Electronic Supplementary Information

for

Combination anti-HIV therapy *via* tandem release of prodrugs from macromolecular carriers

Maarten Danial,*[,a] Sushama Telwatte,[b] David Tyssen,[b] Steffen Cosson,[a,c] Gilda Tachedjian,[b,d,e,f]

Graeme Moad[a] and Almar Postma*[,a]

[a.] *CSIRO Manufacturing, Clayton VIC 3168, Australia.*
[b.] *Centre for Biomedical Research, Burnet Institute, Melbourne, VIC 3004, Australia.*
[c.] *Australian Institute for Bioengineering & Nanotechnology, University of Queensland, St. Lucia, QLD 4072, Australia.*
[d.] *Monash University, Department of Microbiology, Clayton VIC 3800, Australia.*
[e.] *The University of Melbourne, Department of Microbiology and Immunology at the Doherty Institute for Infection and Immunity, Melbourne, VIC 3000, Australia.*
[f.] *RMIT University, School of Science, Melbourne, VIC 3000, Australia.*

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Materials and methods

Chemicals

Acetone (≥99.8%), ammonium chloride (≥ 99.8%), chloroform (for liquid chromatography, ≥99.8%), 4-dimethylaminopyridine (DMAP, ≥99%), *N*,*N*-dimethylformamide (DMF, Seccosolv®), ethyl acetate (EtOAc ≥99.5%), triethylamine (Et_3N ≥99%) were purchased from Merck. Dichloromethane (CH_2Cl_2, HiPerSolv CHROMANORM for HPLC, stabilized, min. 99.8%), magnesium sulfate (100%), *n*-pentane (HiPerSolv CHROMANORM for HPLC, 95%) and sodium sulfate (100%) was obtained from VWR. Ethanethiol (99%), 4,4'-azobis(4-cyanovaleric acid) (Wako V-501, 98%), *N*,*N*-diisopropylethylamine (DIEA, 99.5%), hydroquinone (99.5%), 2-hydroxyethyl disulfide (technical grade, 96%), human serum albumin (99%), methacrylic anhydride (94%), methacryloyl chloride (≥ 97.0%), methanol (CHROMASOLV®, for HPLC, ≥ 99.9%) and 4-nitrophenyl chloroformate (96%) were purchased from Sigma-Aldrich. Carbon disulfide was obtained from BDH Limited. Diethyl ether (Et_2O) was purchased from RCL Labscan. Bis(4-nitrophenyl) carbonate (99%) was purchased from Allicon Pharmaceuticals. Lamivudine (3TC, 99%) and zidovudine (AZT) were obtained from APICHEM. Silica (0.020 – 0.045 mm) was purchased from Davisil. Trimethylsilyl propanoic acid sodium salt (TMSP-d_4) was purchased from Wilmad Glass Company (Buena, NJ, USA). All monomers, monomer precursors, chain transfer agent and V-501 were stored in the freezer (-18 ^oC) prior to use. In-house deionized water was used for all reactions and chemical product washings.

Biological reagents and media

TZM-bl cells. A modified HeLa reporter cell line that expresses the CD4 receptor as well as the CXCR4/CCR5 coreceptors required for HIV entry.1 Infection of these cells leads to expression of beta-galactosidase and luciferase genes driven by the HIV LTR reporter, resulting in either a colorimetric or chemiluminescence readout, respectively, depending upon the substrate. TZM-bl cells were obtained through the NIH AIDS Research and Reference Reagent Program, contributed by Drs John C. Kappes, Xiaoyun Wu and Tranzyme Inc.

HIV-1 Ba-L strain. Virus was isolated from a primary culture of adherent cells grown from explanted infant human lung tissue. HIV-1 Ba-L is a subtype B CCR5-tropic HIV-1 strain.[2] The virus was propagated to high titres in peripheral blood-derived monocytes/macrophages and CD4+ T cells. The strain was obtained from the NIH AIDS Reagent and Reference Reagent Program.

DMEM-10 medium. Medium used to both culture TZM-bl cells and dilute the compounds in the assays, consisting of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) enriched with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM glutamine (Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen).

RPMI-1640 medium. Medium used to culture SupT1 cells, consisting of Roswell Park Memorial Institute (RPMI-1640) (Invitrogen) enriched with 10% FBS and 1% anti-anti (Gibco).

Synthesis

N-(2-Hydroxypropyl) methacrylamide (7)

Methacrylic anhydride (71 g, 0.46 mol) was dissolved in diethyl ether (200 mL) and added dropwise to a solution of 1-amino-2-propanol (34.6 g, 0.46 mol) in diethyl ether/CH_2Cl_2 (192 mL/8 mL). *Caution – this is an exothermic reaction, which can lead to diethyl ether boiling.* After stirring the reaction for 2 hours at room temperature, the mixture was placed in the freezer (-18 ºC) for 16 hours, which yielded crystals of **7**. The crystals were filtered and washed using diethyl ether/CH_2Cl_2 (4/1 v/v). The crystals were dried *in vacuo*. Yield 38.6 g (63 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.35 (br, 1H), 5.71 (dd, J = 1.8 Hz, 1H), 5.33 (dd, J = 2.7 Hz, 1H), 3.93 (m, 1H), 3.46 (m, 1H), 3.15 (m, 1H), 1.95 (s, 3H), 1.18 (d, J = 6.3 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.5, 139.8, 120.2, 67.6, 47.3, 21.2, 18.8 (see Figure S3 for assignments). HRMS (APCI) $[M+H]^+$ $C_7H_{14}NO_2$ calculated 144.1025, found 144.1019.

4-Cyano-4-(((ethylthio)carbonothioyl)thio)pentanoic acid (CTA)

The synthesis of the CTA was performed as described in Convertine et al.[3] Briefly, sodium hydride (60 % in mineral oil, 3.15 g, 0.079 mol) was added to diethyl ether (150 mL) in a 250 mL round bottom flask and was cooled to <10 ºC. Ethanethiol (4.72 g, 0.076 mol) was added dropwise (over 20 minutes) to the suspension containing sodium hydride and the resultant mixture was allowed to stir for 10 minutes. To this solution, carbon disulfide (6 g, 0.079 mol) was then added dropwise. After a further 10 minutes, the resultant ethane trithiocarbonate sodium salt (7.0 g, 0.044 mol) was isolated by filtration by suction using a fritted glass funnel. The ethane trithiocarbonate sodium salt was resuspended in diethyl ether (100 mL) and iodine (5.6 g, 0.022 mol) was added and stirred for 45 minutes at room temperature. Subsequently, the

brown suspension was washed two-fold with $Na_2S_2O_3$ (200 mL of 5 wt. %) solution and once with brine (200 mL) to yield a yellow ether phase which was collected, dried over $MgSO_4$, filtered and concentrated *via* rotary evaporation to yield a viscous yellow liquid (3.07 g, 0.0112 mol). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.30 (q, 4H), 1.34 (t, 6H).

The bis(ethyl trithiocarbonate) was dissolved in ethyl acetate (100 mL) and 4,4'-azobis(4-cyanopentanoic acid) (V-501, 4.57 g, 0.0169 mol) was added. This solution was refluxed under nitrogen at 90-100 ºC for 20 hours. The solution was concentrated and purified by flash silica chromatography (30.0 × 5.0 cm) using *n*-pentane/ethyl acetate/acetic acid (1/1/0.03 v/v/v). After rotary evaporation of the appropriate fractions (R_f = 0.65 in *n*-pentane/ethyl acetate/acetic acid 1/1/0.03 v/v/v), a viscous yellow liquid was obtained. The yellow liquid was placed at -18 ºC, which yielded yellow crystals that were subsequently filtered, washed with ice-cold pentane and dried *in vacuo*. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.33 (q, J = 7.4 Hz, 2H), 2.67 (m, 2H), 2.40 (m, 2H), 1.86 (s, 3H), 1.34 (t, J = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 216.8, 176.5, 119.1, 46.4, 33.7, 31.6, 29.5, 25.0, 12.9 (See Figure S4 for assignments). HRMS (ESI) $[M+H]^+$ $C_9H_{14}NO_2S_3$ calcd 264.0181, found 264.0181.

TZM-bl cytotoxicity assay and data analysis

Cytotoxicity was measured using a colorimetric cell viability assay (Promega CellTiter 96® AQueous One Solution Cell Proliferation Assay - MTS) according to manufacturer's instructions. Absorbance was measured using a Thermo Multiskan Ex plate reader at 492/690 nm and mean background values were subtracted from each mean average test value.

The cell-only and substrate-only wells enabled the determination of the maximum absorbance reading for cell viability and the background level of the substrate, respectively. For both assay

types, mean background levels were subtracted from all sample dilutions and untreated controls. The percentage viability was calculated for all polymer compound dilutions as compared to the untreated control. Using GraphPad Prism (Version 6.0a) or Origin (Version 9.1), the concentrations of each compound was plotted against the corresponding percentage viability determined from the cytotoxicity assay.

