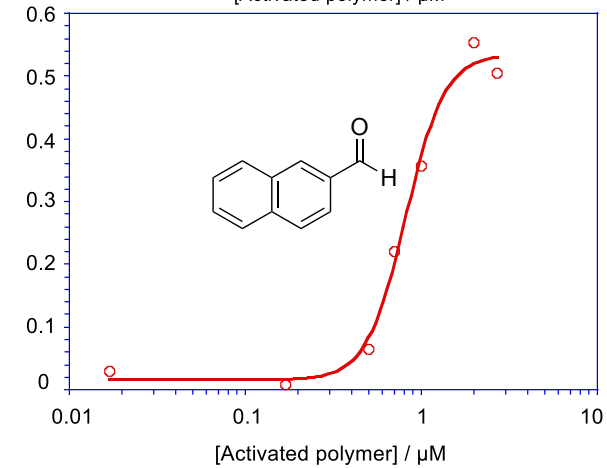
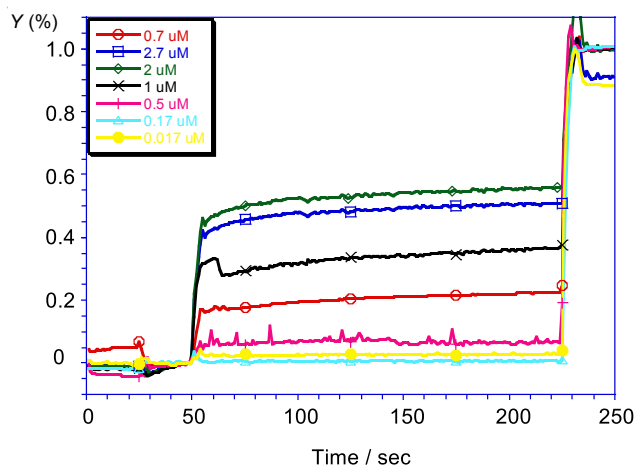
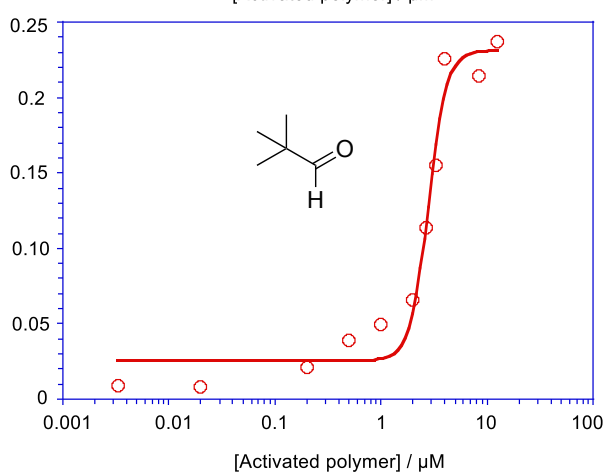
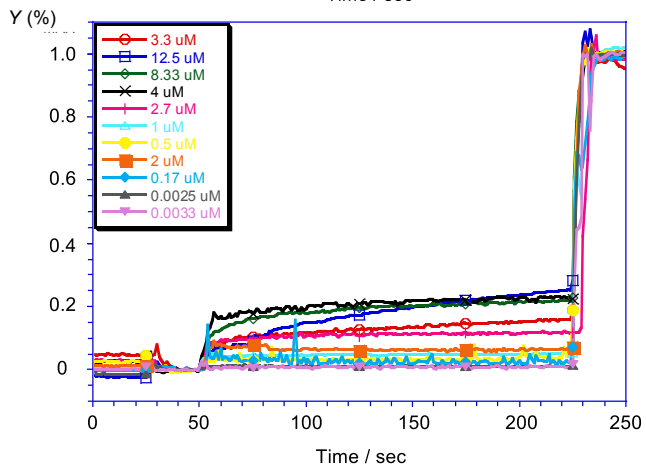
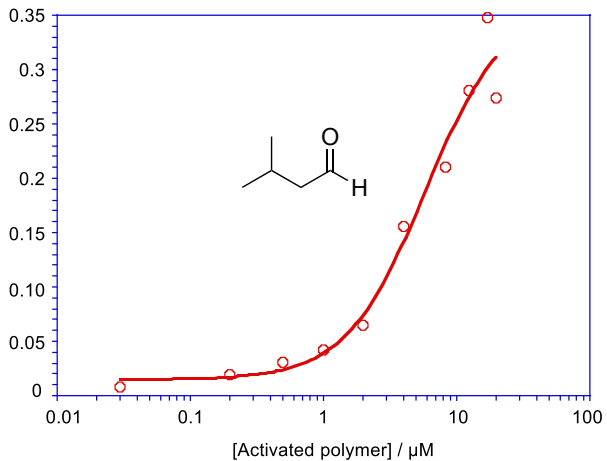
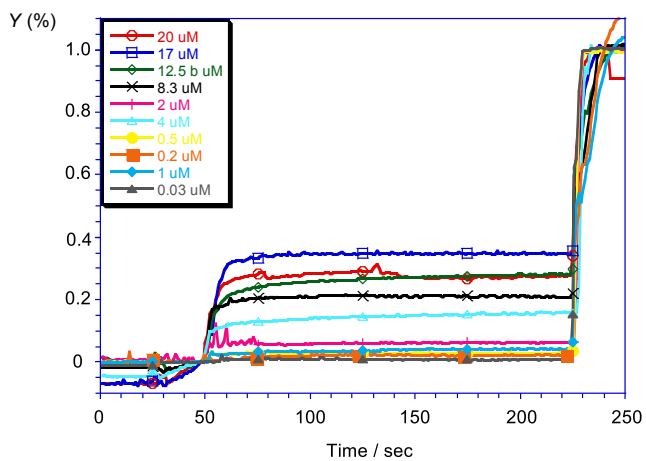
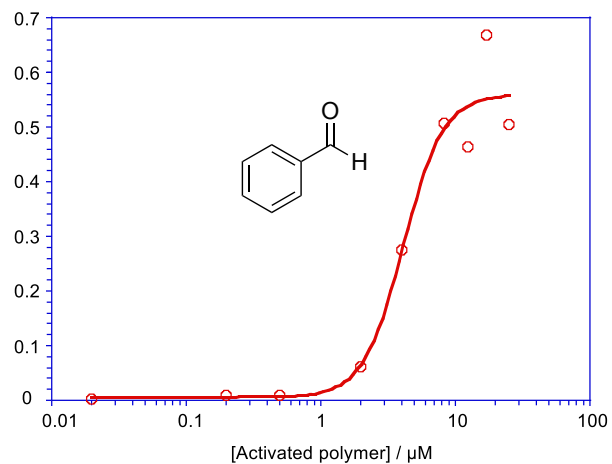
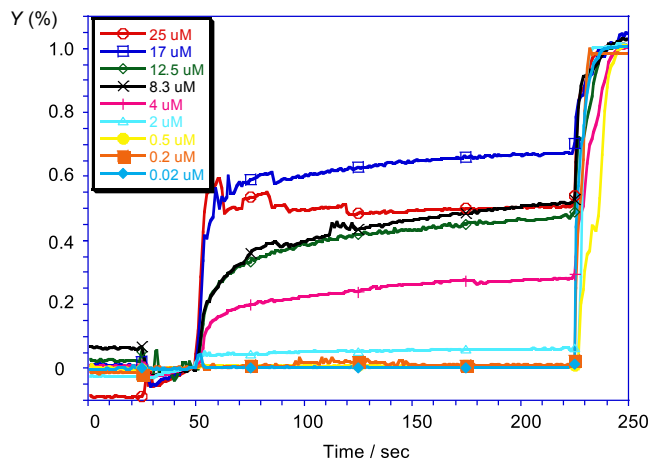
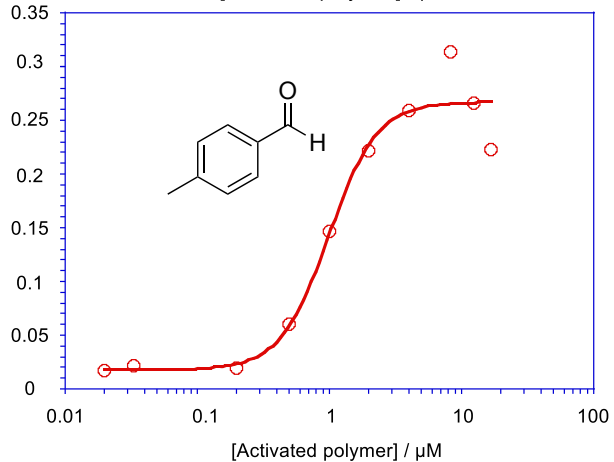
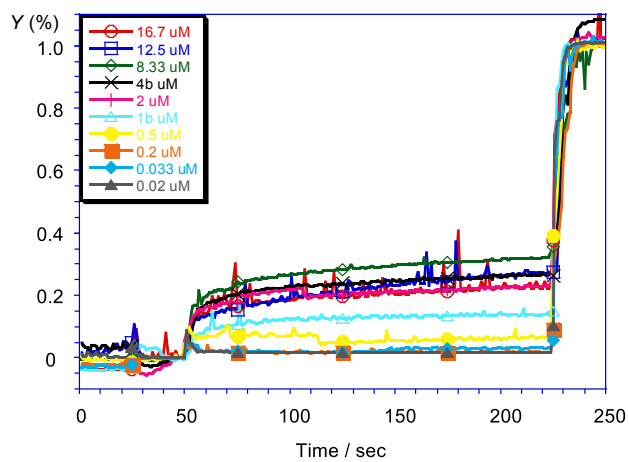
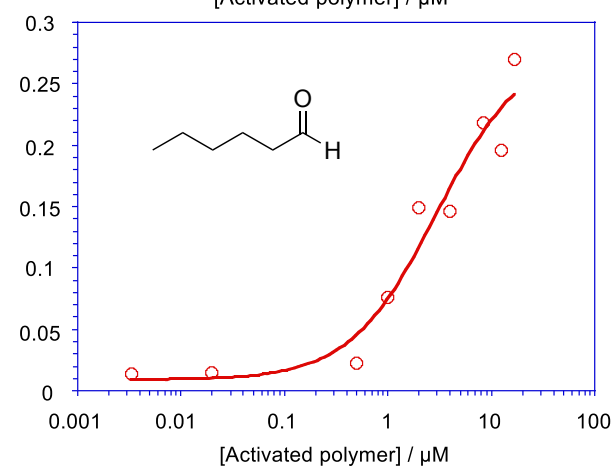
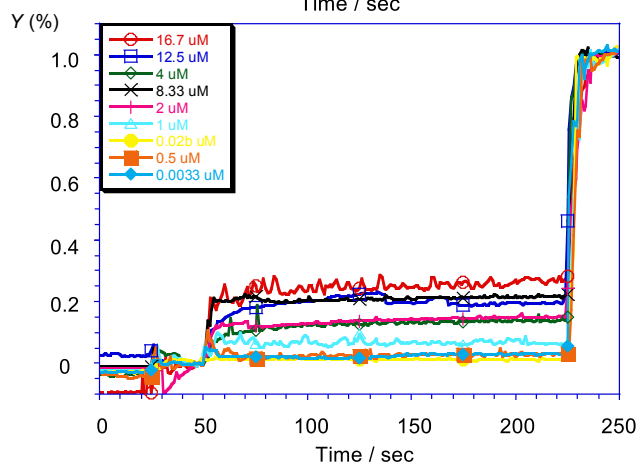
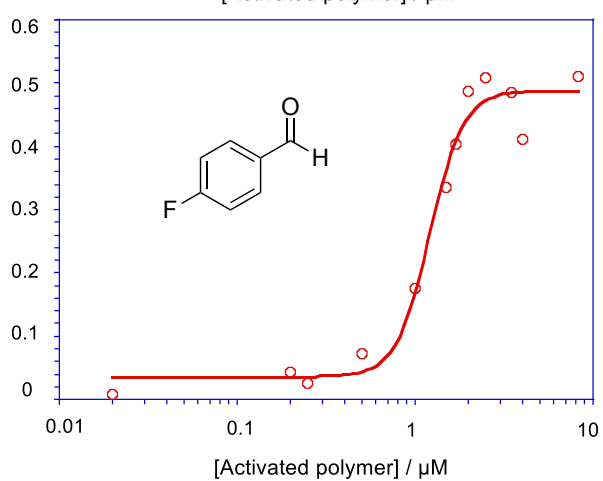
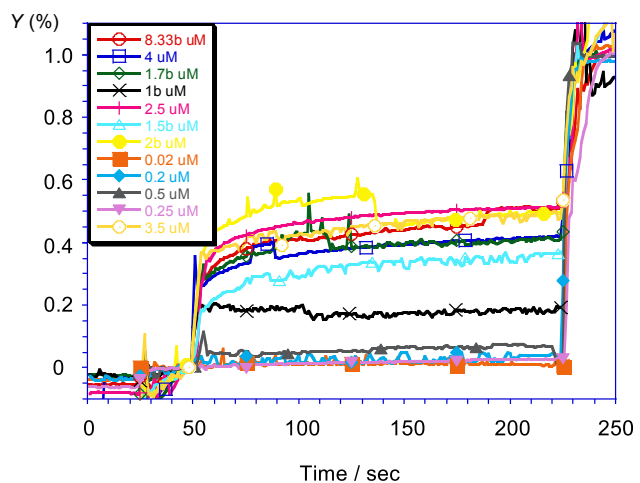
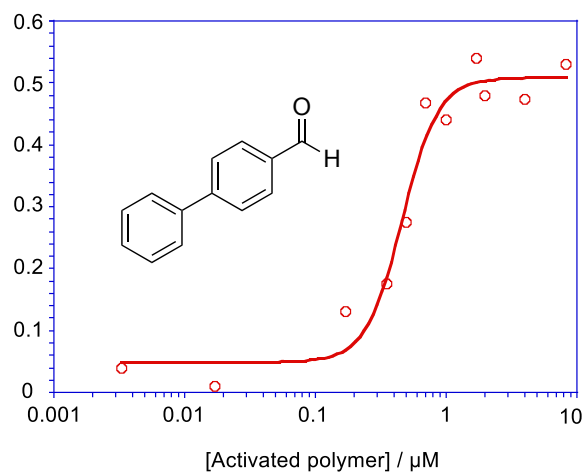
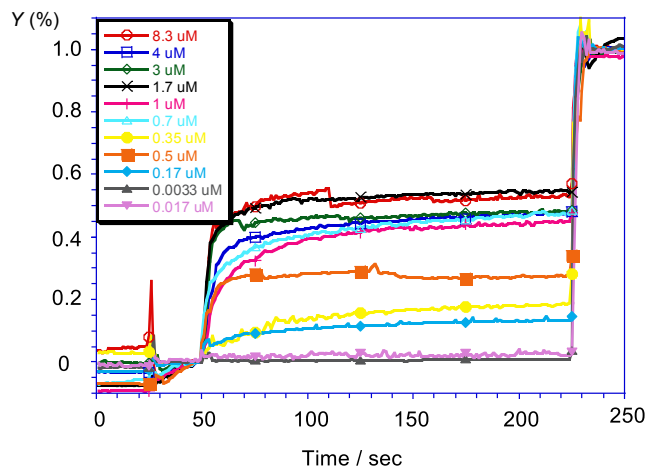


