Supporting Information

Disulfide reshuffling triggers the release of a thiol-free anti-HIV agent to make up fast-acting, potent macromolecular prodrugs

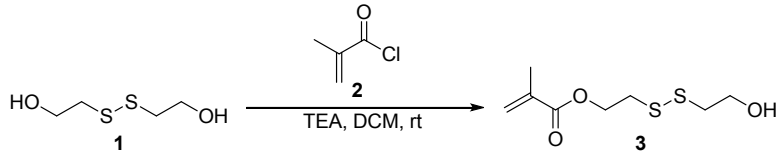
*Anders Kock,[1] Kaja Zuwala,[1,2] Anton A.A.A. Smith,[1] Pau Ruiz-Sanchis,[1] Benjamin M. Wohl,[1,3] Martin Tolstrup,[2] Alexander N. Zelikin[1,3]**

[1] Department Department of Chemistry, Aarhus University, Aarhus, Denmark
[2] Department of Infectious Diseases, Aarhus University Hospital, Denmark
[3] iNano Interdisciplinary Nanoscience Centre, Aarhus University, Aarhus, Denmark

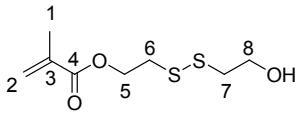
Contents

Experimental Section

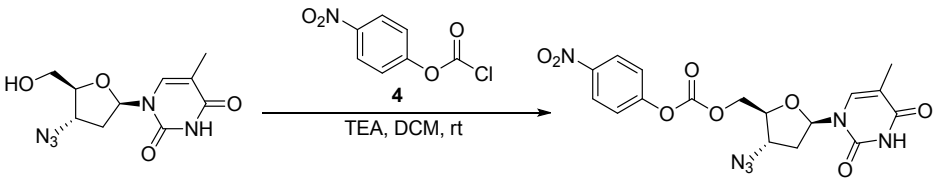
Monomer synthesis



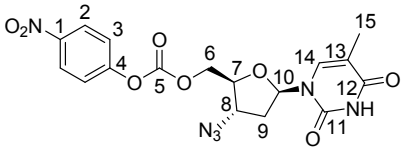
2 (2.31 g, 22.05 mmol) and TEA (6.15 mL, 4.25 mmol) were added over a solution of **1** (6.83 g, 44.25 mmol) in DCM (150 mL) under N_2 atmosphere, giving a pink solution. After 1:30 h, the reaction was quenched with NH_4Cl sat. (300 mL), washed with water (300 mL) and brine (300 mL), and dried over Na_2SO_4 anh. and concentrated *in vacuo* to a pink oil. The crude was purified on silica eluting from 9:1 to 6:4 pentane:EtOAc affording **3** as a colourless oil (2.96 g, 60%).



^{1}H-NMR (400MHz, $CDCl_3$), δ (ppm): 6.12 (1H, s, H_{2a}), 5.57 (1H, s, H_{2b}), 4.41 (2H, t, $J_{H5\text{-}H6}$ = 6.7 Hz, H_5), 3.87 (2H, t, $J_{H8\text{-}H7}$= 5.8 Hz, H_8), 2.95 (2H, t, $J_{H6\text{-}H5}$ = 6.7 Hz, H_6), 2.87 (2H, t, $J_{H7\text{-}H8}$ = 5.8 Hz, H_7), 1.93 (3H, s, H_2). **^{13}C-NMR** (100MHz, $CDCl_3$), δ (ppm): 167.5 (s, C_4), 136.2 (s, C_3), 126.3(t, C_2), 62.8 (t, C_5), 60.4 (t, C_8), 41.9 (t, C_6), 37.2 (t, C_7), 18.5 (q, C_1).

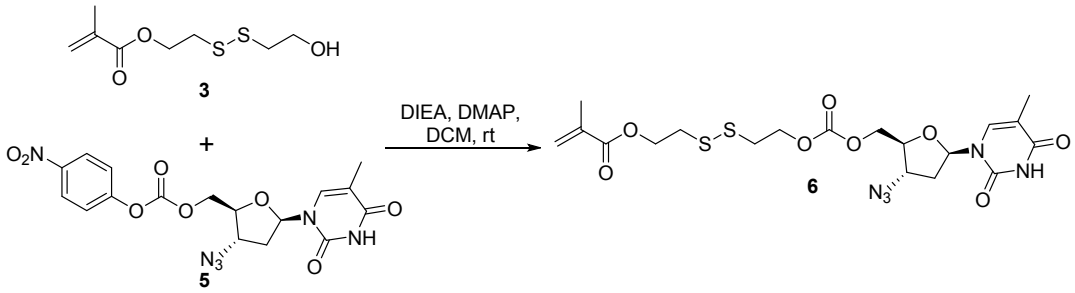


TEA (4.2 mL, 30 mmol) was added to a suspension of AZT (4.0 g, 15 mmol) in DCM (30 mL) under N_2 atmosphere, generating a colour-less cloudy solution. **4** (3.32 g, 16.6 mmol) dissolved in DCM (10 mL) was added, turning the solution clear and yellow. After 17 hours the reaction was quenched with NH_4Cl sat. (300 mL), washed with water (300 mL) and brine (300 mL), dried over Na_2SO_4 anh. and concentrated *in vacuo* to yellow grime. The crude product was purified on silica, eluting with DCM:EtOH from 100:0 to 97:3. Concentration *in vacuo* generated a foam that could be crushed into a colourless powder (3.65 g, 53%).

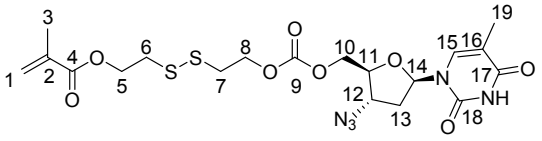


^{1}H-NMR (400MHz, $CDCl_3$), δ (ppm): 9.09 (1H, s, H_N), 8.22 (2H, d, $J_{H2\text{-}H3}$ = 9.6 Hz, H_2), 7.34 (2H, d, $J_{H3\text{-}H2}$ = 9.6 Hz, H_3), 7.22 (1H, s, H_{14}), 6.09 (1H, t, $J_{H10\text{-}H9}$ = 6.2 Hz, H_{10}), 4.46 (2H, d, $J_{H6\text{-}H7}$ = 3.2 Hz, H_6), 4.31 (1H, q, J_{H8} = 6.6 Hz, H_8), 4.06 (1H, dt, $J_{H7\text{-}H6}$ = 3.6 Hz, $J_{H7\text{-}H8}$ = 5.9 Hz, H_7), 2.46 (2H, t, J_{H9} = 6.8 Hz, H_9),

1.84 (3H, s, H_{15}). **^{13}C-NMR** (100MHz, $CDCl_3$), δ (ppm): 163.2 (s, C_{12}), 155.2 (s, C_5), 152.2 (s, C_4), 150.2 (s, C_{11}), 145.8 (s, C_1), 136.9 (d, C_{14}), 125.6 (d, C_2), 121.8 (d, C_3), 111.6 (s, C_{13}), 86.1 (d, C_{10}), 81.4 (s, C_7), 67.6 (t, C_6), 60.1 (s, C_8), 37.6 (t, C_9),12.7 (q, C_{15}). **HRMS** (ESI+) *m/z* calcd for $C_{17}H_{17}N_6O_8$[M+H$^+$]=433.1102, found 433.1105, *m/z* calcd for $C_{17}H_{16}N_6O_8Na$[M+Na$^+$]=455.0922, found 455.0924.

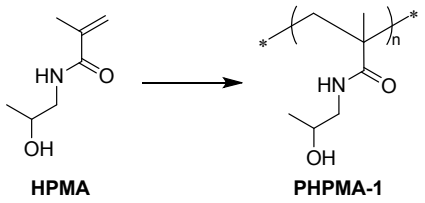


DIEA (174 mg, 1.35 mmol) was added to a solution of **3** (200 mg, 0.9 mmol) in DCM (20 mL) under N_2 atmosphere. **5** (188 mg, 0.45 mmol) dissolved in DCM (10 mL) and DMAP (11 mg, 0.09 mmol) dissolved in DCM (6 mL) were added. The reaction turned yellow upon addition of **5**. After 20h the reaction was quenched with NH_4Cl sat. (50 mL), washed with brine (50 mL), dried over Na_2SO_4 anh. and concentrated *in vacuo* to a yellow gel. The crude product was purified by flash chromatography on silica eluting with pentane:EtOAc from 50:50 to 40:60 as a colourless gel (185 mg, 80% yield).