SupT1 cytotoxicity assay and data analysis

The SupT1 cytotoxicity assay was performed as described in Danial et al.[4] Robotic dispensing was programmed with the WinPrep software (PerkinElmer). High content imaging was programmed using the Harmony software (PerkinElmer). Integration of the assay was programmed with the Plate::Works software (PerkinElmer). One day prior to the cytotoxicity assay, SUPT1 cells were seeded at 10000 cells per well into 96 well plate using an automated liquid handling robot (Janus, PerkinElmer) and placed in a humidified incubator at 37 ^{o}C, 5% CO_2. Custom-made polymer library (in DMSO solution at 100 mg mL^{-1}, stored at -20 ^{o}C) was dispensed (100 μL) in a 96 well plate and stored at -20 ^{o}C.

The cytotoxicity assay was performed on a Cell:Explorer automated workstation (PerkinElmer) comprised of a STX-44 incubator (Liconics), an automated liquid handling robot (Janus 4-varispan + 96-MDT/gripper, PerkinElmer), a robot arm (Plate::Handler II, PerkinElmer), a HEPA filtered enclosure and a high content screening imager (Operetta, PerkinElmer). At the onset of the automated assay, assay plates (96 well plate containing the cells) were placed into the automated incubator (37 ^{o}C, 5% CO_2, 95% humidity), while the polymer library assay plate was placed on the liquid handling robot deck. Assays were run as follows: (i) assay plates were transferred from the incubator to the liquid handling robot, and 2

μL of polymer solution was dispensed in each well of the assay plate (96) from the reagent plate (96) using the varispan pipettor-arm (to yield a 0.1 mg/mL final concentration, 6 repeats for each compound distributed in different two assay plates); (ii) the assay plates were transferred to the incubator and incubated for 24 hours; (iii) A staining reagent solution, containing Hoechst 33342 (Life Technologies) and ethidium homodimer (Life Technologies), was prepared at a final 1:1000 dilution (respectively) in PBS, dispensed in a 96 well plate (100 μL) and stored at 4 ºC. The reagent plates (96 well plates containing the staining reagent solutions), and a 1-well trough was filled with media (80 mL) were placed on the Janus liquid-handling robot deck. The assay plates were transferred from the incubator to the liquid handling robot, 20 μL of staining solution was dispensed in each well, then the assay plates were transferred into the incubator for 15 minutes prior to wash with media; (iv) assay plate were then transferred on the high content imager for imaging.

Image processing and analysis to quantify cell viability was performed using the Columbus software (PerkinElmer). Total cell number was quantified by counting nuclei (Hoechst channel). Fluorescent intensity of the Ethidium homodimer staining (Alexa 546 channel) was measured for each individual detected nuclei and a threshold was applied to select living cells. Viability for each well was calculated for each well by dividing the number of living cells over the total number of cells. The effects of a positive control (PBS), vehicle control (DMSO) and negative control (Triton X100) on the cell viability was performed along the polymer library.

Combination index

The combination index (CI) of the polymers consisting of AZT SIL and 3TC SIL was determined using the CalcuSyn software (version 2.11) through the method of Chou and Talalay.[5] To do the analysis, the EC_{50} values with respect to each prodrug was calculated as follows:

$$EC_{50,AZT} = n_{AZT} \cdot EC_{50,polymer} \quad (1)$$

$$EC_{50,3TC} = n_{3TC} \cdot EC_{50,polymer} \quad (2)$$

Where n_{AZT} is the amount of AZT released from the polymer and n_{3TC} is the amount of 3TC released from the polymer. The total prodrug EC_{50} required for the CalcuSyn software was then calculated as follows:

$$EC_{50} = EC_{50,AZT} + EC_{50,3TC} \quad (3)$$

The CI for the polymers consisting of AZT SIL and 3TC SIL was compared to pristine AZT and 3TC respectively at the total prodrug concentration EC_{50} values. The composition and CI values of these polymers shown in Figure S15 and are tabulated in Table S3.

Supplementary Tables

Table S1. Polymerisation feed and conditions.

Polymer	Polymerisation feed (mg)					Added solvent volume	Time
	AZT SIL 3	3TC SIL 5	HPMA 7	CTA	V-501	(μL)	(h)
P1	178.9	0.0	447.4	15.2	1.60	1553	5
P2	357.9	0.0	397.8	4.6	1.60	1603	8
P3	559.3	0.0	466.1	2.8	0.30	1534	12
P4	0.0	165.8	447.4	15.2	1.60	1553	5
P5	0.0	331.5	397.8	9.1	0.97	1602	8
P6	0.0	331.5	397.8	4.6	0.49	1603	8
P7	357.9	165.8	348.0	4.6	4.79	1652	5
P8	179.0	165.8	397.8	9.1	0.97	1602	12
P9	179.0	165.8	348.0	4.6	0.49	1602	12
P10	357.9	165.8	348.0	9.1	0.97	1652	6
P11	357.9	165.8	348.0	2.3	0.24	1652	6
P12	179.0	331.5	348.0	4.6	0.49	1652	6
P13	0.0	0.0	497.2	4.9	4.88	1503	5
P14	0.0	0.0	497.2	4.6	0.49	1503	5
P15	0.0	0.0	497.2	2.3	0.49	1503	5

Table S2. Polymerisation molar feed ratio and final experimental molar ratios.

Polymer	Polymerisation feed (mol %)[a]			Monomer composition (mol %)[b]		
	AZT SIL 3	3TC SIL 5	HPMA 7	AZT SIL 3	3TC SIL 5	HPMA 7
P1	10	0	90	2	0	98
P2	20	0	80	2	0	98
P3	25	0	75	3	0	97
P4	0	10	90	0	2	98
P5	0	20	80	0	5	95
P6	0	20	80	0	6	94
P7	20	10	70	2	4	94
P8	10	10	80	1	3	96
P9	10	10	80	1	2	97
P10	20	10	70	2	2	96
P11	20	10	70	3	3	94
P12	10	20	70	1	9	90
P13	0	0	100	0	0	100
P14	0	0	100	0	0	100
P15	0	0	100	0	0	100

[a] mole percent monomer composition in the polymerization feed. [b] mole percent monomer composition in polymer determined by ^{1}H-NMR.

Table S3. Combination index (CI) determined at f_a=0.5.

Polymer	Drug Ratio[a]		CI	Property
	AZT SIL	3TC SIL		
P7	0.82	1.58	0.072	Strong synergism
P8	0.84	2.02	0.064	Strong synergism
P9	1.30	3.08	0.093	Strong synergism
P10	1.78	1.93	0.069	Strong synergism
P11	2.64	3.14	0.025	Strong synergism
P12	1.19	10.69	0.341	Synergism

[a] Average number of AZT SIL and 3TC SIL per polymer determined by ^{1}H NMR.