Figure S7: A) Changes to fractional emission intensity $I(t)$ for the transport of Herring DNA (2 μM) in EYPC-LUVs \Rightarrow HPTS/DPX in the presence of activated polymer (17 μM) prepared from χ_2 (0.9-0.1) in the absence of cationic aldehyde ($\chi_1 = 0$). B) Same plot for activated polymers (17 μM) prepared with higher molar fractions of **2** ($\chi_2 = 0.5, 0.75$ or 0.9) and $\chi_1 = 1 - \chi_2$. C) Same control showing no activity in vesicles for the purely cationic activated polymer ($\chi_1 = 1$).





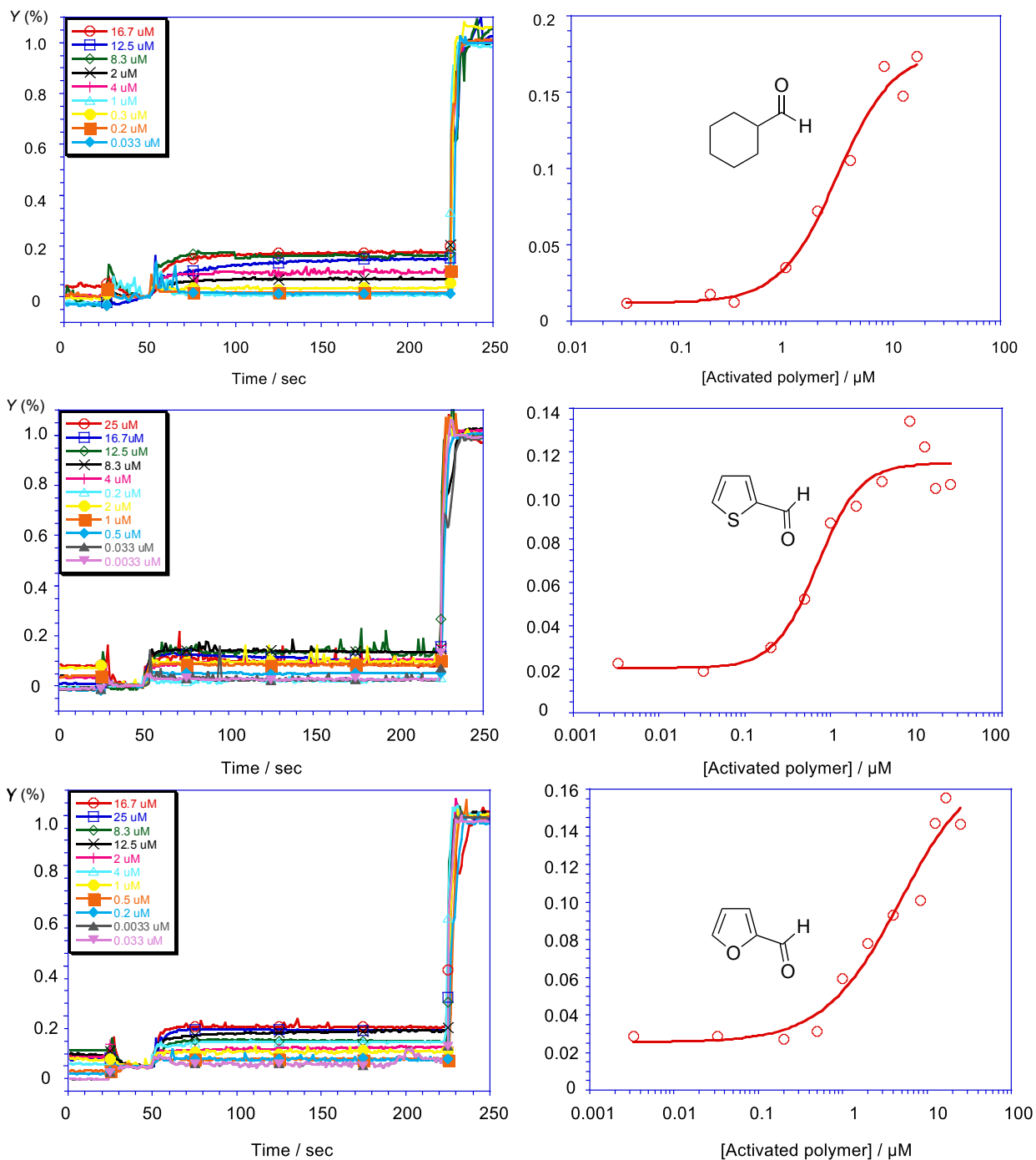

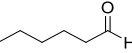




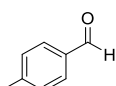
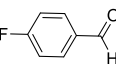
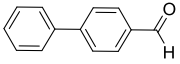




Figure S8: Changes to fractional emission intensity $I(t)$ (A) and dose-response curve (B) for the transport of Herring DNA ($2 \mu\text{M}$) in EYPC-LUVs \supset HPTS/DPX with increasing concentrations of activated polymer (χ_1 : 0.85 and $\chi_{\text{Hydrophobic}}$: 0.15) (Table S2).

Table S2: EC_{50} (μM), Y_{MAX} (%) and n for the transport of Herring DNA (125 μM) in EYPC-LUVs \Rightarrow HPTS/DPX with increasing concentrations of activated polymer **P1(1)₈₅(n)₁₅** prepared from 15% of hydrophobic aldehyde (**2-18**) and 85% of **1**. All experiments were done in triplicate. **P1(1)₈₅(n)₁₅** stands for **P1**: Polymer 1; **(1)₈₅**: guanidinium aldehyde at a $\chi_1 = 0.85$; **(n)₁₅**: hydrophobic aldehyde at a $\chi_n = 0.15$.

Polymer	Aldehyde Number	Aldehyde	EC_{50} (μM)	Y_{max} (%)	n
P1(1)₈₅(2)₁₅	2		5.73 ± 2.36	35.94 ± 7.80	1.47 ± 0.61
P1(1)₈₅(3)₁₅	3		2.95 ± 2.25	28.00 ± 8.4	1.04 ± 0.57
P1(1)₈₅(4)₁₅	4		0.81 ± 0.05	53.50 ± 2.70	3.91 ± 0.94
P1(1)₈₅(5)₁₅	5		4.10 ± 0.74	56.00 ± 5.00	2.92 ± 1.48
P1(1)₈₅(12)₁₅	12		5.10 ± 1.40	23.10 ± 2.30	2.80 ± 0.17
P1(1)₈₅(13)₁₅	13		3.01 ± 0.61	17.74 ± 1.68	1.64 ± 0.50
P1(1)₈₅(14)₁₅	14		0.98 ± 0.17	26.71 ± 1.51	2.37 ± 0.89
P1(1)₈₅(15)₁₅	15		1.21 ± 0.08	48.80 ± 1.90	4.60 ± 1.19
P1(1)₈₅(16)₁₅	16		0.46 ± 0.05	50.9 ± 2.60	3.13 ± 1.02
P1(1)₈₅(17)₁₅	17		4.83 ± 3.30	17.58 ± 4.10	0.96 ± 0.39
P1(1)₈₅(18)₁₅	18		0.70 ± 0.16	11.50 ± 0.56	1.75 ± 0.66

Cells Lines and Culture

HeLa cells stably expressing enhanced green fluorescent protein (HeLa-EGFP) were maintained in Dulbecco's Modified Eagle's Medium from Life Technologies™ (DMEM, high glucose, GlutaMAX™, pyruvate) supplemented with 10% (v/v) of fetal bovine serum (FBS) from Hyclone™ (Thermo Fisher Scientific Inc) and 500 µg·mL⁻¹ of Geneticin® (Life Technologies™). Transfection of HeLa-EGFP was performed in the same medium, free of serum and antibiotics. Cells incubations were performed in a water-jacketed 37 °C/5% CO₂ incubator.