^{1}H-NMR (400MHz, $CDCl_3$), δ (ppm): 8.30 (1H, s, H_N), 7.34 (1H, s, H_{15}), 6.20 (1H, t, $J_{H14\text{-}H13}$ = 6.8, H_{14}), 6.13 (1H, s, H_{1a}), 5.60 (1H, s, H_{1b}), 4.41 (6H, m, H_{10}, H_5, H_8), 4.27 (1H, q, J_{H12} = 7.3 Hz, H_{12}), 4.07 (1H, dt, $J_{H11\text{-}H10}$ = 3.1 Hz, $J_{H11\text{-}H12}$ = 5.3 Hz, H_{11}), 2.98 (4H, m, H_6, H_7), 2.46 (1H, m, H_{13}), 1.93 (6H, m, H_3, H_{19}). **^{13}C-NMR** (100MHz, $CDCl_3$), δ (ppm): 167.3 (s, C_4), 163.3 (s, C_{17}), 154.6 (s, C_9), 150.1 (s, C_{18}), 136.1 (s, C_2), 135.4 (d, C_{15}), 126.3 (t, C_1), 111.5 (s, C_{16}),85.2 (d, C_{14}), 81.73 (d, C_{12}), 66.6 (t, C_8), 66.3 (t, C_5), 62.59 (t, C_{10}), 60.13 (d, C_{11}), 37.82 (t, C_{13}), 37.31 (t, C_6), 37.06 (t, C_7), 18.43 (q, C_3), 12.76 (q, C_{19}). **HRMS** (ESI+) *m/z* calcd for $C_{19}H_{26}N_5O_8S_2$ [M+H$^+$]=516.1217, found 516.1220, *m/z* calcd for $C_{19}H_{25}N_5O_8S_2Na$ [M+Na$^+$] = 538.1037, found 538.1040.

Polymer synthesis

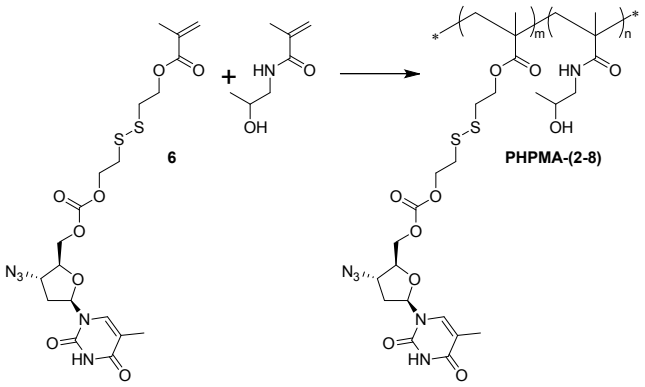


PHPMA-1

4-cyano-4[(dodecylsulfanylthiocarbonyl)sylfanyl]pentanoic acid (6.31 mg, 15.6 μmol), and 4,4′-azobis(4-cyanovaleric acid) (1.10 mg, 3.9 μmol) were dissolved in 1.45 mL of DMSO in an ampule. After the addition of *N*-(2-hydroxypropyl) methacrylamide (250 mg, 1.75 mmol), the solution was degassed through four freeze−pump−thaw cycles. The reaction was left to proceed for 14:55h at 70°C, followed by precipitation into acetone: Et_2O 2:1 to obtain the product (75 mg).

^{1}H-NMR (DMSO-d_6), δ (ppm): 7.17 (bs, HPMA N-H) 4.69 (bs, 1H, HPMA O-H), 3.66 (bs, 1H, HPMA C-H), 2.89 (bs, HPMA CH_2).

General protocol and characterization for PHPMA-(2-8)



6, 4-cyano-4[(dodecylsulfanylthiocarbonyl)sylfanyl]pentanoic acid, and 4,4′-azobis(4-cyanovaleric acid) were dissolved in DMSO in an ampule. After the addition of *N*-(2-hydroxypropyl) methacrylamide, the solution was degassed through four freeze−pump−thaw cycles. The reaction was left to proceed about 16 hours at 70°C, followed by precipitation into acetone:Et_2O 2:1 to obtain the product **PHPMA-(2-8)**.

^{1}H-NMR (DMSO-d_6), δ (ppm): 11.36 (bs, 1H, AZT N-H), 7.47 (bs, 1H, AZT H_{15}), 7.17 (bs, HPMA N-H) 6.13 (bs, 1H, AZT H_{14}), 4.69 (bs, 1H, HPMA O-H), 4.47 (bs, 1H, AZT H_{11}), 4.36 (bs, 4H, linker H_5, H_8), 4.12 (bs, 2H, AZT H_{10}), 4.01 (bs, 1H, AZT H_{12}), 3.66 (bs, 1H, HPMA C-H), 3.01 (bs, 1H, linker H_6, H_7) 2.89 (bs, HPMA CH_2), 2.33 (bs, 2H, AZT H_{13}).

Table S1. Amounts of materials used for the syntheses of the polymers.

	HPMA m (mg)	6 m (mg)	ACVA m (mg)	RAFT agent[1] m (mg)	DMSO V (mL)
PHPMA-1	250	0	1.10	6.31	1.45
PHPMA-2	250	18.4	1.15	6.64	1.45
PHPMA-3	250	47.4	2.58	14.9	1.45
PHPMA-4	250	47.4	1.24	7.13	1.45
PHPMA-5	250	100	1.38	7.95	1.45
PHPMA-6	250	70.0	1.38	7.95	1.45
PHPMA-7	250	47.4	0.239	1.38	1.45
PHPMA-8	250	47.4	0.239	1.38	1.45

[1]RAFT agent: 4-cyano-4-[(dodecylsulfanyl-thiocarbonyl)sulfanyl]pentanoic acid

PHPMA-(9-13) were synthesized as previously reported[1] and were chosen to serve as controls, i.e. similar molar mass and AZT loading. See Table S1 for polymer characterization.

Polymer characterization

Size-exclusion chromatography (SEC) was performed using a system comprising a LC-20AD Shimadzu HPLC pump, a Shimadzu RID-10A refractive index detector and a DAWN HELEOS 8 light scattering detector along with a SPD-M20A PDA detector, equipped with a HEMA-Bio Linear column with 10 µm particles, a length of 300 mm and an internal diameter of 8 mm from MZ-Analysentechnik in series with a OHpak SB-803 HQ Shodex column with the dimensions 8.0 × 300 mm a particle size of 6 µm. The solvent used was 0.01 M PBS filtered through a 0.1 µm filter with 300 ppm sodium azide. The dn/dc used to calculate the molecular weights of the polymers was that of the carrier polymer (0.168 mL/g, measured with MALS assuming full mass recovery).

Polymer samples were incubated with 10 mg DTT for 10 minutes to cleave off SIL groups, as the SIL groups made the polymers greasy and negatively interact with the column material. SEC was attempted on the intact polymers in H_2O, PBS, DMF and DMAc, with no resolution of the problem. In order to compensate for the lost polymer mass, polymer weights were calculated by the equation:

$$M_n^{reported} = M_n^{measured} \cdot \frac{\alpha \cdot M_{HPMA} + M_{SIL\ uncleaved}}{\alpha \cdot M_{HPMA} + M_{SIL\ cleaved}}$$

With α being the ratio between HPMA and SIL $\left(\frac{n_{HPMA}}{n_{SIL}}\right)$ on the polymer as measured by ^{1}H-NMR, and M_{HPMA}, $M_{SIL\ uncleaved}$ and $M_{SIL\ cleaved}$ are molar masses of the monomers.

[1] K. Zuwala, A.A.A. Smith, A. Postma, C. Guerrero-Sanchez, P. Ruiz-Sanchis, J. Melchjorsen, M. Tolstrup, and A.N. Zelikin, **2014**, *Advanced Healthcare Materials*, doi: 10.1002/adhm.201400148.

Table S2. Characteristics of polymers used in this study.

	AZT feed (%)	AZT content (%)	M:RAFT	Conversion (%)	Mn (GPC)	PDI	DP (GPC)
PHPMA-1	0	0	112	-	10.29	1.16	76
PHPMA-2	2	3.1	108	-	10.68	1.21	69
PHPMA-3	5	3.6	50	64	7.54	1.15	48
PHPMA-4	5	7.7	104	47	11.15	1.19	65
PHPMA-5	10	18.0	98	43	11.11	1.14	53
PHPMA-6	10	7.1	96	55	11.58	1.14	68
PHPMA-7	5	8.8	538	30	26.49	1.22	151
PHPMA-8	5	13.7	538	26	21.50	1.32	111
PHPMA-9	0	0	250	54	20.0	1.26	140
PHPMA-10	10	11	50	76	9.9	1.03	
PHPMA-11	10	12	125	69	18.5	1.08	
PHPMA-12	15	19	50	83	9.3	1.04	
PHPMA-13	15	18	125	72	17.5	1.10	

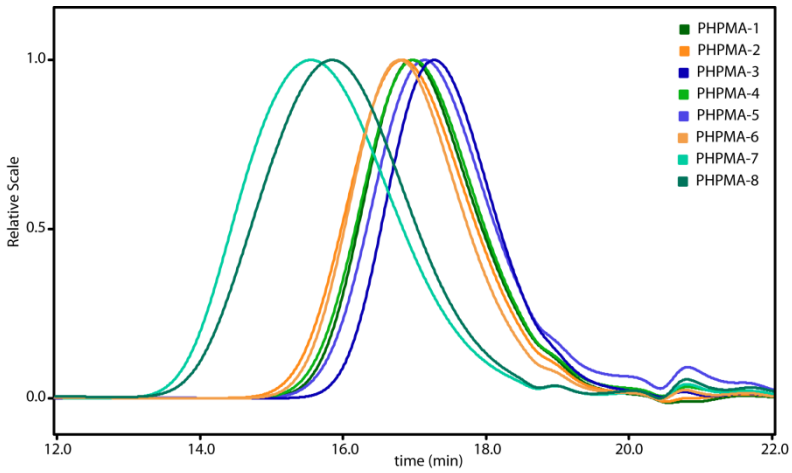


Figure S1. Size exclusion chromatography elution profiles (refractive index spectra) of polymeric prodrugs of AZT with SIL linkage.