Supplementary Figures

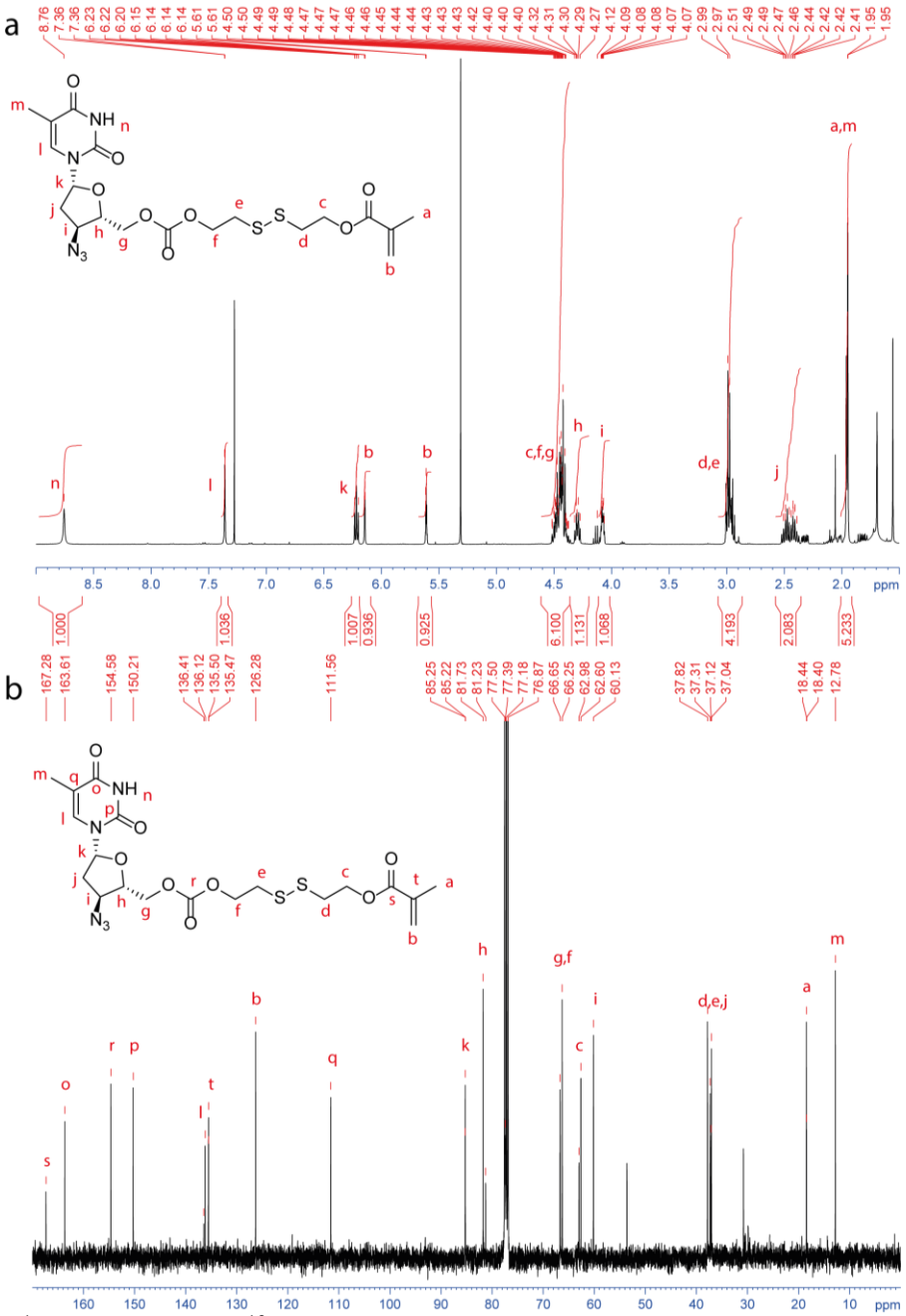

Figure S1. (a) ^{1}H-NMR and (b) ^{13}C-NMR spectra of zidovudine (AZT SIL) methacrylate monomer (**3**) in $CDCl_3$.

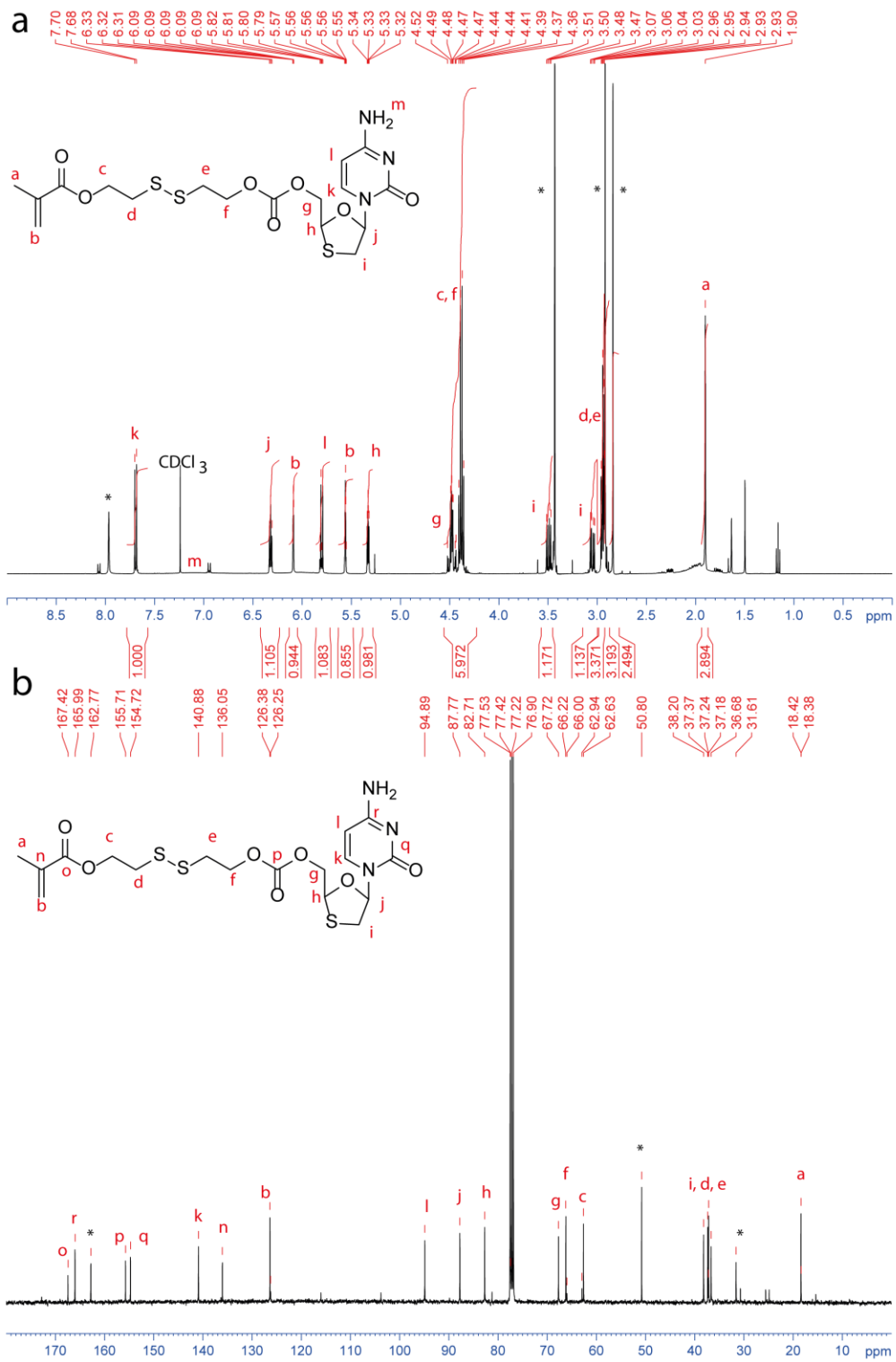

Figure S2. (a) ^{1}H-NMR and (b) ^{13}C-NMR spectra of lamivudine (3TC SIL) methacrylate monomer (**5**) in $CDCl_3$. * indicates residual solvents DMF and MeOH.

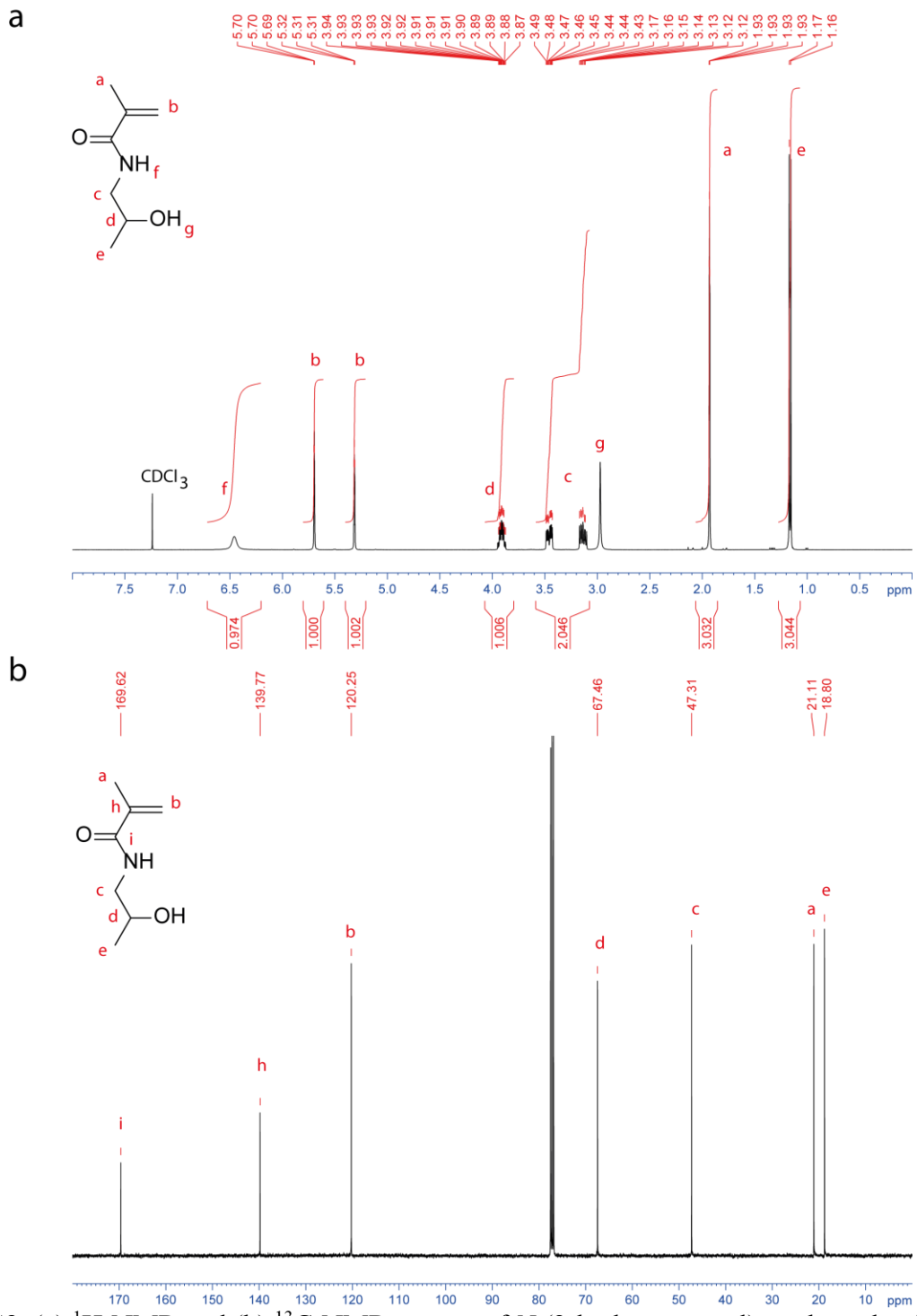

Figure S3. (a) ^{1}H-NMR and (b) ^{13}C-NMR spectra of *N*-(2-hydroxypropyl) methacrylamide (**7**) in $CDCl_3$.