- ***In Vitro* Screening for siRNA Delivery**

Activated polymer stock solutions were prepared in DMSO/AcOH_{aq} (v/v) as described above and diluted with DMSO to afford a range of stock solutions concentrations of (3-0.1 mM). These stock solutions were then sequentially diluted with DMEM medium free of serum and antibiotics to afford the final concentration in cells (17-0.3 µM). The solutions of siRNA/ activated polymer polyplexes were freshly prepared prior to the transfection experiments. 10 µl of the siRNA solution (1 µM in DMEM) and 8 µl of activated polymer solution at variable concentrations in DMEM, high Glucose, GlutaMAX™, 10% (v/v) DMSO, were added to 190 µl DMEM, high glucose, GlutaMAX™, and the mixture was homogenized by pipetting. Then, cell medium was aspirated from 96-well plate and 50 µl of the mixture was added in each well. The final concentration of DMSO in each well was 0.125% (v/v). After 4 hours of transfection the medium was aspirated and replaced by 100 µl of fresh DMEM, high glucose, GlutaMAX™, pyruvate, supplemented with 10% (v/v) FBS. The total fluorescence knockdown was quantified after 72 hours in a microwell plate reader (Infinite F2000pro Tecan). For the best performing activated polymers, at the most efficient and less toxic concentration, siRNA solutions were prepared at different concentrations (Table S3). For control and normalization experiments forward transfection with Lipofectamine® RNAiMAX was performed according to the supplier instructions. All experiments were done in triplicate.

Table S3: Conditions employed in the optimization of the transfection experiments. All experiments were done at a final concentration of activated polymer **P1(1) χ 1(2) χ 2** of 4 μ M.

χ 2 ^a	[siRNA] (nM) ^b	siRNA (pmol)
0.05	14	1.5
	0	0
	1.75	0.2
	3.5	0.4
	7	0.8
	14	1.5
0.15	28	3
	14	1.5
0.3	14	1.5
0.4	14	1.5

^a Molar fraction of χ 1 = 1 - χ 2. ^b [siRNA] refers to the final concentration of siRNA.

- **Transfection in HeLa-EGFP**

HeLa-EGFP were transfected either with Ambion® Silencer® GFP (EGFP) siRNA (siEGFP) from Life Technologies™ or scramble RNA (siMock, All Star Negative Control) from Qiagen. 72 h post siRNA transfection, cell supernatant was removed and EGFP expression was measured by fluorimetry (λ_{ex} 489nm; λ_{em} 509nm). The percentage of EGFP knockdown was calculated as the percentage of fluorescence decrease observed in cells transfected with siEGFP compared to transfection with siMock with the same reagents at the same conditions. Percentage of cell viability was calculated as the percentage of remaining fluorescence in samples transfected with siMock compared to non-transfected cells in DMEM, high glucose, GlutaMAX™ and pyruvate, supplemented with 0.125% (v/v) DMSO.

Lipofectamine® RNAiMAX was used as a positive control of siRNA transfection in the *in vitro* screening of activated polymers in HeLa-EGFP. The quality of the transfection experiments was assessed calculating the *Z-factor* using Equation S3,

$$\text{Equation S3: } Z\text{-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

In where μ stands for the mean value and σ for the corresponding standard deviation of relative fluorescence units (RFU) of both the positive (p = cells transfected with mixture of siEGFP and activated polymers or Lipofectamine® RNAiMAX) and negative (n = non-transfected cells in medium supplemented with 0.125% (v/v) DMSO) controls (μ_p , σ_p , and μ_n , σ_n). A *Z-factor* between 0.5 and 1.0 indicates an excellent assay, 0.5 is equivalent to a separation of 12 standard deviations between μ_p and μ_n (**Figure S9**).

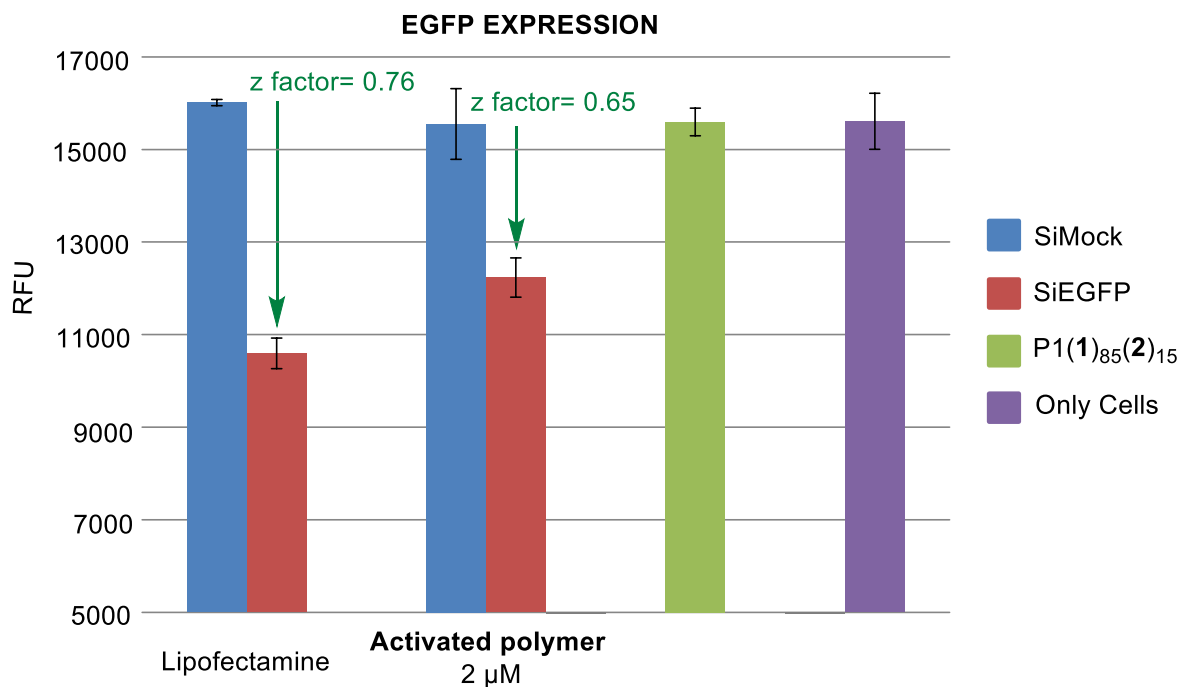


Figure S9: RFUs and *Z-factor* for the knock-down of EGFP in HeLa-EGFP with activated polymer (χ_1 : 0.85 and $\chi_{\text{Hydrophobic}}$: 0.15) or Lipofectamine® RNAiMAX.

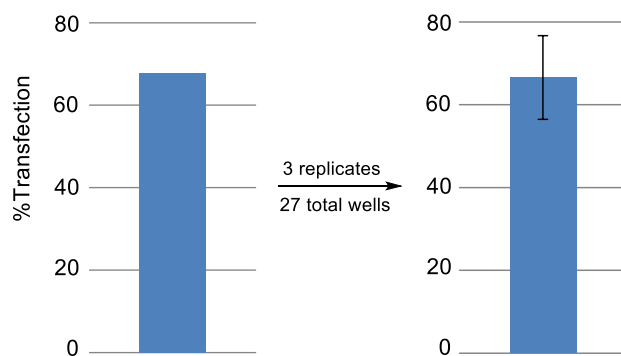


Figure S10: Transfection efficiency (percent of EGFP Knockdown) in HeLa-EGFP by 2 μM activated polymer (χ_1 : 0.85 and $\chi_{\text{Hydrophobic}}$: 0.15).