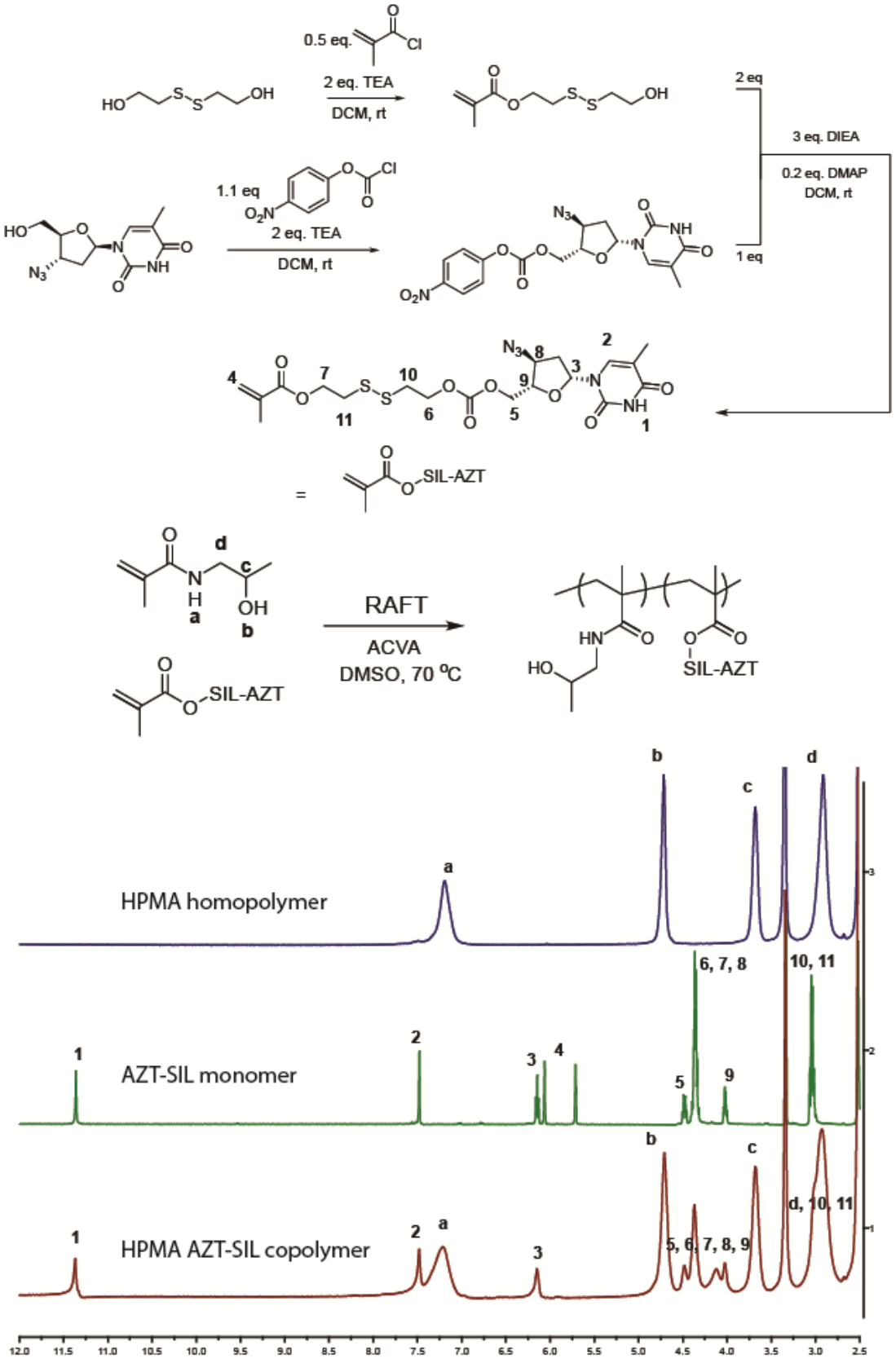


Figure SI2 : NMR characterization of the monomer and MP derived thereof with signal assignment.

HPLC release study

To quantify release of AZT under physiological relevant conditions from MP bearing an ester or an SIL linkage samples were prepared at 2 g/L of polymer in 200 mM sodium phosphate buffer (pH 7.4). PHPMA-10 and **PHPMA-6** were chosen as representative polymers with matching molar mass and drug loading. Both polymers were subjected to 5 mM GSH, cell lysate (see below), 10% FBS, and just phosphate buffer over 24 hours at 37°C.

Cell lysate was prepared by growing TZM-bl cells (see below for culture conditions) to 80% confluency in a T75 flask. Cell were subsequently washed twice with ice-cold phosphate buffer and dislodged through with a cell scaper into 700 µL ice-cold phosphate buffer. The cell suspension was transferred to a microtube and kept on ice. Mechanical cell lysis was achieved by repeated passage through a syringe needle (25G) and cell debris subsequently removed through centrifugation (15400 rcf, 4°C, 20 min). A 14 g/L polymer solution was diluted to 2 g/L using the clear supernatant and incubated in parallel with the remaining samples at 37°C for 24 hours.

Subsequent to the incubation all protein containing samples (i.e. FBS, lysate) were purified with a centrifugation filter MWCO 3k, 14000 rcf, 25°C, 30 min). AZT levels were quantified through analytical HPLC on a Shimadzu LC-2010A HT equipped with a Tosoh Bioscience TSK-GEL G1000PW column (300x7.5 mm, 10 µm) using sodium phosphate buffer (200 mM, pH 7.4) as mobile phase at 0.7 mL/min.

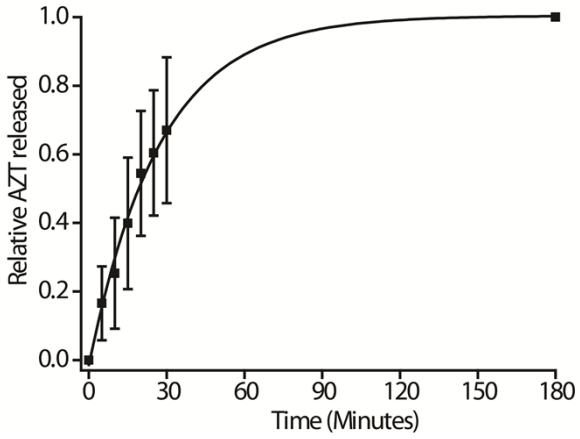


Figure SI 3 : HPLC characterization of release of AZT from the SIL containing macromolecualr prodrugs in the phosphate buffer in the presence of 5 mM GSH.

Half-life determination

Solutions of 0.4 mM **PHPMA-4** (1.78 mg, 2 mL), 10 mM GSH (6.15 mg, 2 mL) and 2.5 mM Ellman's reagent (7.93 mg, 8 mL) were prepared in 10 mM PBS. GSH solution was neutralized with 1 eq NaOH. 0.5 mL **PHPMA-4** and 0.5 mL GSH were mixed, then 0.1 mL was transferred to 0.9 mL Ellman's at 0, 5, 10, 15, 20, 25, 30 and 180 min. These samples were analysed by GPC, with the 180 minute data point representing full release, then fitted with an exponential model.

Cell culture

TZM-bl HeLa cells were maintained in Dulbecco's Modified Essential Medium (DMEM) (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated fetal calf serum (FCS), 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen, Glostrup, Denmark). Cells were grown on T75 bottles (Nunc, Roskilde, Denmark) at 37 °C with 5% CO_2.

Viruses

HIV-1 Bal strain (NIH AIDS Research and Reference Reagent Program, Bethesda, USA) was generated by transfection of HEK293T cells using calcium phosphate precipitation. Briefly, HEK293T cells were seeded at 4.5×10^4 per cm^2 on T75 bottle (Nunc, Roskilde, Denmark) and 10 µg of HIV-1 plasmid was mixed with 450 µL sterile water, 50 µL 2.5 M $CaCL_2$ and then 500 µL HEPES was added dropwise. 24 h after transfection the cell media was renewed and 48 h post transfection virus-containing supernatant was harvested, filtered through a 0.20 µm filter and stored at -80° C. TCID50 was determined by infecting TZM-bl cells and measuring luminescent signal. The calculations of TCID50 were done using Reed-Muench formula.