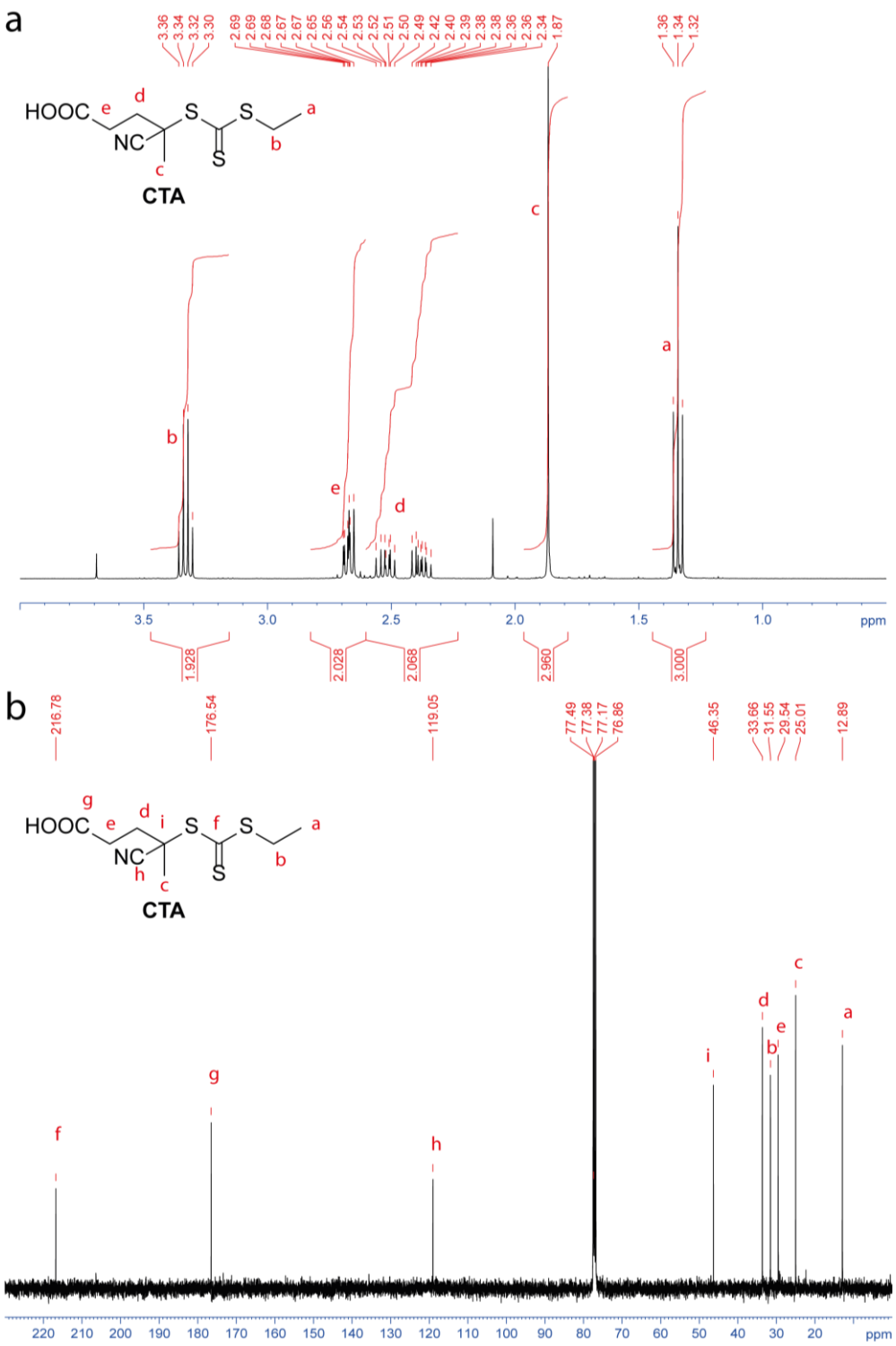

Figure S4. (a) ^{1}H-NMR and (b) ^{13}C-NMR spectra of 4-cyano-4-(((ethylthio)carbonothioyl)thio)pentanoic acid (**CTA**) in $CDCl_3$.

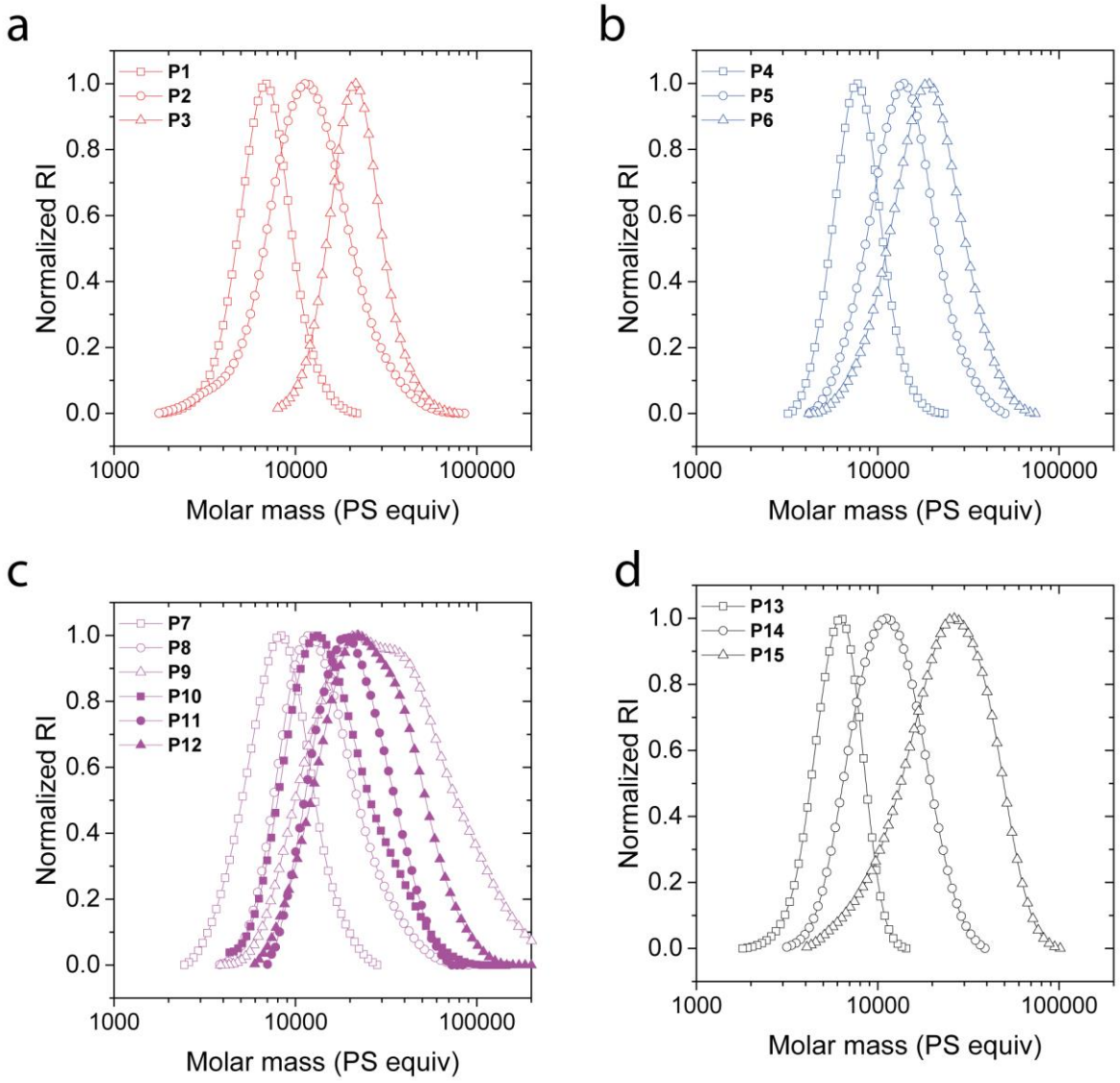

Figure S5. Size exclusion chromatograms of polymers (a) **P1 – P3**, (b) **P4 – P6**, (c) **P7 – P12**, and (d) **P13 – P15**.