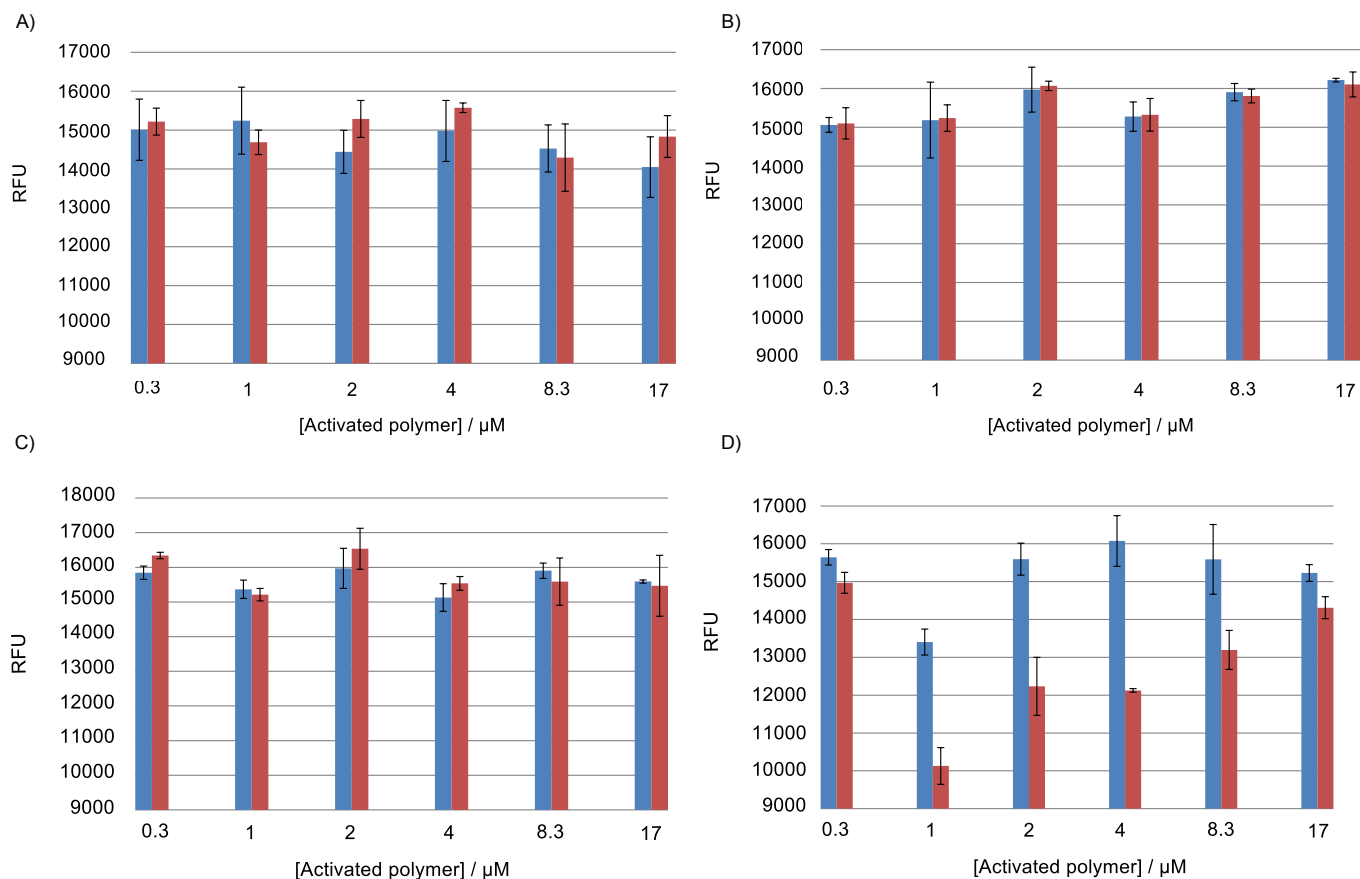


Figure S11: Relative fluorescent units (RFUs) for the siRNA transfection experiments. In all cases and in all concentrations, transfection experiments were performed with siRNA (red) and *siMock* (negative control experiment in blue). In all cases the molar fractions were $\chi_1 = 0.85$ and $\chi_{\text{Hydrophobic}} = 0.15$. a) Hexanal (**3**), **P1(1)₈₅(3)₁₅**, b) Naphthaldehyde (**4**), **P1(1)₈₅(4)₁₅**, c) Benzaldehyde (**5**), **P1(1)₈₅(5)₁₅**, d) Knock-down of EGFP in HeLa-EGFP is only observed for isovaleraldehyde (**2**), **P1(1)₈₅(2)₁₅**. A satisfactory Z factor was obtained for the different concentrations: 0.3 μM: 0.91, 1 μM: 0.89, 2 μM: 0.65, 4 μM: 0.46; 8 μM: 0.79, 17 μM: 0.88.

To optimize the concentration of activated polymer, HeLa-EGFP were treated with siEGFP/ activated polymer polyplexes with different final concentrations of activated polymer or different molar fractions of **2** (**Figure S12**).

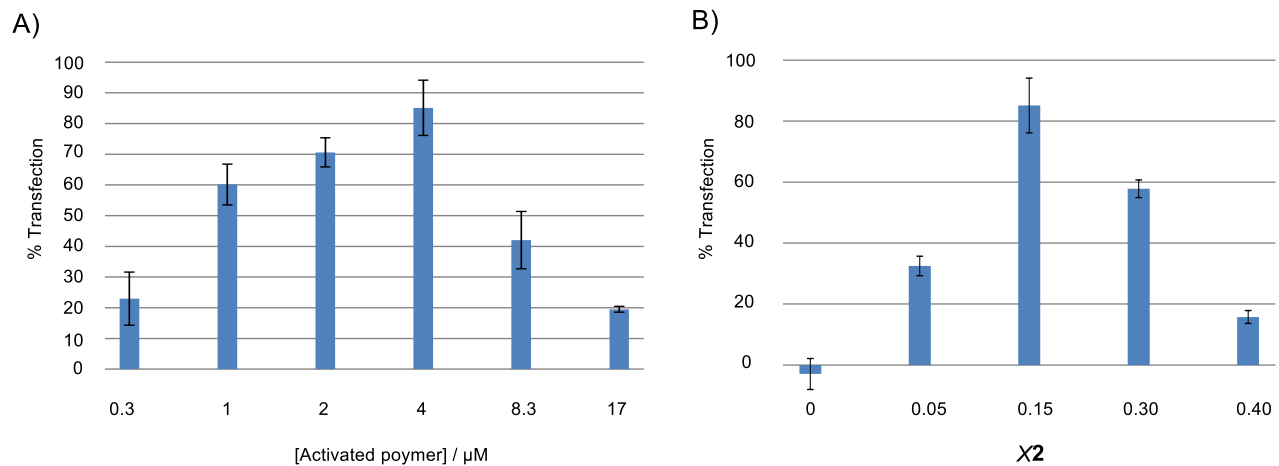


Figure S12: A) Transfection efficiency in HeLa-EGFP at a constant siRNA concentration (14 nM) and increasing concentrations of activated polymer ($\chi_1 = 0.85$ and $\chi_{\text{Hydrophobic}} = 0.15$). B) Transfection efficiency in HeLa-EGFP at a constant siRNA concentration (14 nM) and constant concentration of activated polymer (4 μM) prepared from different molar fractions of 2 ($\chi_1 = 1 - \chi_2$).

To optimize the concentration of siRNA, HeLa-EGFP were treated with siEGFP/ activated polymer polyplexes, at a constant activated polymer concentration (12.25 μM) and with variable siEGFP concentrations (**Figure S13**).

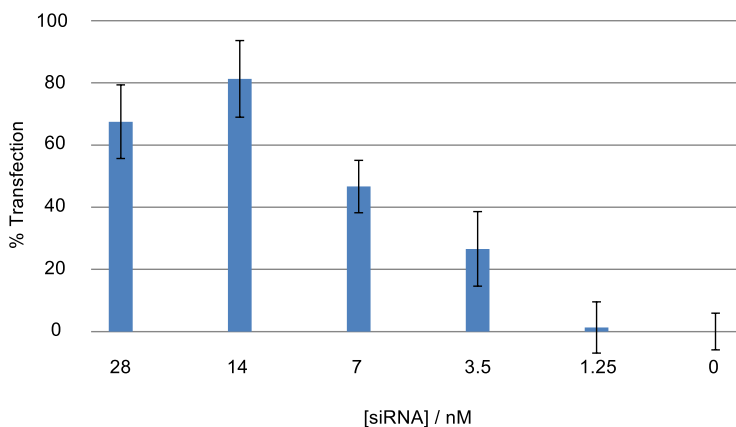


Figure S13: Transfection efficiency in HeLa-EGFP at a constant concentration of activated polymer (4 μM , $\chi_1 = 0.85$ and $\chi_{\text{Hydrophobic}} = 0.15$) and decreasing concentrations of siEGFP.

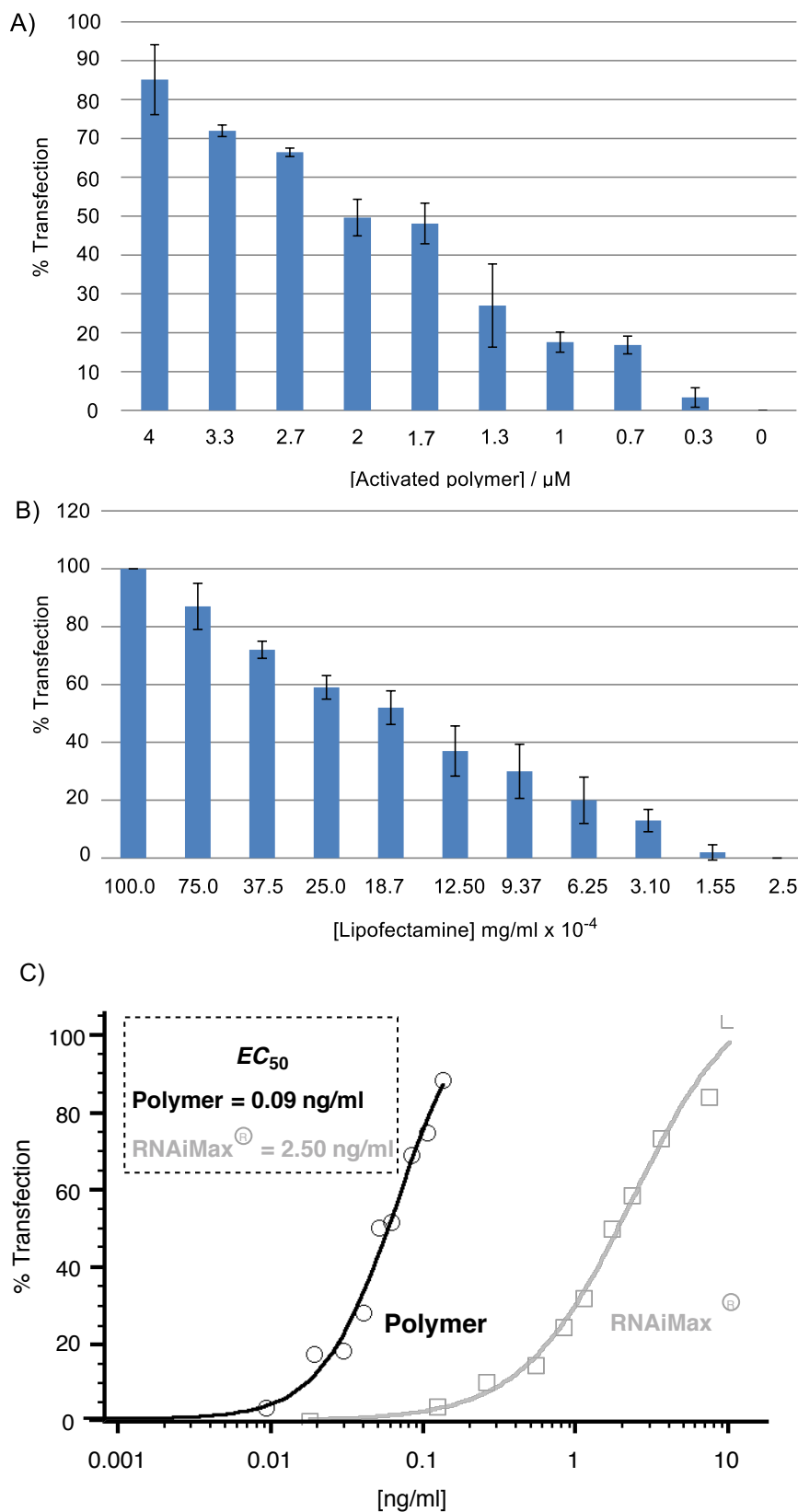


Figure S14: Transfection efficiency in HeLa-EGFP at a constant concentration of activated polymer ($4 \mu\text{M}$, $\chi_1 = 0.85$ and $\chi_{\text{Hydrophobic}} = 0.15$) and increasing concentrations of siEGFP.

- **Cell viability: MTT Assay**^{S4}

Cell viability was established by a standard MTT assay (Fig. S15).^{S4} One day before the assay, a suspension of HeLa-EGFP cells was plated in 96-well tissue culture plates (Costar 96 Flat Bottom Transparent Polystyrol) by adding 100 μ l (~30.000 cells) per well. The next day, the medium was aspirated and cells were incubated in DMEM containing 10% Fetal Bovine Serum (FBS) in the presence of activated polymer (50 μ l/well). After 4 h of incubation at 37°C, the medium was aspirated and replaced by fresh medium (DMEM) containing 10% FBS (100 μ l). Control cells were incubated with cell culture medium (100 μ l final medium). The viability was measured by quantifying the cellular ability to reduce the water-soluble tetrazolium dye 3-(4,5-dimethylthiazole-2,5-diphenyl tetrazolium bromide (MTT) to its insoluble formazan salt as follows. At 72 h, MTT (5 mg/ml in PBS, 10 μ l/well) was added to the wells and the cells were further incubated for 4 h. The supernatant was carefully removed and the water-insoluble formazan salt was dissolved in DMSO (100 μ l/well). The absorbance at 560 nm was measured. Data points were collected in triplicate and expressed as normalized values for untreated control cells (100%).