TZM-bl HIV infectivity assay

HeLa-derived TZM-bl cells (obtained from NIH AIDS Reagent Program, catalogue no. 8129) were used to evaluate HIV infectivity. TZM-bl cells express the HIV receptor CD4 and coreceptors CCR5 and CXCR4 and harbor a luciferase β-galactosidase reporter system under the control of the HIV-1 long terminal repeats (LTRs). TZM-bl cells were seeded in 96-well flat-bottomed culture plates (Sarstedt, Newton, USA) at a density of 5000 cells per well and cultured overnight. MP were added to the cells at indicated concentrations and subsequently infection with HIV-1 Bal strain was performed, or MP were pre-incubated with cells for 24 h and then cells were infected with HIV-1 strain Bal (10 x TCID50). After 48 h viral infection media was removed and cells were lysed in 90 µl 0.5% Nonidet P-40 (Struers Kebo Lab, Aalborg, Denmark) in PBS supplemented with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ for at least 45 min in order to inactivate the virus. Luciferase activity proportional to the level of infection was measured by adding 80 µl of Britelite plus reagent (Perkin-Elmer, Skovlunde, Denmark) per well. After mixing, 150 µl of the solution was transferred to white 96-well plates (Perkin-Elmer, Skovlunde, Denmark). Luciferase activity was quantified by measuring luminescent signal on a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany).

β-galactosidase assay

TZM-bl cells were seeded on 96-well plate at initial density of 5000 cells/well. After overnight incubation media was replaced with media containing PHPMA LMw 19% AZT or SIL PHPMA LMw 18% AZT, both at conc. 4 mg/L. After 24 h of incubation cells were infected with HIV-1 Bal (57 x TCID50). After 48 h from infection β-galactosidase assay for staining of infected cells was performed. Briefly, media was removed and cells were fixed in solution containing 4% formaldehyde (Polysciences Inc., USA) and 0.5% glutaraldehyde (Sigma-Aldrich) in PBS for 5 min. The cells were washed twice in PBS and stained in staining solution containing 5 mM $K_3Fe(CN)_6$ (Sigma-Aldrich, USA), 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$ (Sigma-Aldrich, Japan), 2 mM $MgCl_2$ (Merck, Germany) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma-Aldrich) added to staining solution just before use to final conc. of 1 mg/ml. Cells were stained in 37°C until full development of colour and then washed in PBS. Visualization of the infected cells was performed using an inverted microscope (Axio Observer Z1, Zeiss, Germany).

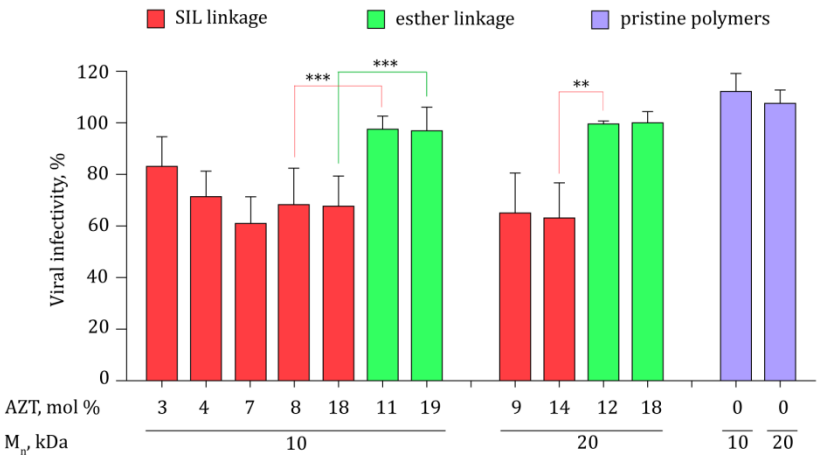


Figure SI 4. Viral replication inhibited by HPMA based macromolecular prodrugs of AZT administered at concentration 1 mg/L. Viral replication was quantified in TZM-bl cells with no pre-incubation of the polymers with the cells and allowing further 48 h for viral proliferation. Results are average of five independent experiments and are presented as average ± standard deviation.

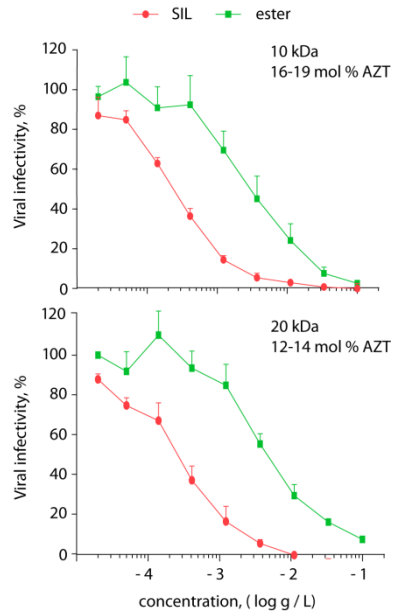
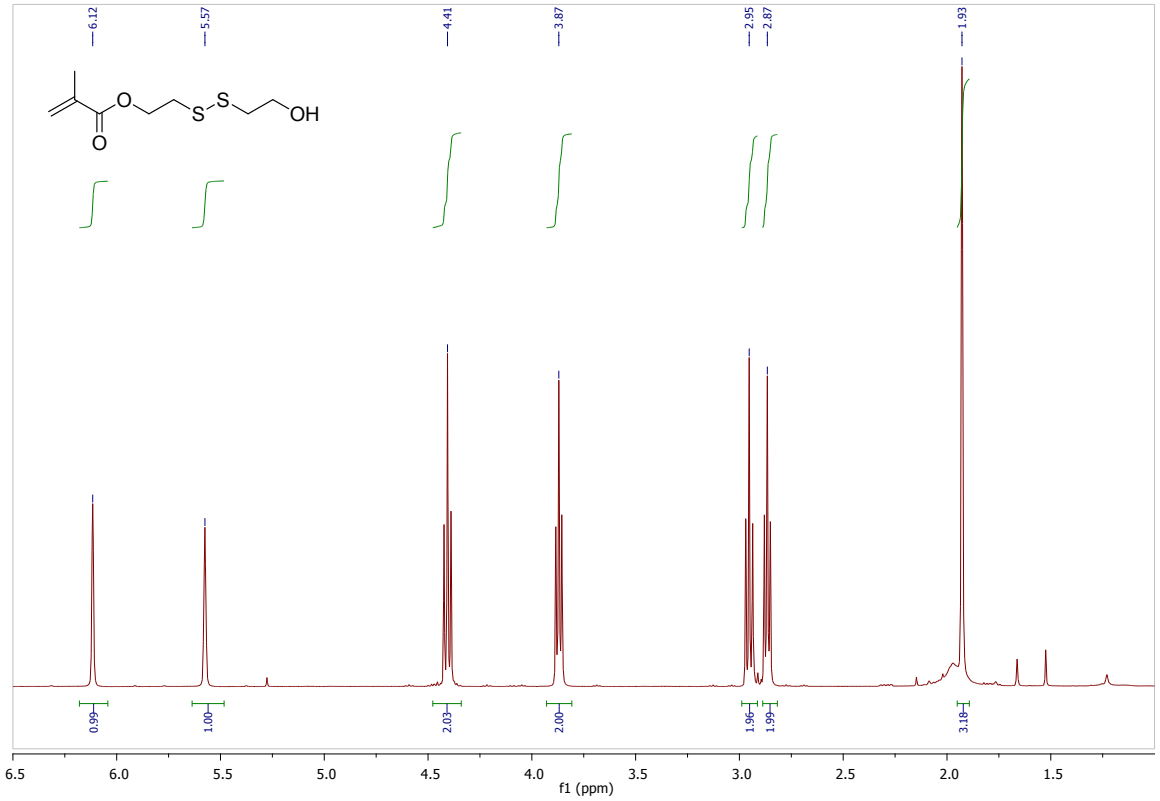
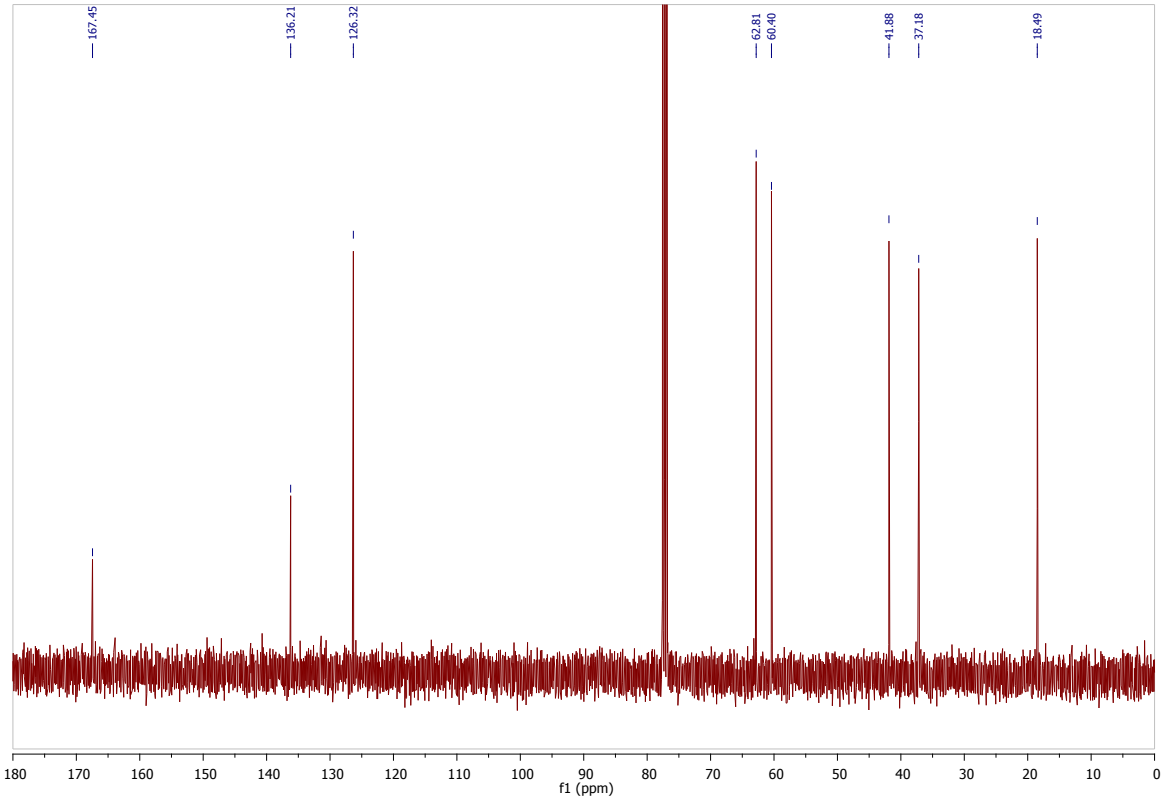
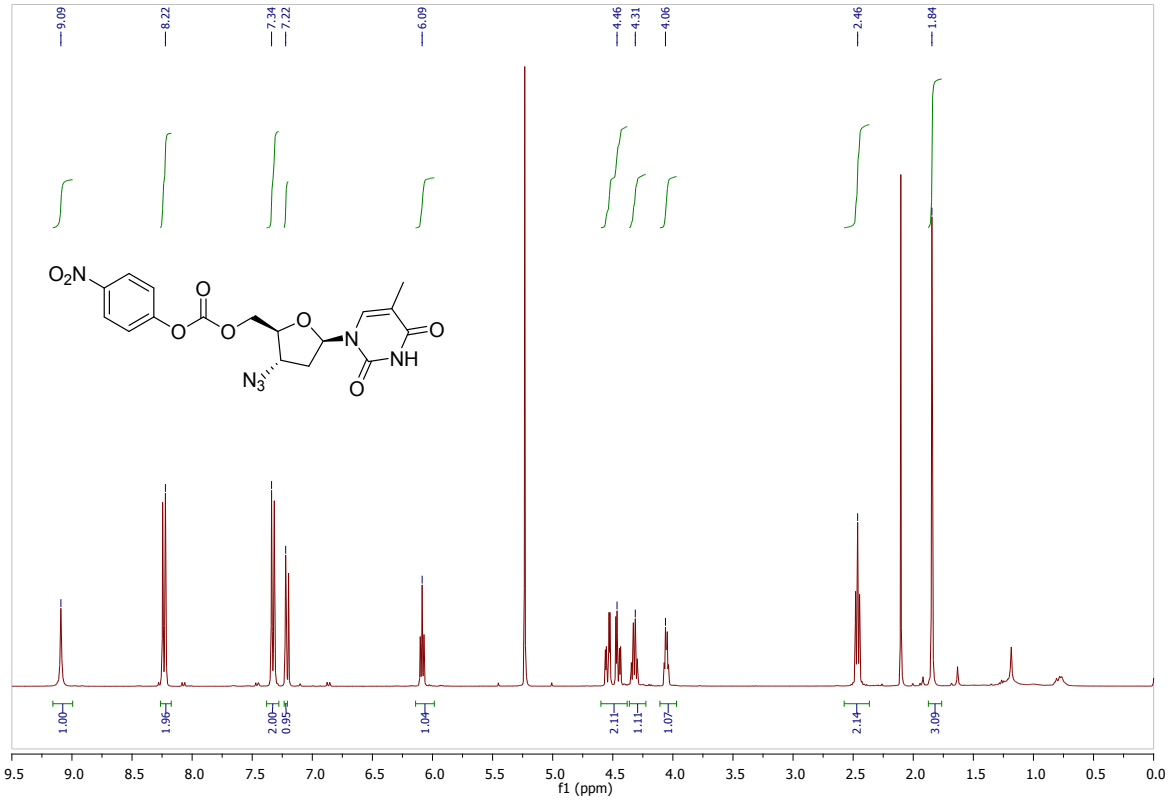