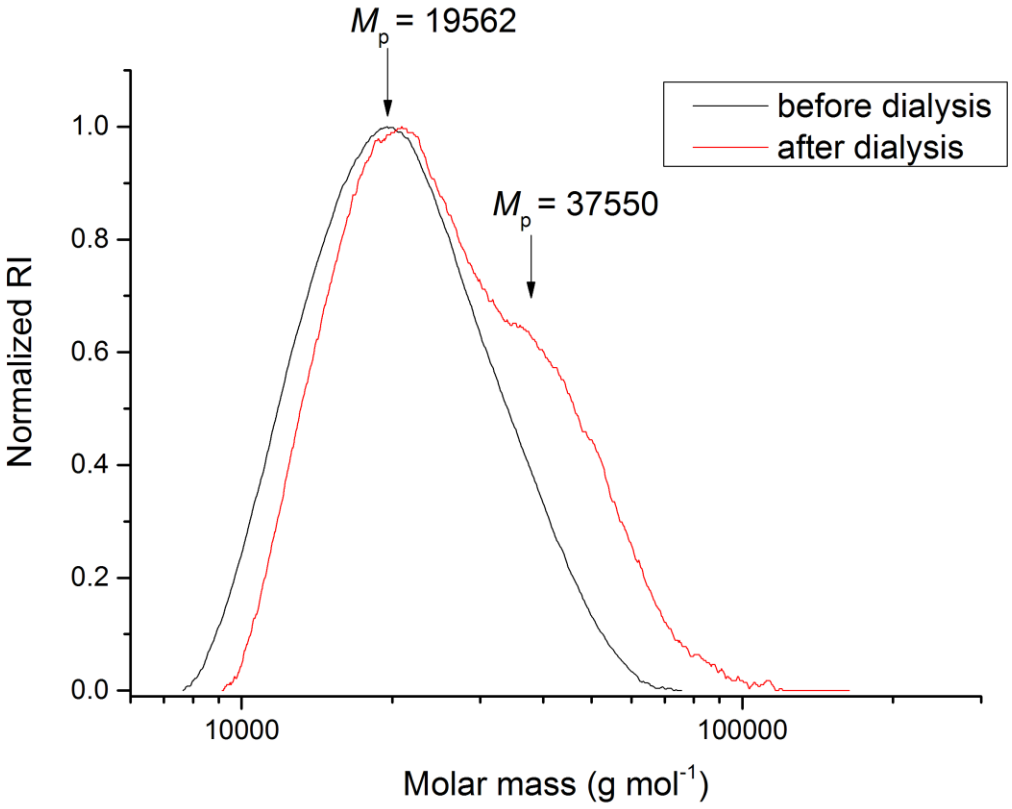

Figure S6. Size exclusion chromatogram of polymer before and after dialysis for 3 days in water / methanol (9/1 v/v).

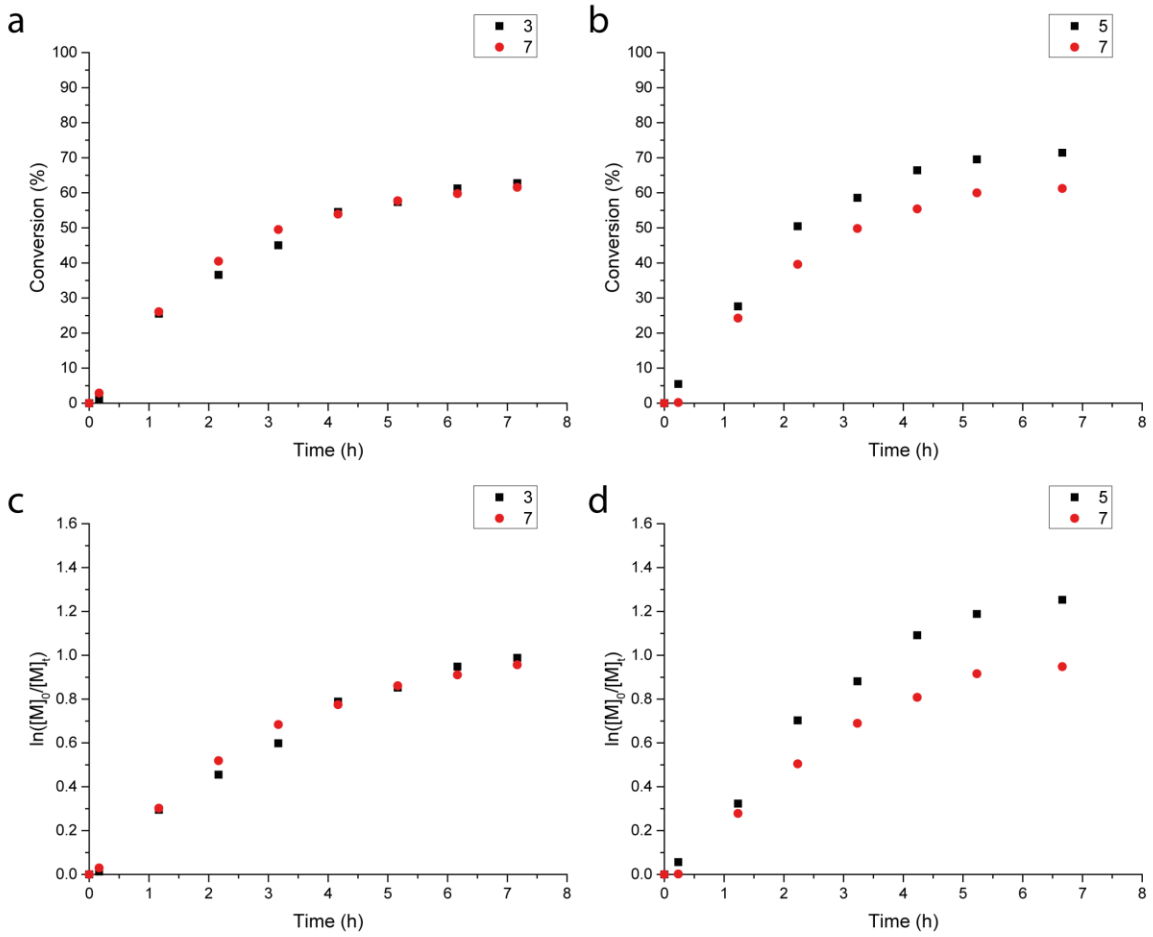

Figure S7. (a) Monomer conversion in a copolymerization consisting of AZT SIL (**3**) and HPMA (**7**) as a function of time. (b) Monomer conversion in a copolymerization consisting of 3TC SIL (**5**) and **7** as a function of time. (c) Pseudo-first order kinetic copolymerization of **3** and **7**. (d) Pseudo-first order kinetic copolymerization of **5** and **7**.