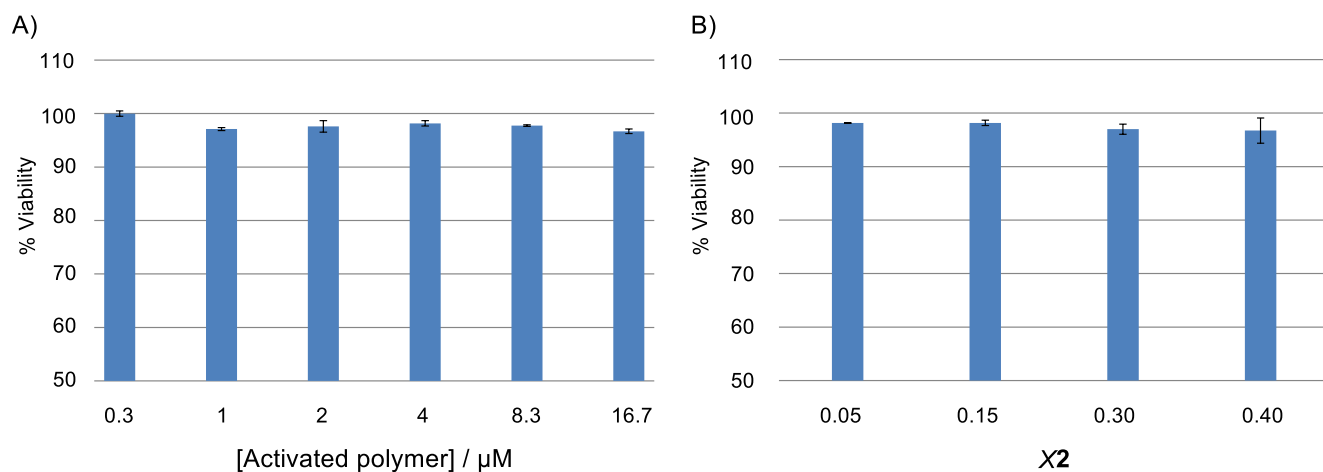


Figure S15: Cell viability from MTT assay in HeLa-EGFP cells at a constant siRNA concentration (14 nM). A) Increasing concentrations of activated polymer ($\chi_1 = 0.85$ and $\chi_{\text{Hydrophobic}} = 0.15$). B) Constant concentration of activated polymer (4 μ M) and with different molar fractions of **2** ($\chi_1 = 1 - \chi_2$).

To further investigate cell viability in the presence of the parent polymer **P1**, the activated polymer **P₁(1)₈₅(2)₁₅** and the Lipofectamine® RNAiMAX we performed additional viability experiments (in HeLa cells) at the working concentrations of the transfection experiments. Following the same protocol described above, but without medium replacement after the initial 4 hours, cells were incubated in the presence of either Lipofectamine® RNAiMAX **P1**, the activated polymer **P₁(1)₈₅(2)₁₅** (**Figure S16**).

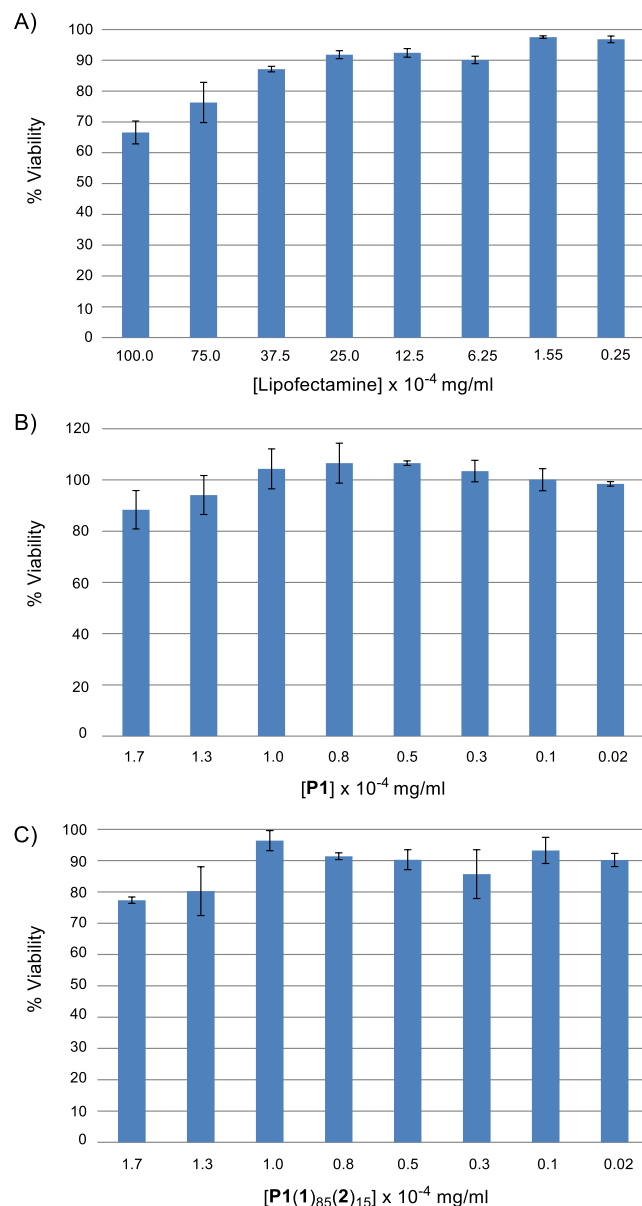


Figure S16: Comparison of cell viability (MTT assay in HeLa cells) at the working concentrations of the transfection experiments for the polymer and for the Lipofectamine® RNAiMAX (A), **P1** (B) and Activated polymer = **P₁(1)₈₅(2)₁₅** (C). In all cases [siRNA] = 14 nM.

Characterization of Polyplexes

• Hydrodynamic Radius and ζ -potential

10 μl of activated polymer's stock solutions were diluted in MilliQ water to afford the desired final concentrations (67-4 μM) and were mixed with a solution of siRNA (10 nM, 995 μl in MilliQ water) before measuring. To measure activated polymer alone, the 995 μl of siRNA solution was replaced by the same amount of bi-distilled water. Bi-distilled water was filtered through a nylon syringe filter (0.45 μm) before use. All experiments were performed at 25 $^{\circ}\text{C}$ and the mean values and standard deviations obtained from triplicates.

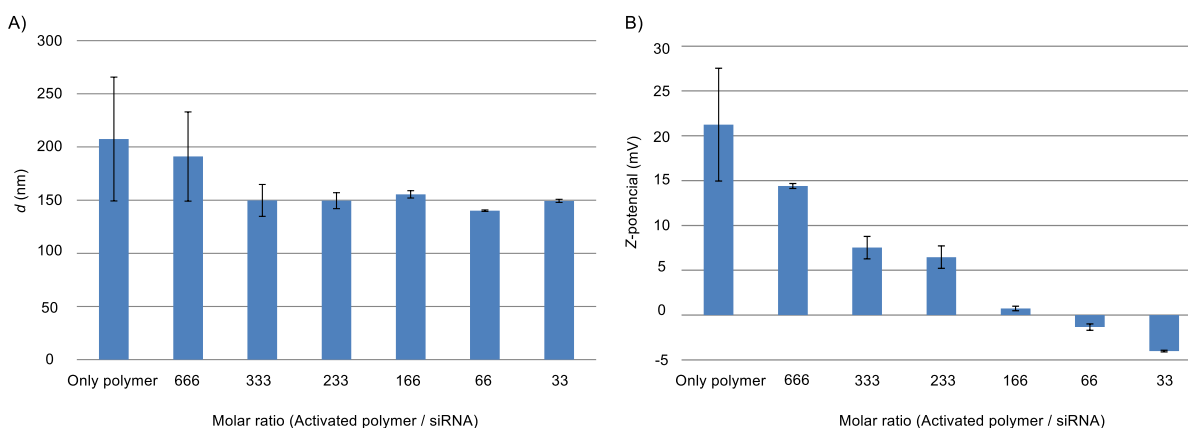


Figure S17: Diameter (A) and ζ -potential (B) for representative siMock/Activated polymer polyplexes. [siMock] = 14 nM. Activated polymer ($\chi_1 = 0.85$ and $\chi_2 = 0.15$).

• Gel Retardation Assay

Pre-mixed siRNA/Activated polymer polyplexes (3 pmol) were loaded onto a 2% agarose gel. The gels were run at 100 V for 60 min in TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA) (**Figure S18**).



Figure S18: Gel retardation assay. siMock (lane 1) and siMock/Activated polymer polyplexes with molar ratios of 2 (lane 2), 4.3 (lane 3), 8.3 (lane 4), 16.7 (lane 5), 33.3 (lane 6) and 66.7 (lane 7) were loaded. [siRNA] = 14 nM in all cases. [Activated polymer] = [$\text{P}_1(1)_{85}(2)_{15}$] = 28 nM (lane 2), 60 nM (lane 3), 0.12 μM (lane 4), 0.23 μM (lane 5), 0.46 μM (lane 6), 0.93 μM (lane 7).