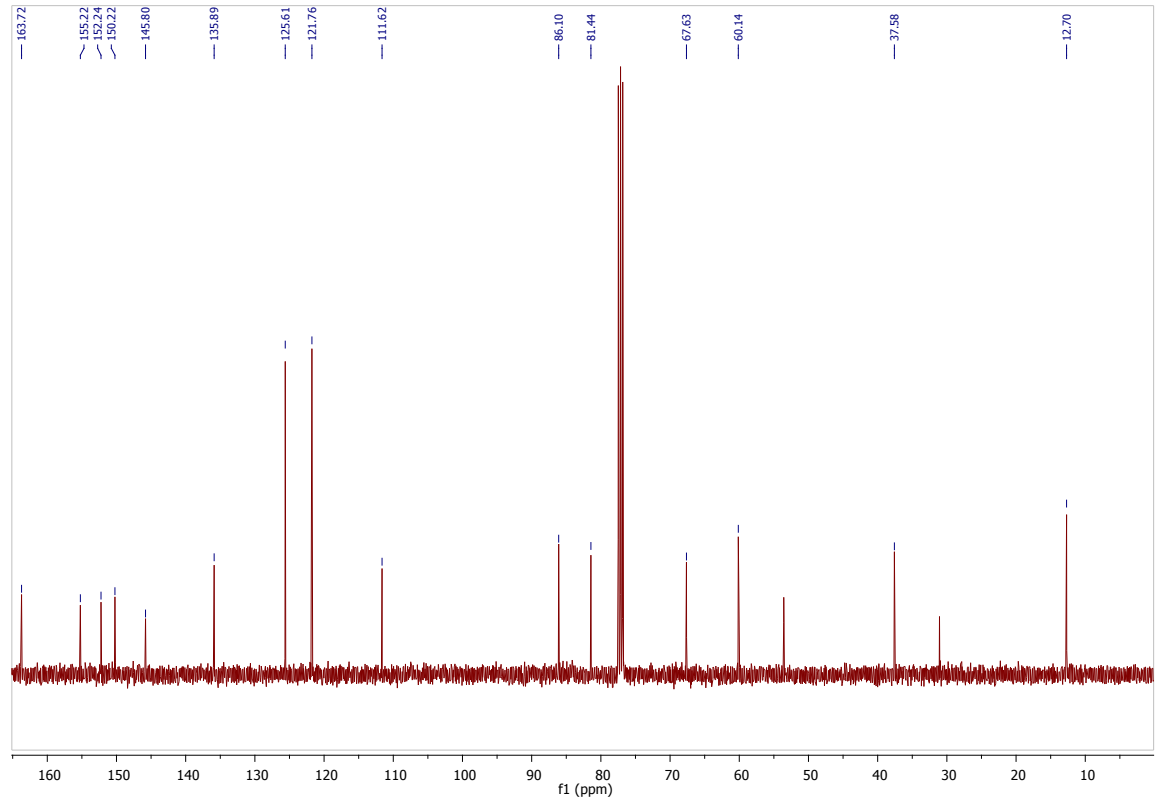
Figure SI 5. Dose response curves for macromolecular prodrugs based on PHPMA conjugated to AZT with ester or SIL linkage with regard to the inhibition of proliferation of HIV-1 Bal strain in the TZM-bl cells. Results are average of three independent experiments and are presented as average ± standard deviation.