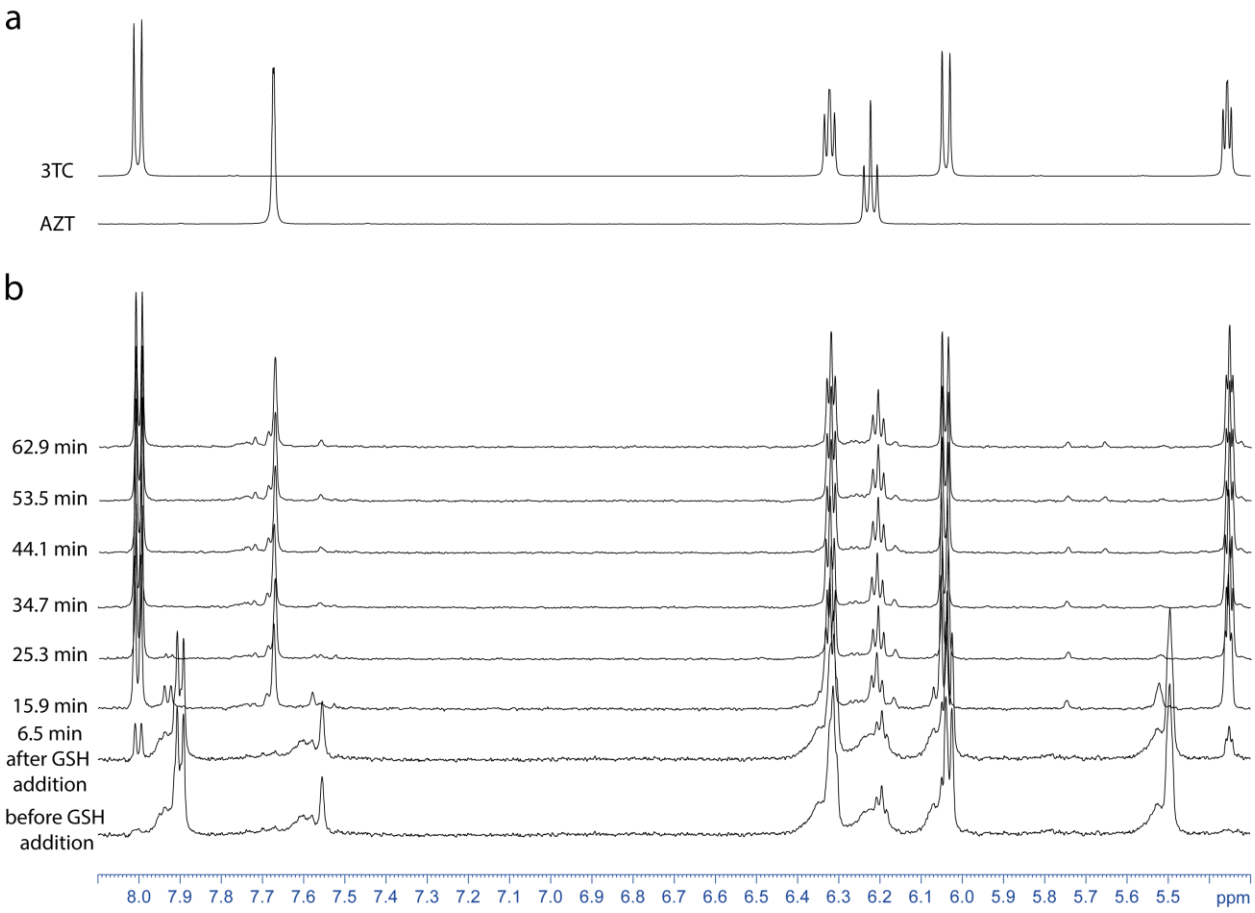

Figure S8. An expanded region of the solvent suppressed ^{1}H-NMR spectra of (a) 3TC and AZT in 10 vol % MeOD in deuterated PBS (10 mM, pD 7.8) and (b) time series of polymer **P7** in the presence of 5 mM GSH in 10 vol % MeOD in deuterated PBS (10 mM, pD 7.8) at 37 ºC. All integrals were determined relative to the internal reference trimethylsilyl propanoic acid sodium salt (TMSP-d_4, -0.02 ppm).

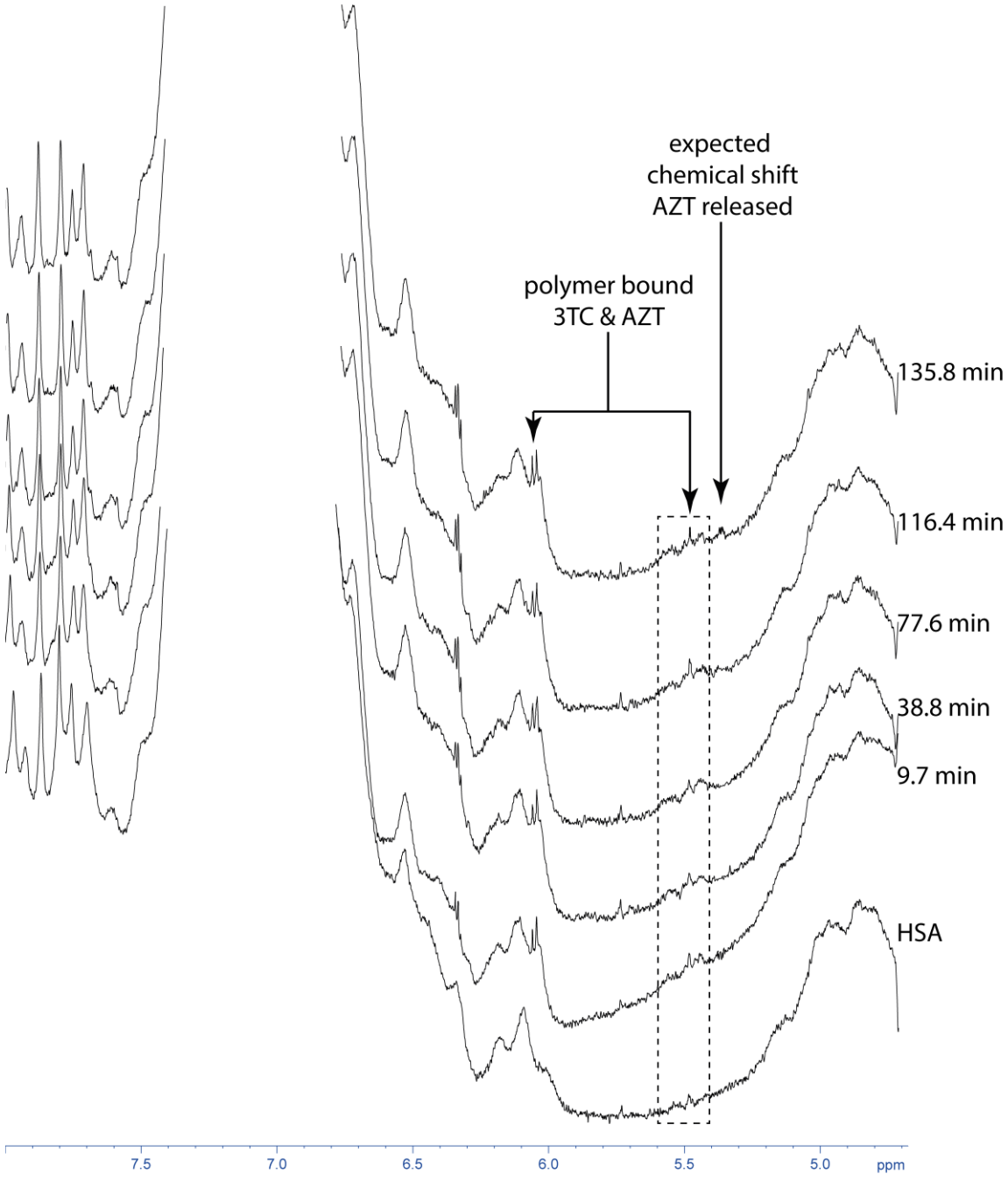

Figure S9. Time series of 1 mg·mL^{-1} polymer **P7** in the presence of 500 μM human serum albumin in 10 vol % MeOD in deuterated PBS (10 mM, pD 7.8) at 37 ^{o}C.

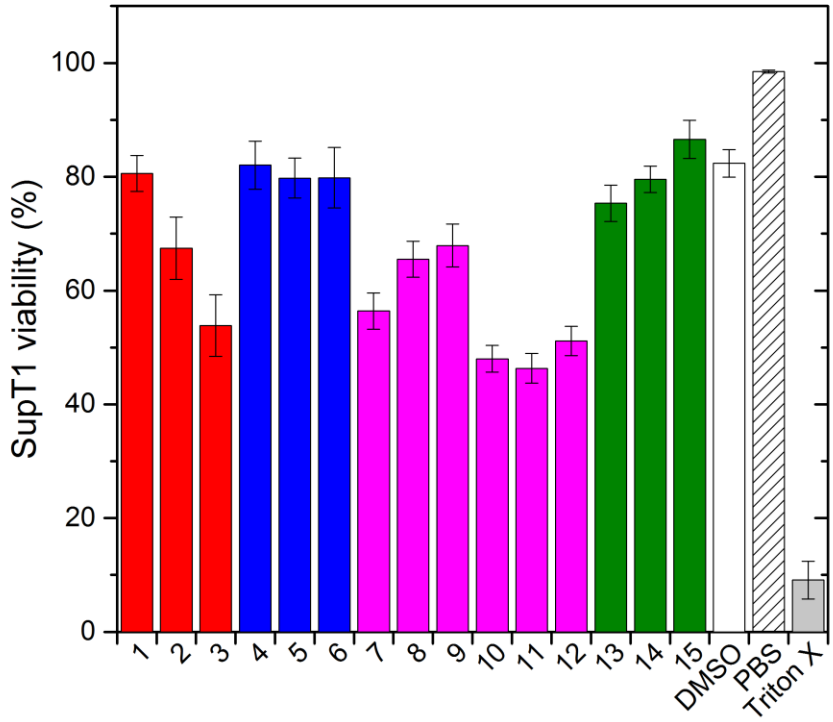

Figure S10. SupT1 viability in the presence of 100 μg·mL^{-1} polymer. Polymers are colour coded according to monomer composition: Red: AZT SIL (**3**) and HPMA (**7**); Blue: 3TC SIL (**5**) and **7**; Magenta: **3**, **5** and **7**; Green: only **7**. Control viability experiments included 1 vol. % DMSO in PBS (final concentration in assay, white), PBS (positive control, striped) and Triton X (negative control, grey). The error bars represent the standard error obtained from the experiments (n = 6).