Additional Figures

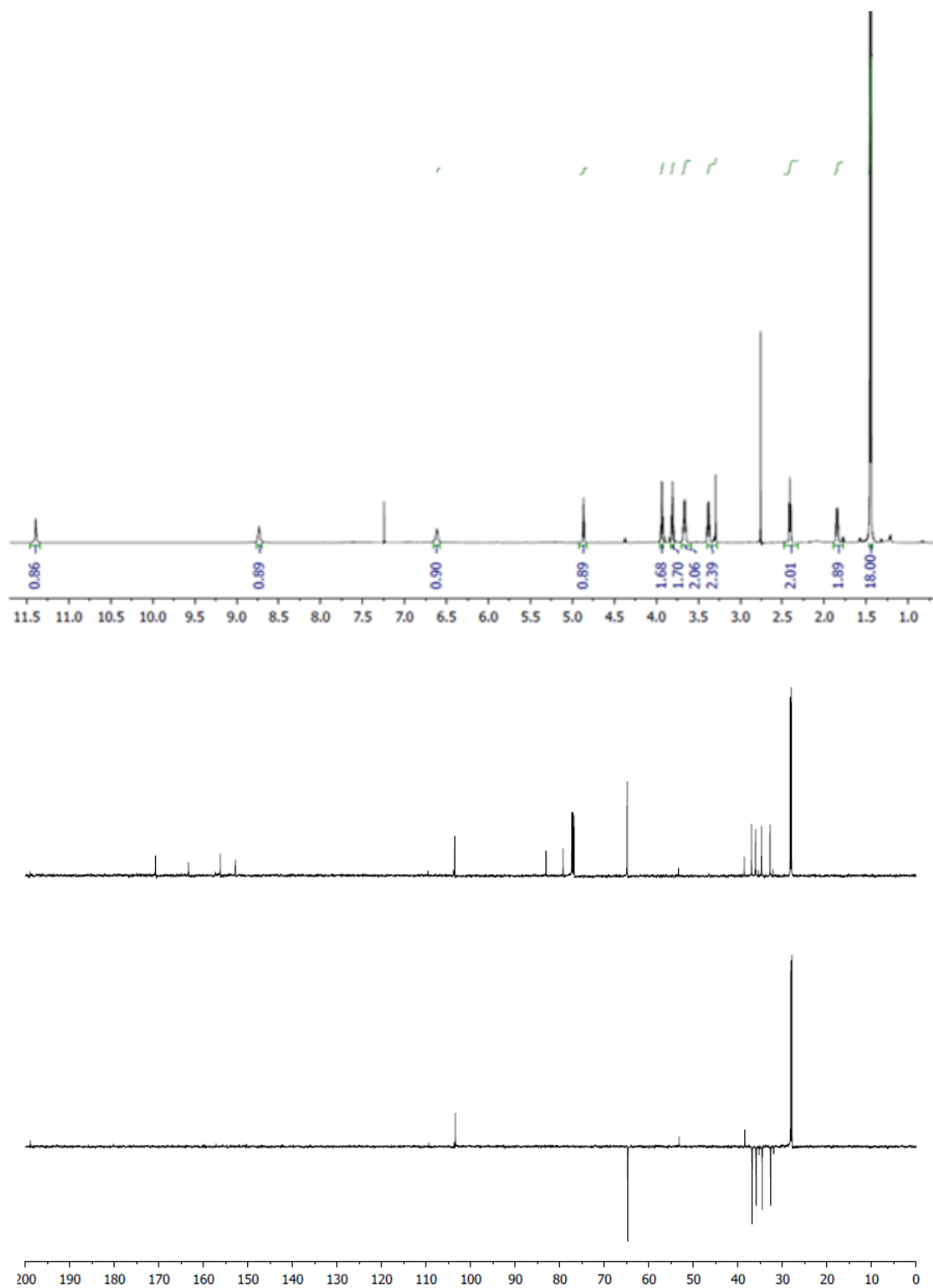
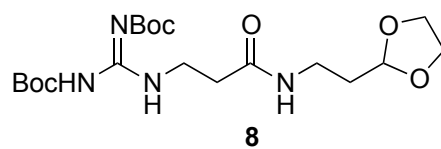


Figure S19: $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ in CDCl_3 spectra of compound **8**.

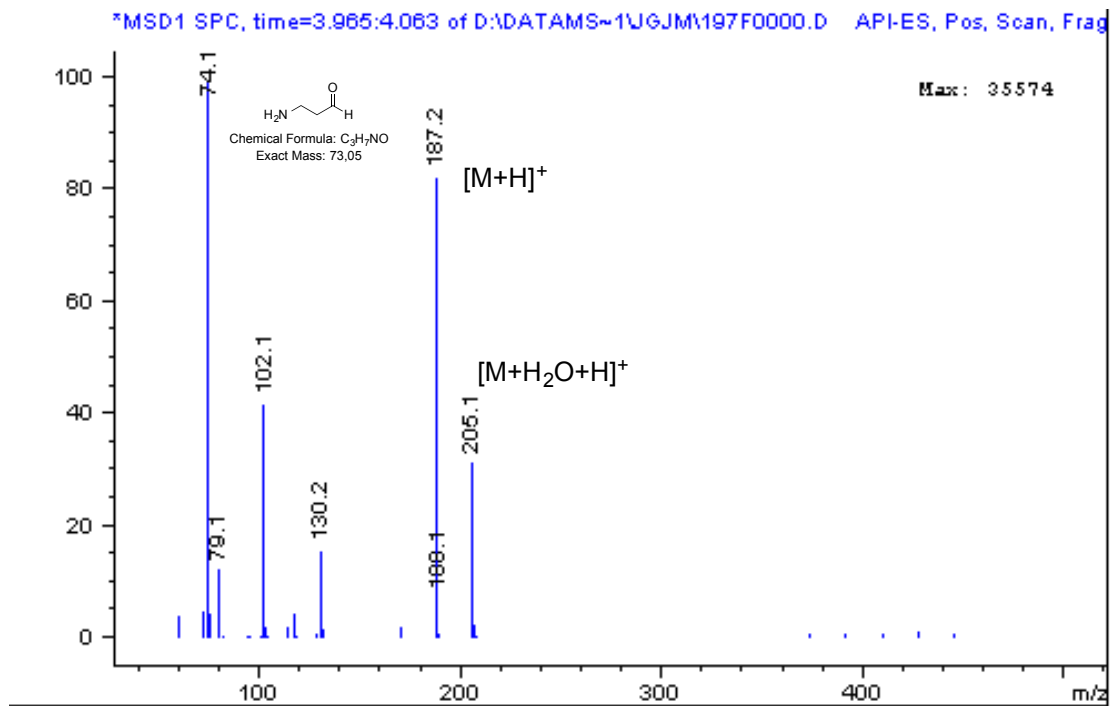
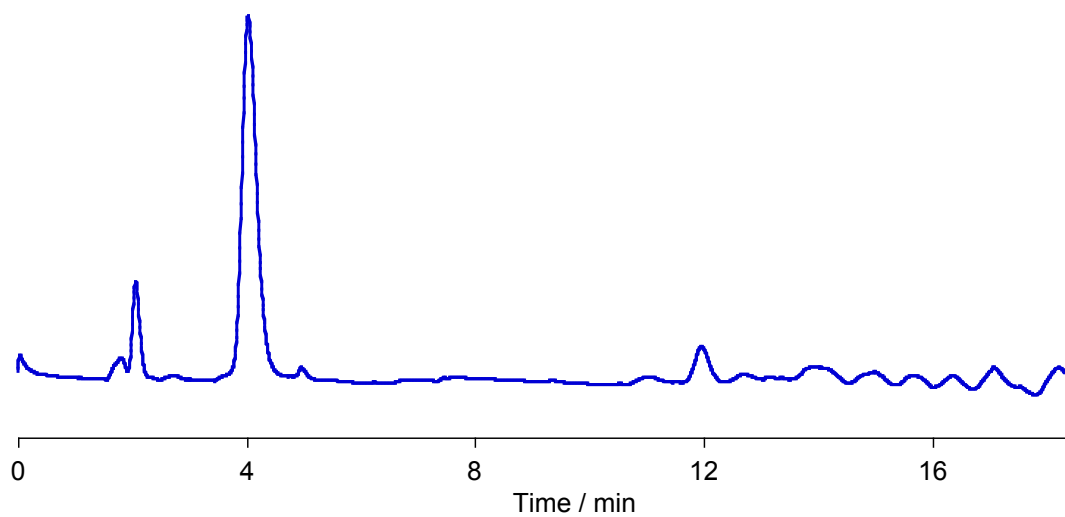
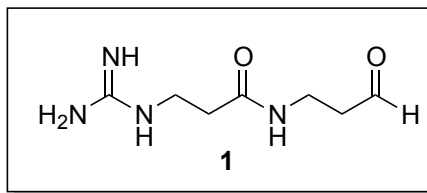


Figure S20: RP-HPLC [Nucleosil 100-7 C18, H₂O (0.1% TFA)/CH₃CN (0.1% TFA) 100:0 (0→10 min), 100:0→75:35 (5→35 min), 0:100 (>35 min)] (*R_t* 4.0 min) and ESI-MS for compound **1**.

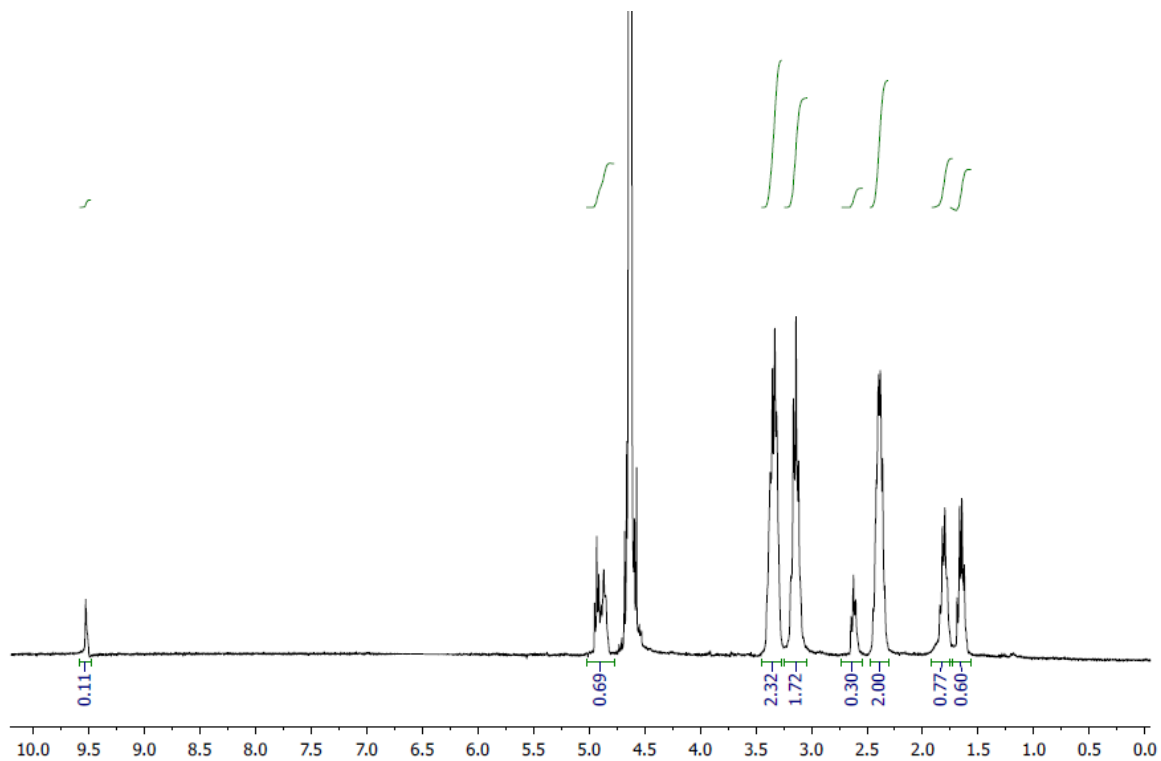
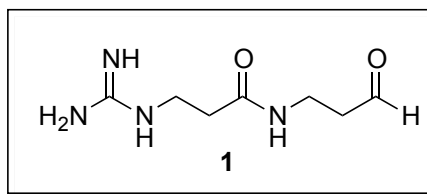


Figure S21: ¹H-NMR spectra in D₂O of compound 1.

