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Triple Activity of Lamivudine Releasing Sulfonated Polymers against HIV-1

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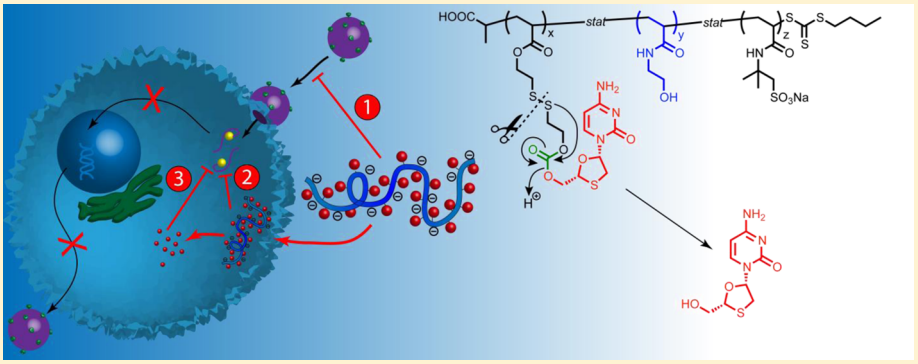
Supporting Information



ABSTRACT: In this article a library of polymeric therapeutic agents against the human immunodeficiency virus (HIV) is presented. The library of statistical copolymers of varied molar mass was synthesized by reversible addition−fragmentation chain transfer (RAFT) polymerization. The synthesized polymers comprise pendent hydroxyl and sulfonated side chains as well as the reverse transcriptase prodrug lamivudine (3TC) attached via a disulfide self-immolative linker. The glutathione mediated release of 3TC is demonstrated as well as the antiviral efficacy against HIV entry and polymerase activity. Although a high degree of polymer sulfonation is required for effective HIV entry inhibition, polymers with approximately ∼50% sulfonated monomer demonstrated potent kinase independent reverse transcriptase inhibition. In addition, the sulfonated polymers demonstrate activity against DNA−DNA polymerase, which suggests that these polymers may exhibit activity against a broad spectrum of viruses. In summary, the polymers described provide a triple-active arsenal against HIV with extracellular activity via entry inhibition and intracellular activity by kinase-dependent lamivudine-based and kinase-independent sulfonated polymer based inhibition. Since these sulfonated copolymers are easily formulated into gels, we envision them to be particularly suited for topical application to prevent the mucosal transmission of viruses, particularly HIV.

KEYWORDS: *HIV-1, antiviral, inhibitor, RAFT, drug delivery, self-immolative linker*

INTRODUCTION

According to the World Health Organization (WHO), approximately 37 million people are infected with the human immunodeficiency virus type 1 (HIV-1).[1] Despite the urgency of finding a lasting solution to this global problem, an effective remedy against the virus that can cause acquired immune deficiency syndrome (AIDS) remains elusive. Nevertheless, mitigation of AIDS in infected individuals can be achieved using a range of 27 drugs targeting the virus, including reverse transcriptase inhibitors, protease inhibitors, and integrase inhibitors, as well as fusion and entry inhibitors.[2−4] Despite the high efficacy demonstrated for many of these drugs, unfavorable properties such as low circulation half-life and adverse side effects often become additional burdens on the patient.[4] For example, AZT (azidothymidine, also known as zidovudine) has a circulation half-life of 0.5−3 h, and a 300 mg oral dose requires twice daily administration.[5] In addition, AZT has been implicated in hematologic toxicity including neutropenia and anemia[6] and has been associated as the