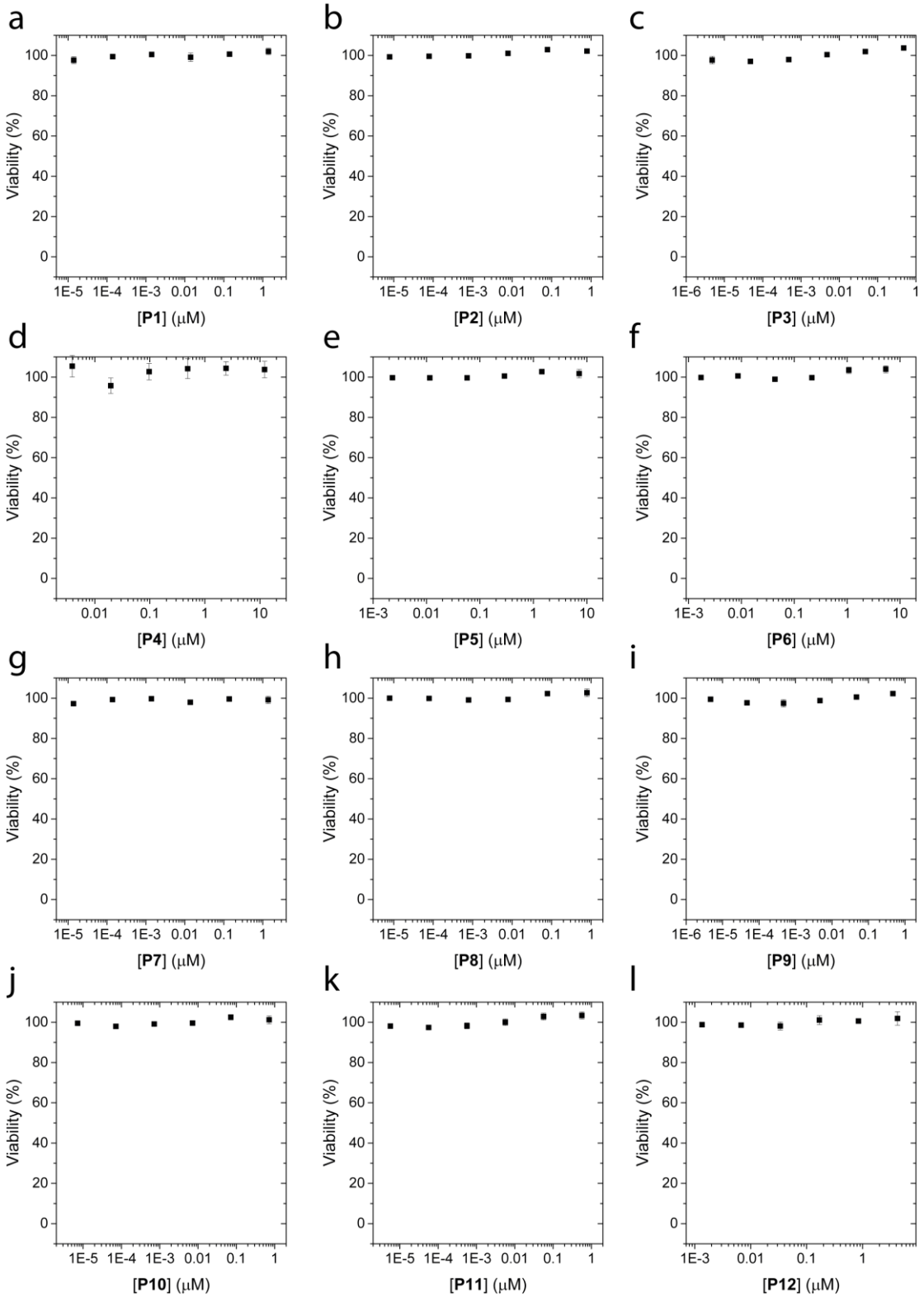

Figure S11. Viability of TZM-bl cells in the presence of polymers (a) **P1**, (b) **P2**, (c) **P3**, (d) **P4**, (e) **P5**, (f) **P6**, (g) **P7**, (h) **P8**, (i) **P9**, (j) **P10**, (k) **P11**, and (l) **P12**.

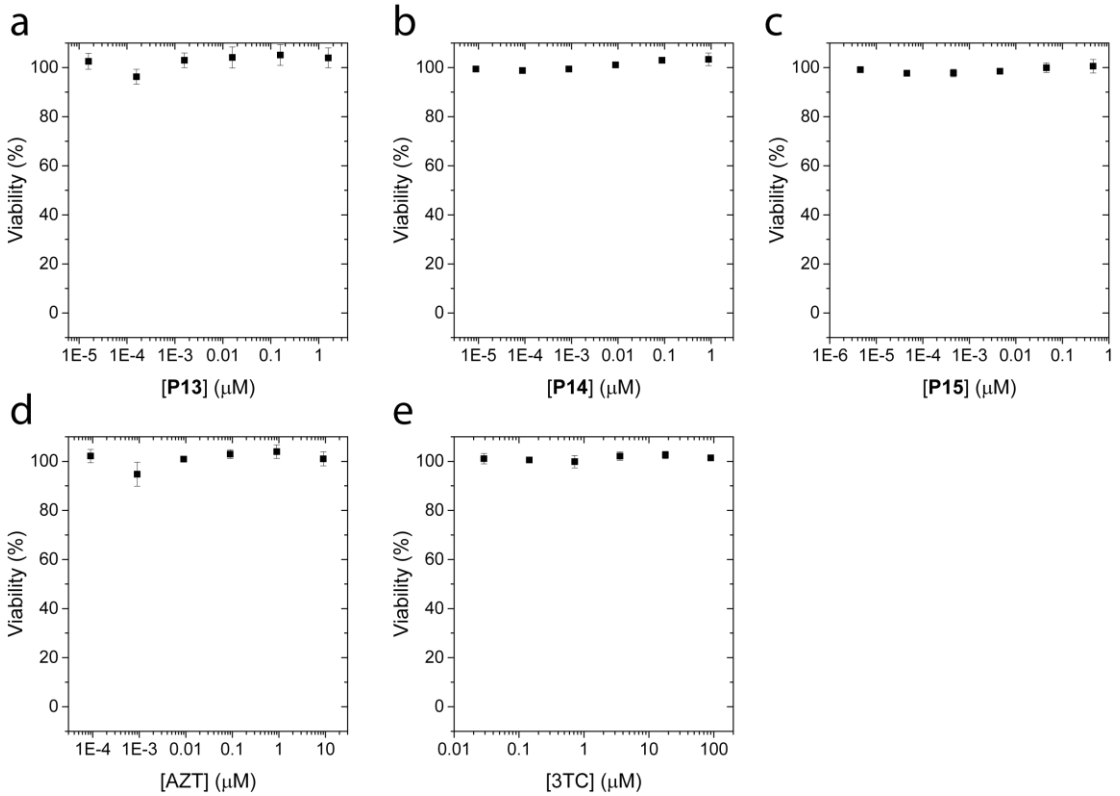

Figure S12. Viability of TZM-bl cells in the presence of control polymers (a) **P13**, (b) **P14**, (c) **P15**, and pristine prodrugs (d) AZT, and (e) 3TC.