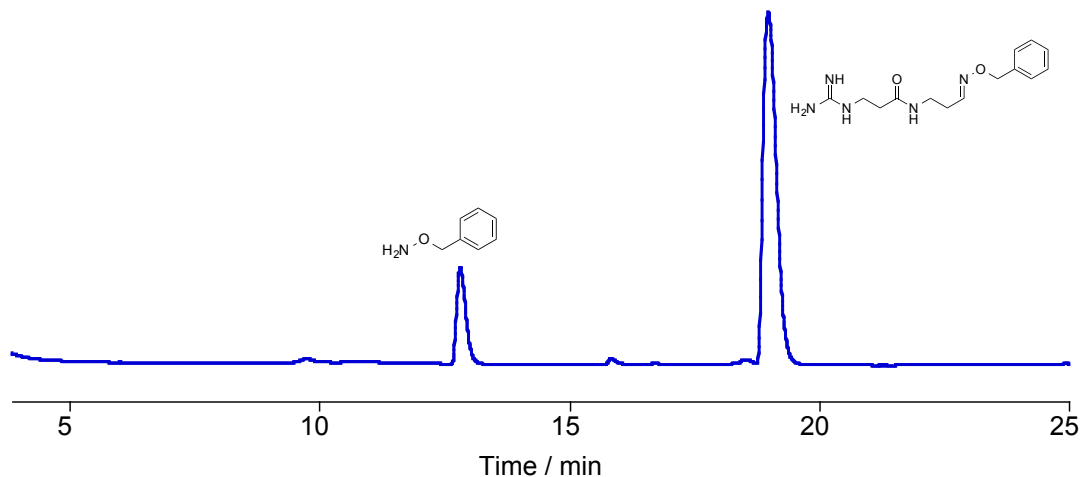
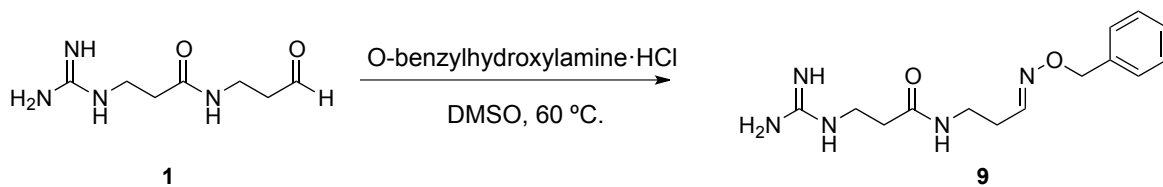


Figure S22: RP-HPLC [Nucleosil 100-7 C18, H₂O (0.1% TFA)/CH₃CN (0.1% TFA) 100:0→80:20 (5→35 min), 0:100 (>35 min)] for the reaction of **1** with benzyl hydroxylamine. The chromatogram after oxime formation shows the presence of the final compound **9** and the excess of hydroxylamine.

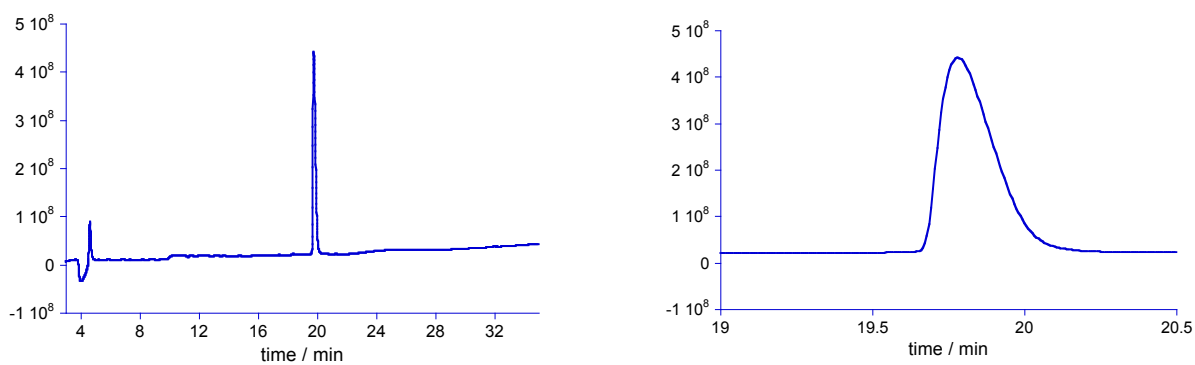


Figure S23: RP-HPLC [Nucleosil 100-7 C18, H₂O (0.1% TFA)/CH₃CN (0.1% TFA) 100:0 (0→5 min), 100:0→35:75 (5→35 min), 0:100 (>35 min)] of compound **9**.

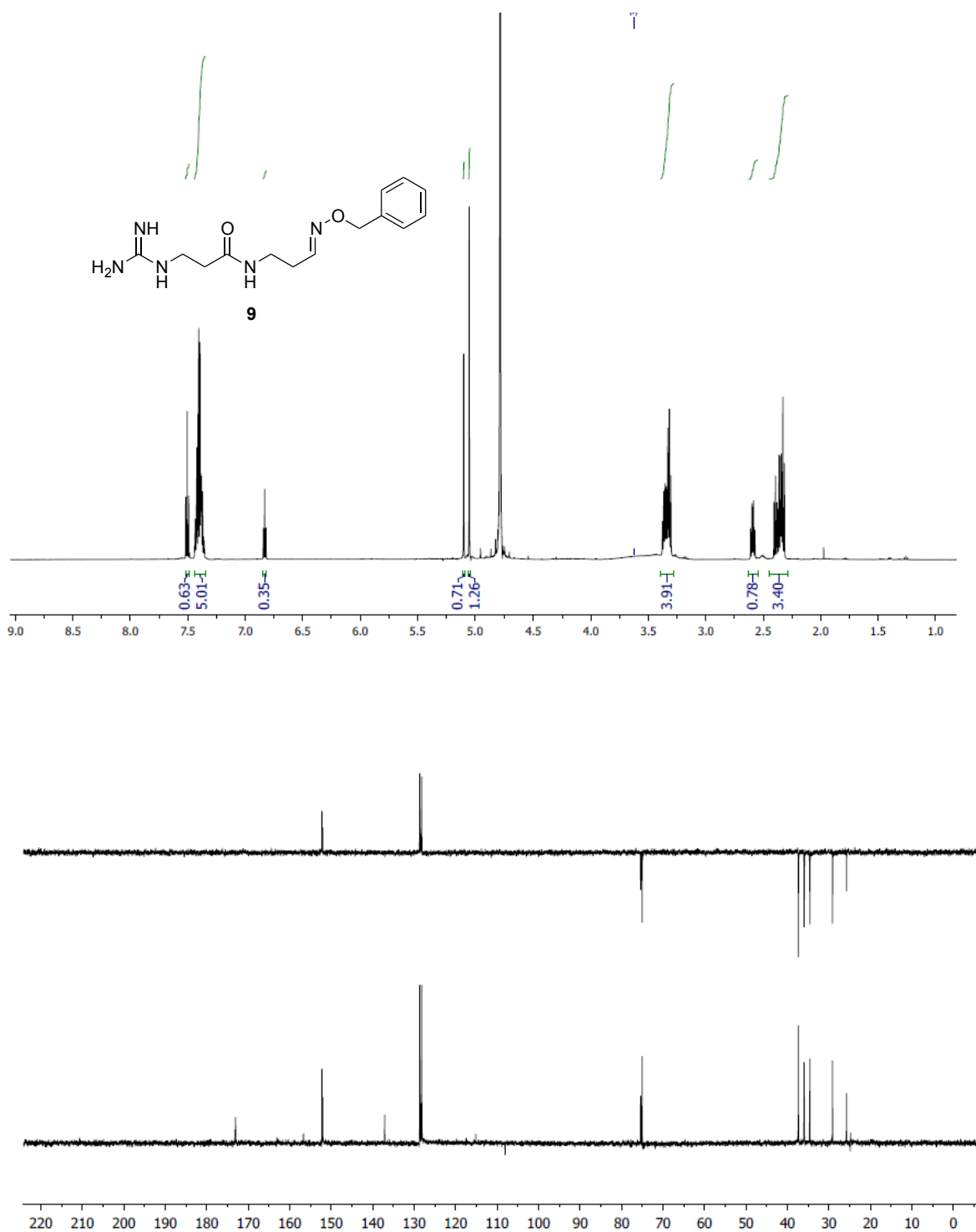


Figure S24: $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra in D_2O of compound 9.