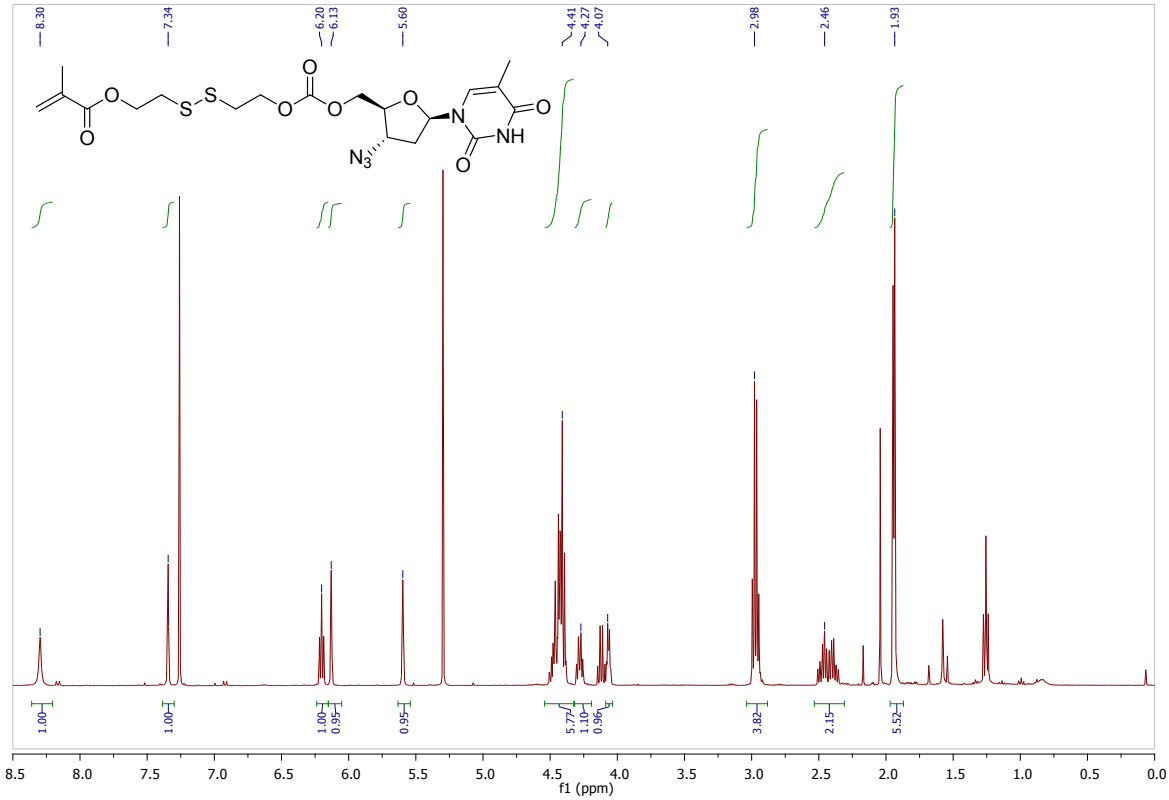
NMR Spectra

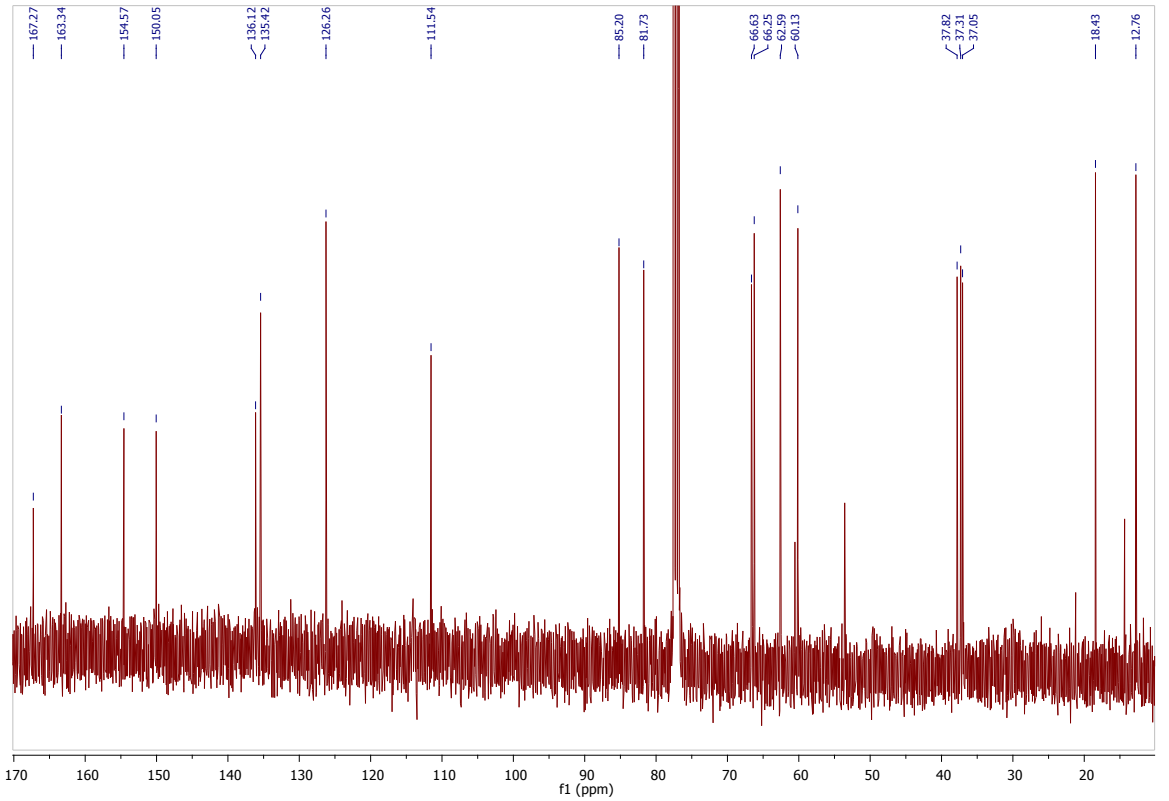

6.12
5.57
4.41
3.87
2.95
2.87
1.93
O
O
S
S
OH
O
0.99
1.00
2.03
2.00
1.96
1.99
3.18
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
f1 (ppm)


167.45
136.21
126.32
62.81
60.40
41.88
37.18
18.49
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

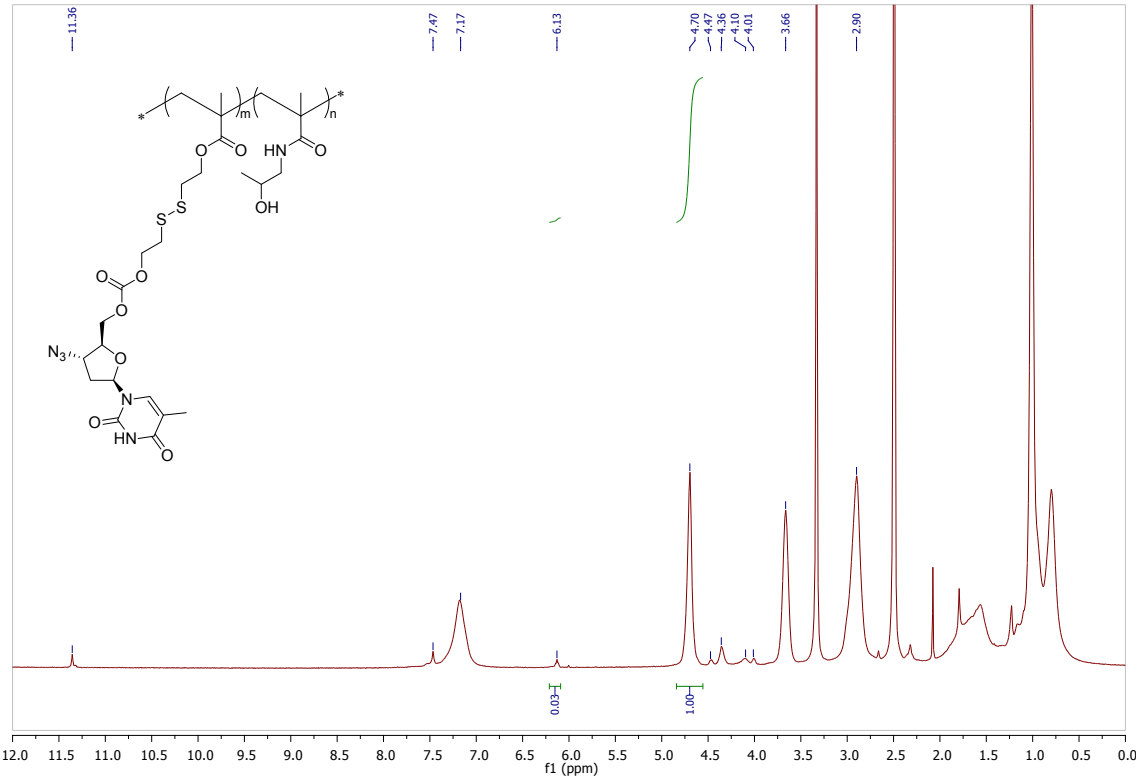

9.09
8.22
7.34
7.22
6.09
4.46
4.31
4.06
2.46
1.84
1.00
1.96
2.00
0.95
1.04
2.11
1.11
1.07
2.14
3.09
f1 (ppm)


163.72
155.22
152.24
150.22
145.80
135.89
125.61
121.76
111.62
86.10
81.44
67.63
60.14
37.58
12.70
f1 (ppm)