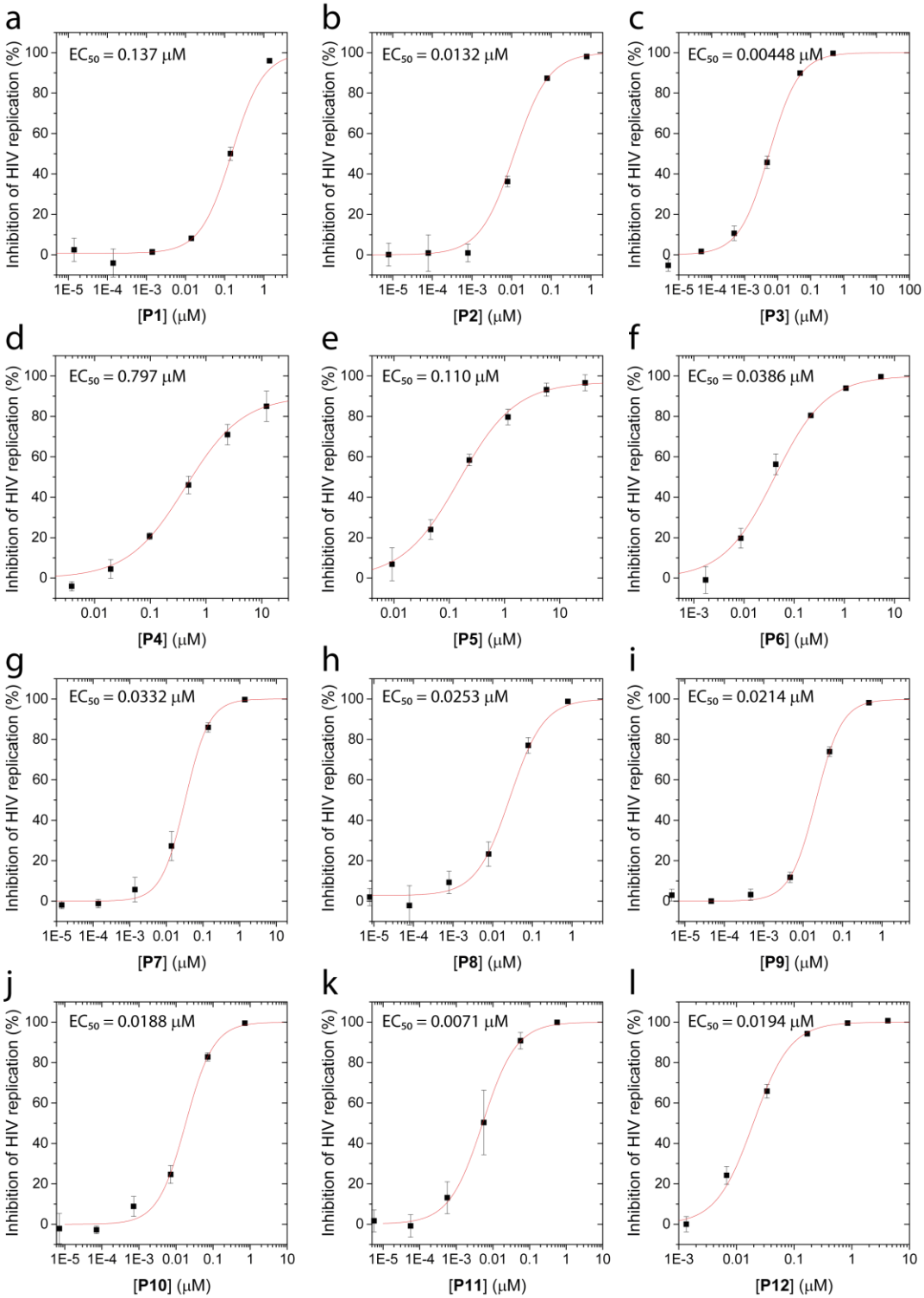

Figure S13. Dose-response curves of polymers (a) **P1**, (b) **P2**, (c) **P3**, (d) **P4**, (e) **P5**, (f) **P6**, (g) **P7**, (h) **P8**, (i) **P9**, (j) **P10**, (k) **P11**, and (l) **P12**. The red lines indicate the fit from which the EC_{50} (per polymer) was determined. The error bars represent the standard error obtained from the independent experiments (n = 3 or 4).

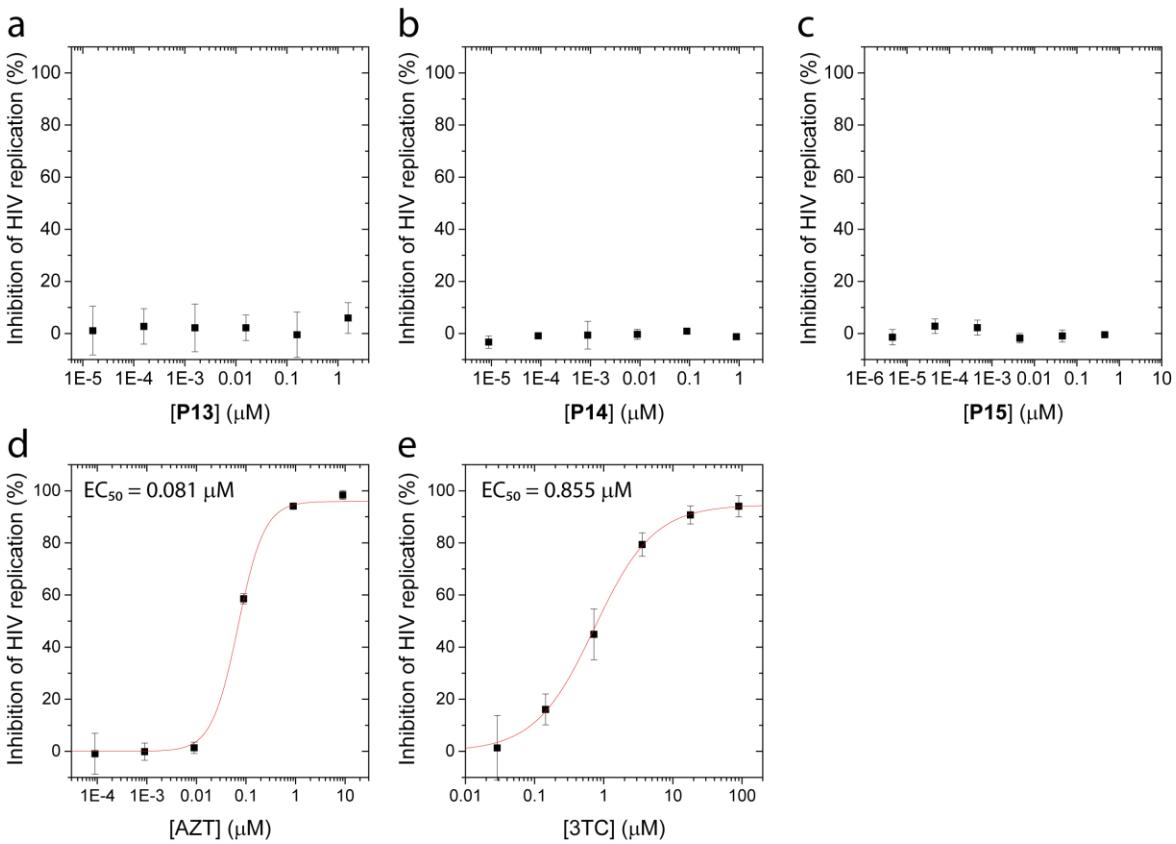

Figure S14. Dose-response of control poly(HPMA) polymers (a) **P13**, (b) **P14**, (c) **P15**, (d) AZT, and (e) 3TC. The red lines indicate the fit from which the EC_{50} was determined. The error bars represent the standard error obtained from the independent experiments (n = 3 or 4).

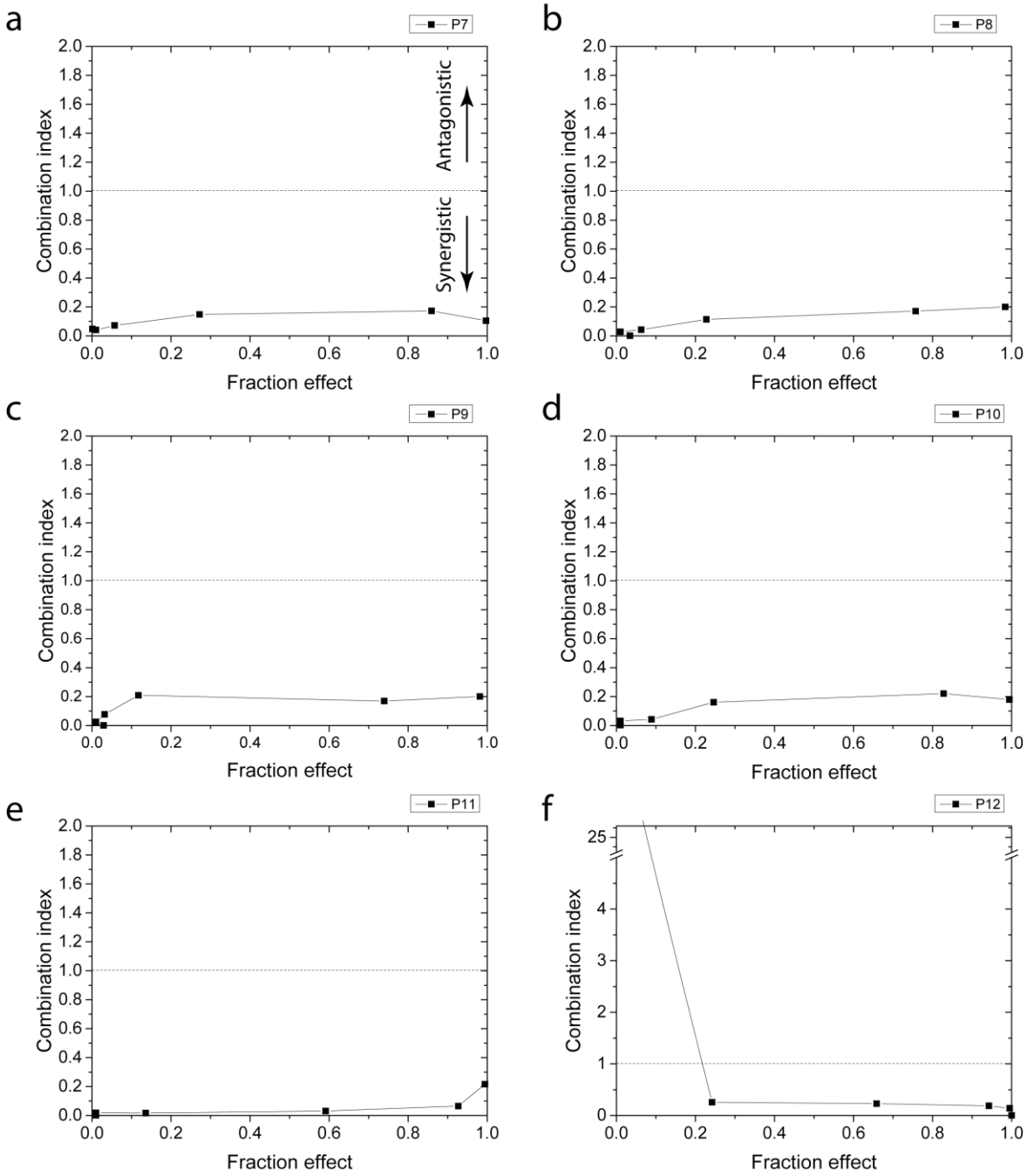

Figure S15. Combination index of (a) **P7**, (b) **P8**, (c) **P9**, (d) **P10**, (e) **P11**, and (f) **P12** as a function of fraction effect. The dotted line at unity indicates additivity.

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