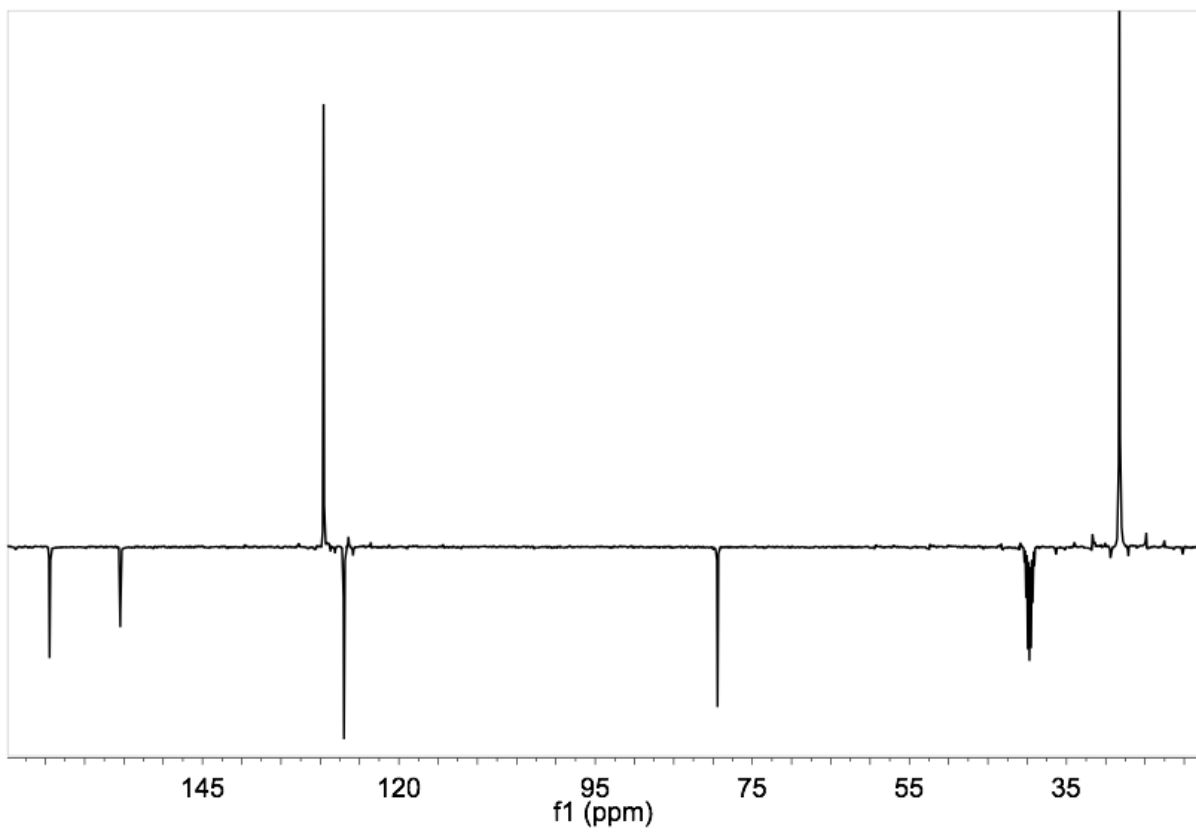
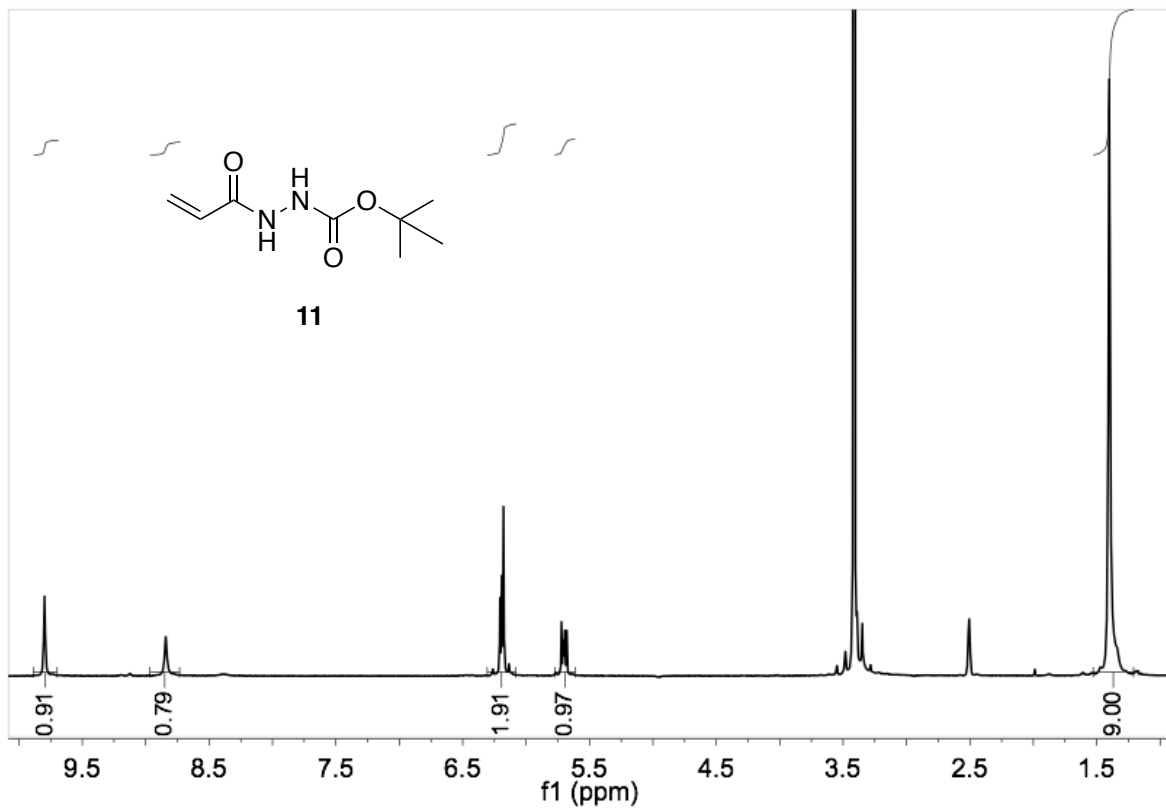


Figure S25: ¹H-NMR and ¹³C-NMR spectra in DMSO-*d*₆ for compound 11.

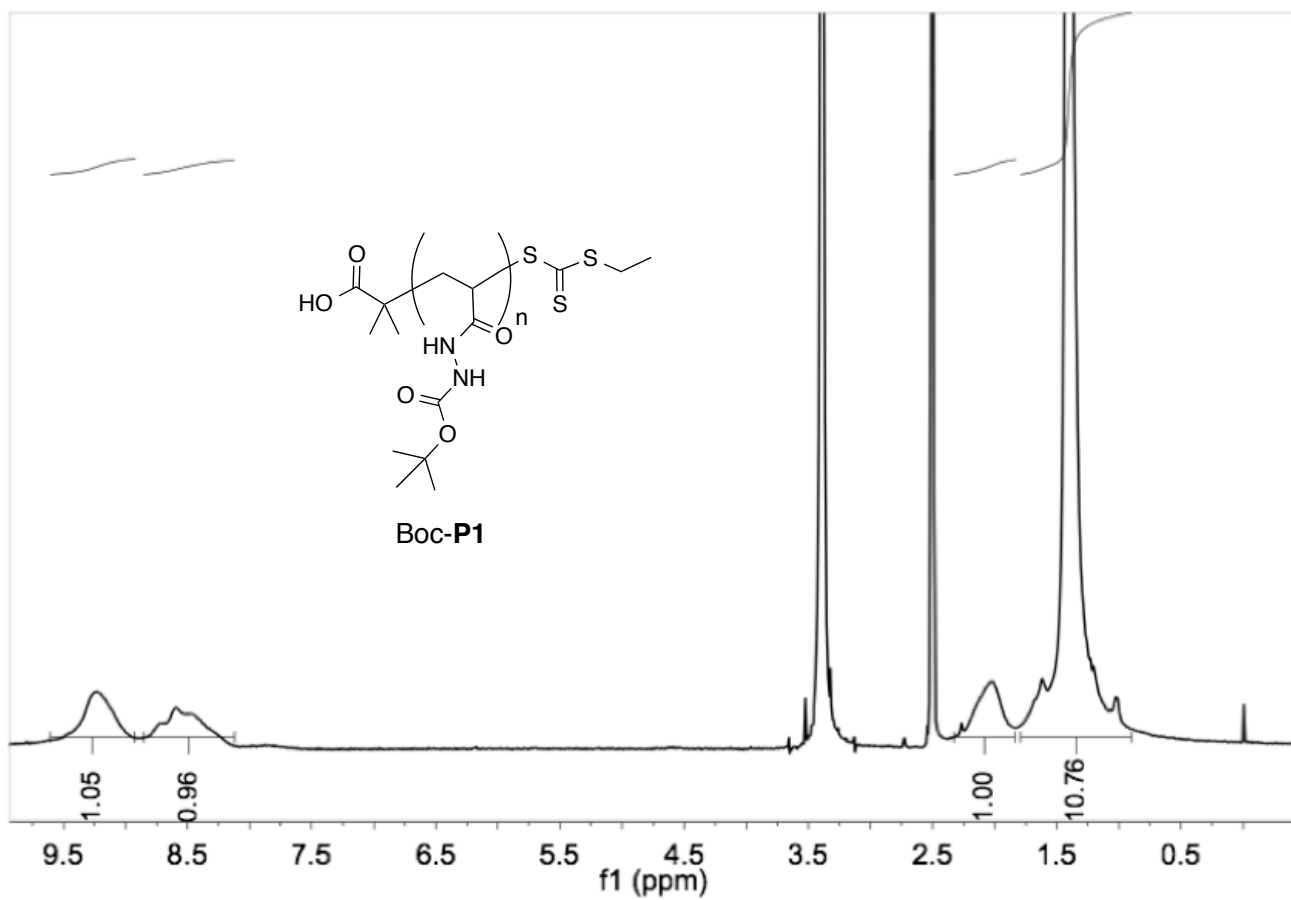


Figure S26: ¹H-NMR spectra in DMSO-*d*₆ of **Boc-P1**.

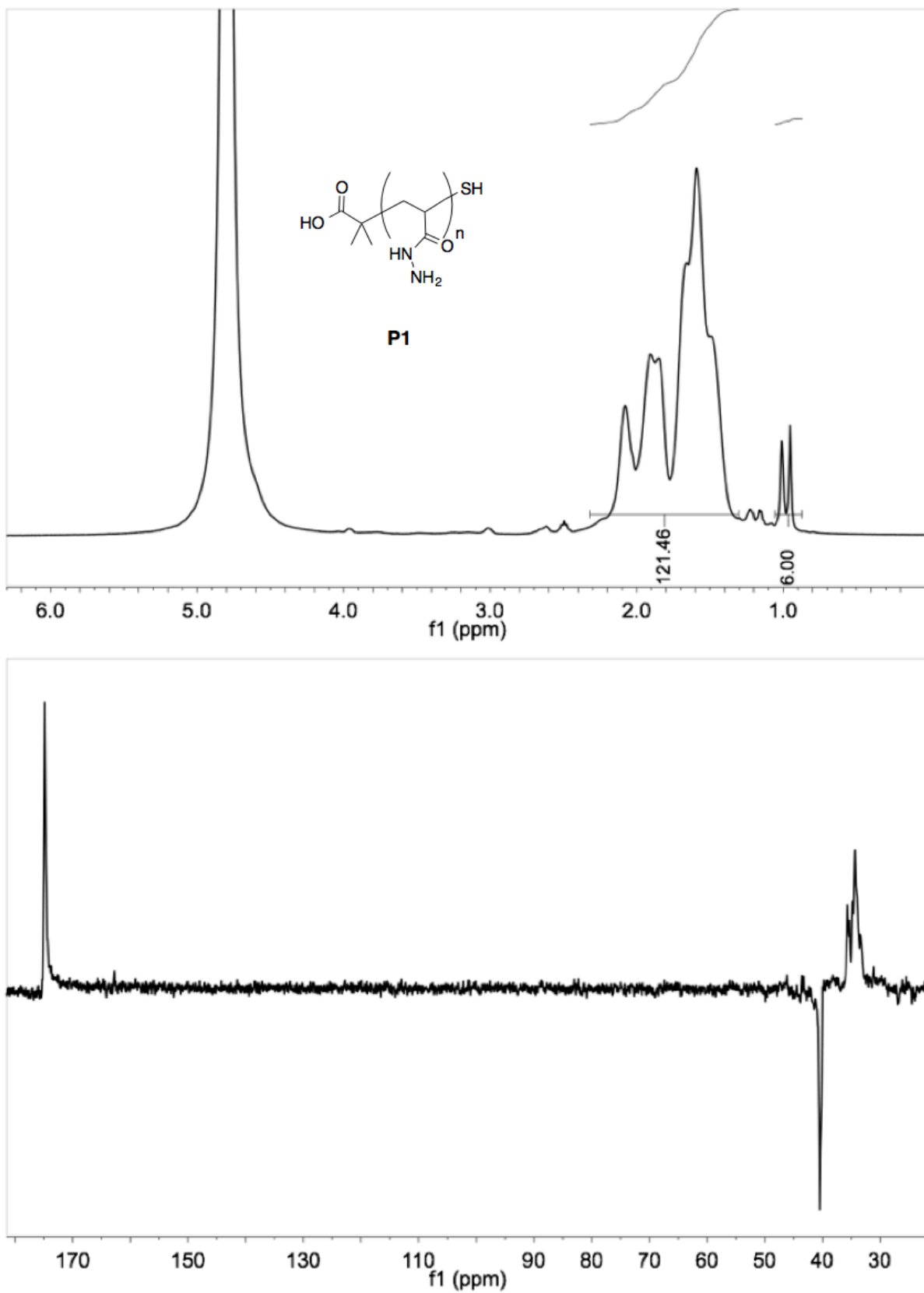


Figure S27: ¹H-NMR and ¹³C-NMR spectra in DMSO-*d*₆ of P1.

Supporting References

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