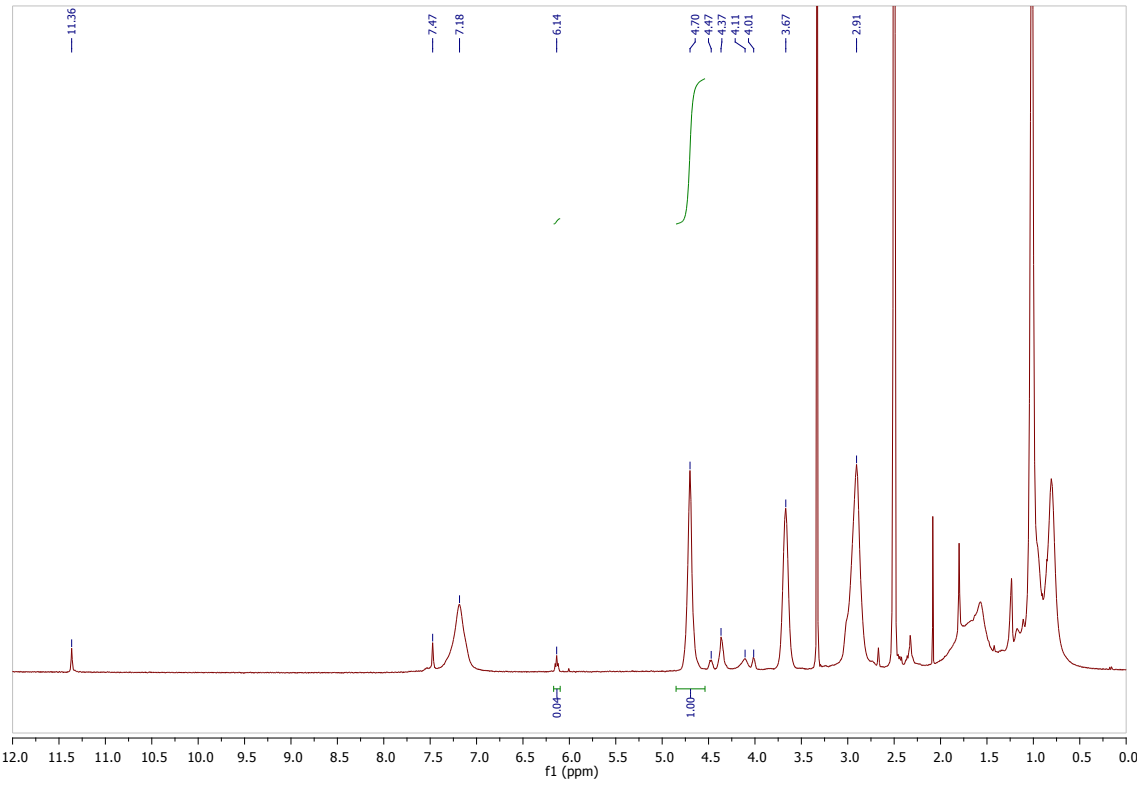

8.30
7.34
6.20
6.13
5.60
4.41
4.27
4.07
2.98
2.46
1.93
1.00
1.00
1.00
0.95
0.95
5.77
1.10
0.96
3.82
2.15
5.52
f1 (ppm)


167.27
163.34
154.57
150.05
136.12
135.42
126.26
111.54
85.20
81.73
66.63
66.25
62.59
60.13
37.82
37.31
37.05
18.43
12.76
f1 (ppm)

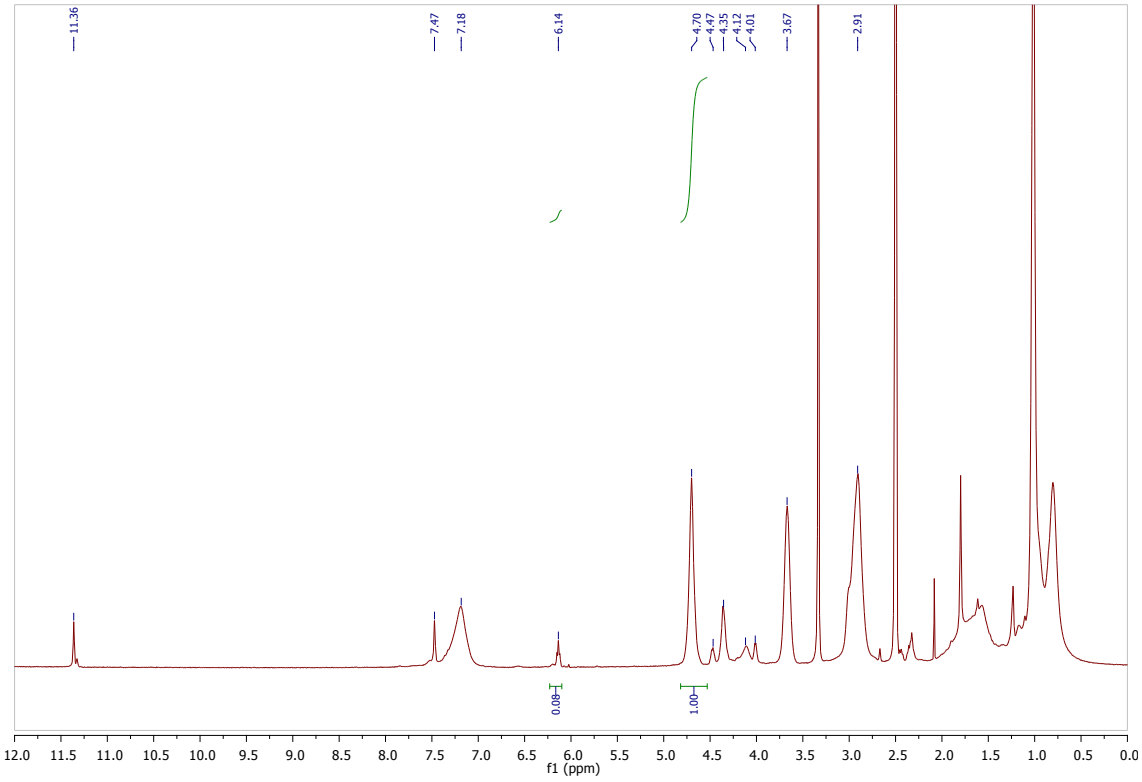
PHPMA-2



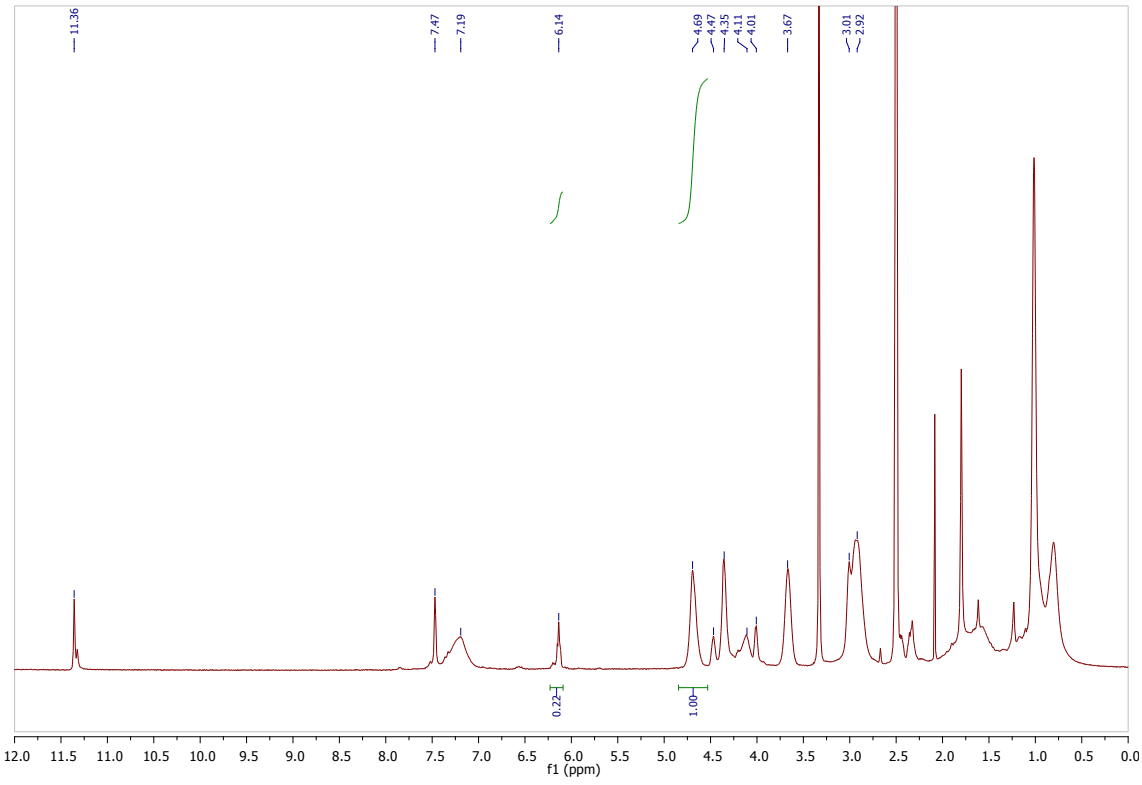
PHPMA-3



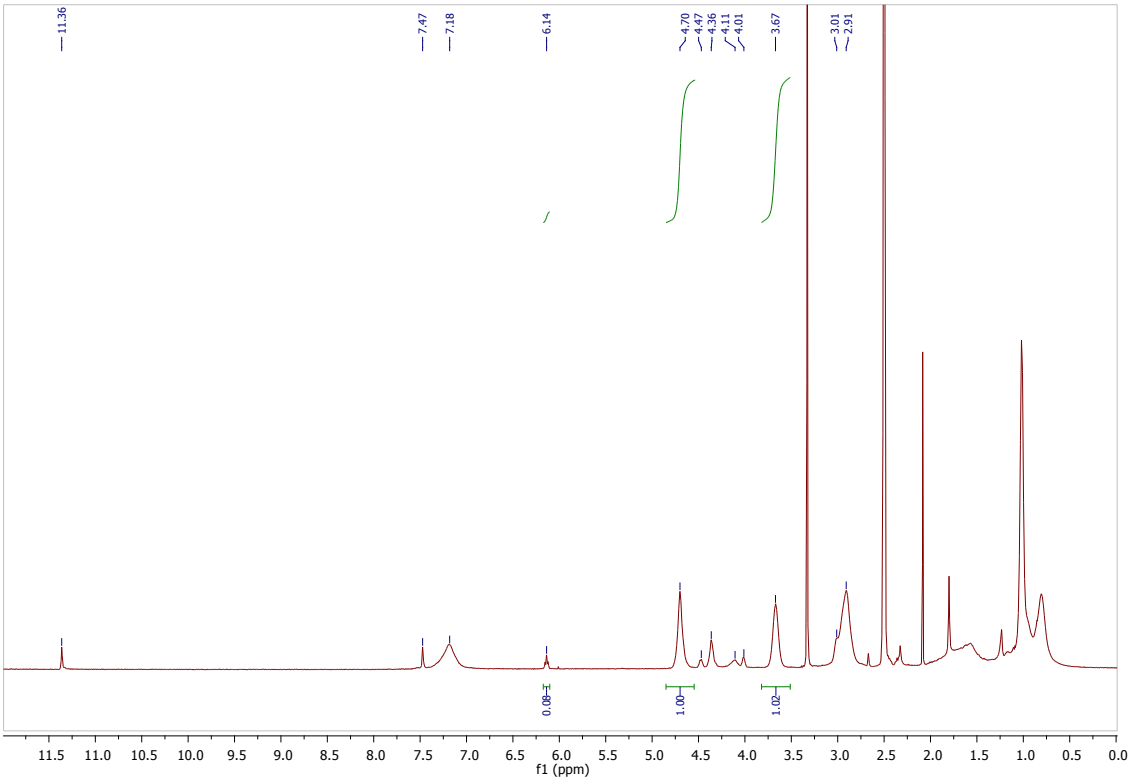
PHPMA-4



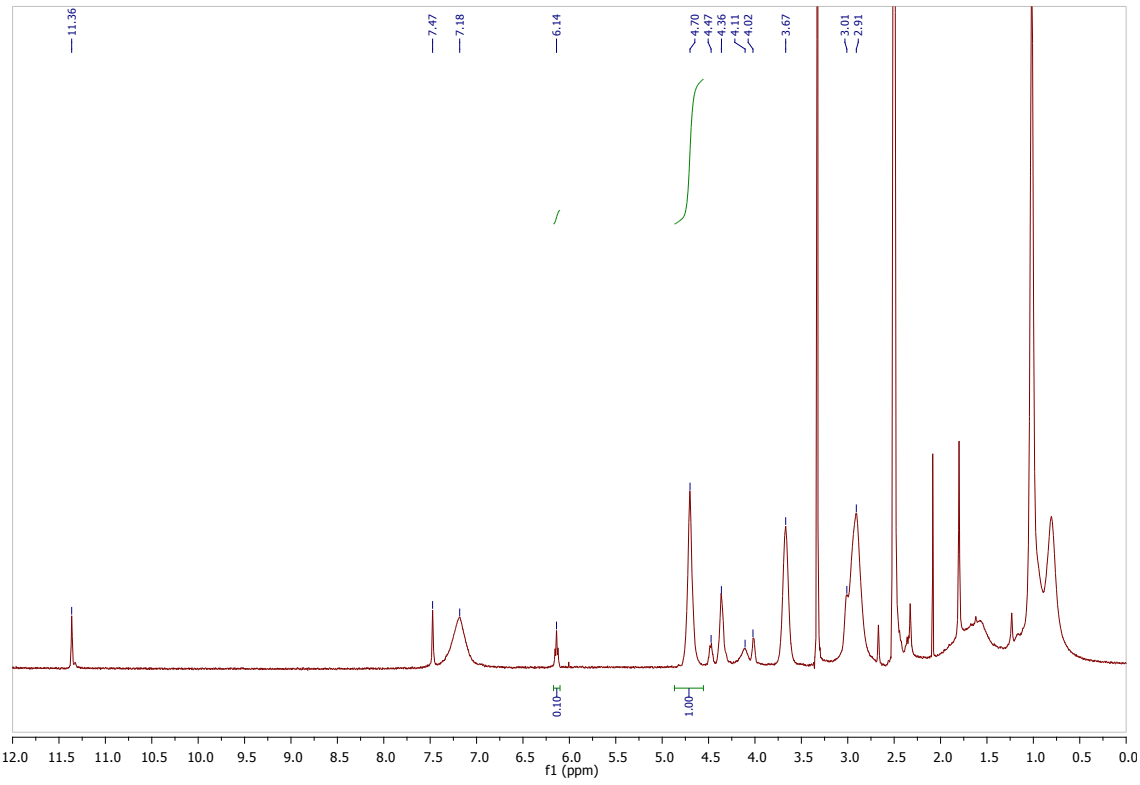
PHPMA-5



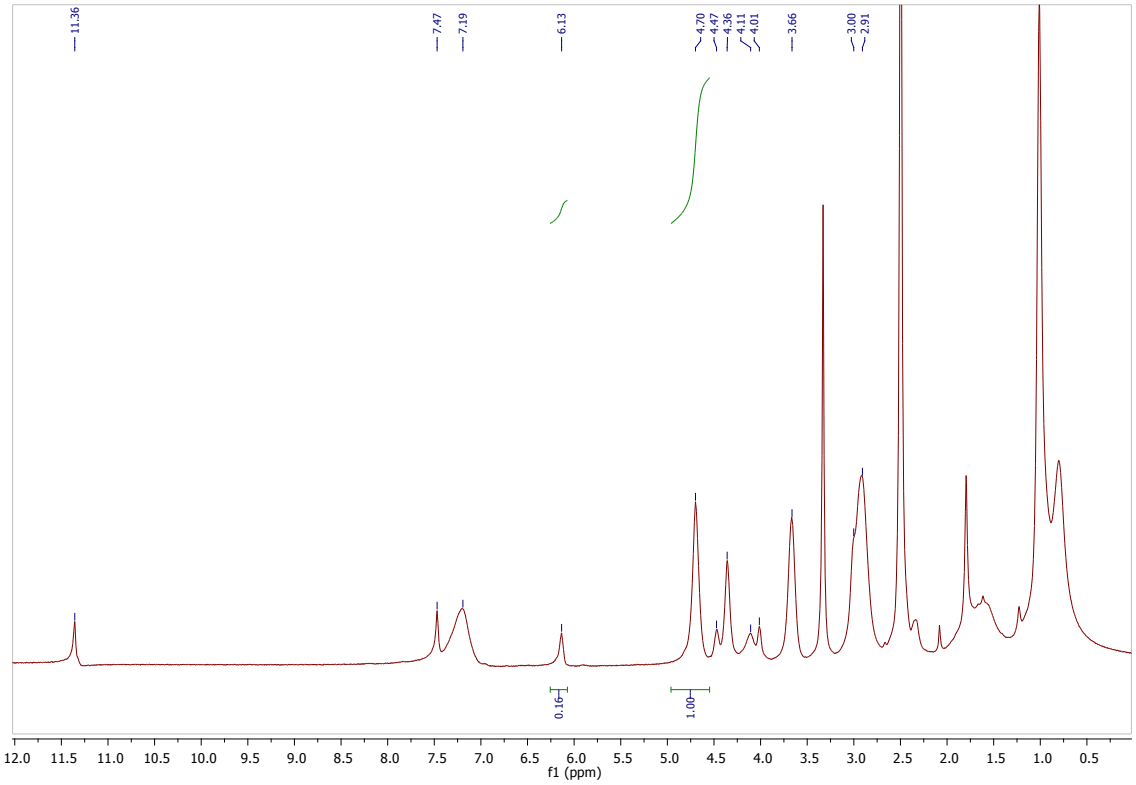
PHPMA-6



PHPMA-7



PHPMA-8


11.36
7.47
7.19
6.13
4.70
4.47
4.36
4.11
4.01
3.66
3.00
2.91
0.16
1.00
12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5
f1 (ppm)