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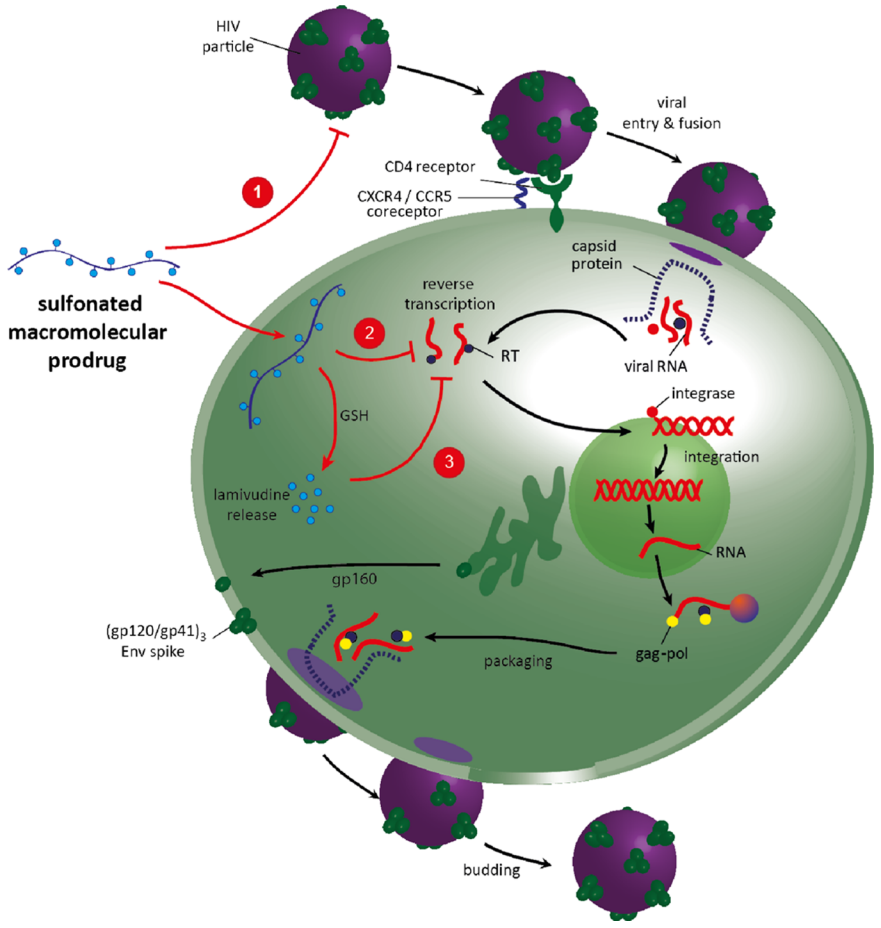

Figure 1. Illustration of the triple mode of anti-HIV action exhibited by the polymers presented including (1) entry inhibition, (2) kinase-independent reverse transcription inhibition mediated by the polymer, and (3) kinase-dependent reverse transcriptase inhibition mediated by the release of lamivudine. Adapted with permission from Danial and Klok.[29] Copyright 2013 American Chemical Society.

cause of myopathy.[7] For these reasons, AZT is often not recommended for first-line treatment of HIV.[8]

Release of antiretroviral drugs from nanogels,[9] hydrogels,[10] or polymer conjugates[11−14] have all provided viable options for prolonging the circulation half-life and alleviating the cytotoxic side effects. While these systems effectively prolong the action of the antiviral released, drug release mechanisms rely on the presence of bonds that are intermediately stable to hydrolysis, such as ester linkages, to attach the drug to the polymer.[11,12] While this provides a higher therapeutic window between antiviral activity and cytotoxicity, these systems do not provide complete release and usually results in a lower efficacy against HIV infectivity.[11−14] Recently, a macromolecular prodrug anti-HIV system was devised that was capable of a rapid intracellular triggered release of an antiviral drug. In this system, the AZT drug was attached through a self-immolative, disulfanyl ethylcarbonate linker between the drug and a methacrylate polymer side chain.[15] The self-immolation of the linkage relies on the presence of intracellular glutathione (GSH) that mediates the release of AZT from the polymer side chains.[15] This technology offers a means to conjugate and control the release of any hydroxyl-, carboxylic acid-, or amine-functionalized drug from a polymer and is mediated by a thiol such as GSH.[16,17]

One of the limitations of drug delivery systems devised to date is the emergence of AZT drug resistant strains of HIV. For this reason, we hypothesized that an antiviral system that possesses inhibition efficacy against multiple stages of viral replication,[18] which additionally comprises a different reverse transcriptase inhibitor, lamivudine, would exhibit increased efficacy against HIV. A similar strategy has been exploited against hepatitis C, where copolymers consisting of poly(acrylic acid) and pendent ribavirin were designed to create polymer therapeutics with concerted activity.[19] Here, multiple modes of anti-HIV activity may also act as multiple selection pressures to help reduce the potential emergence of resistant HIV strains.[2,3] By analogy, a combination of antivirals, which are usually administered to HIV infected individuals as part of highly active antiretroviral therapy (HAART) that is now standard of care, exhibits synergy and is therefore highly effective in reducing viral loads and hence superior to single drug antiretroviral therapies.[3]

This analogy suggests that polymers that carry multiple antiviral cargos could make effective anti-HIV active agents that could be used as virustats[20] (also known as microbicides). However, while pendent anionic groups on polymers have been demonstrated to electrostatically bind to the positively charged V3 loop on the HIV glycoprotein gp120 and form effective polymeric anti-HIV therapeutics,[21−23] these effects have only recently been combined with reverse transcriptase inhibitor AZT to provide multimodal antiretroviral activity at the entry and reverse transcription phase of HIV replication.[24] Although the antiviral effect induced by polyanionic side-chains, such as acrylic acid or methacrylic acid side chains has shown to lead to

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an increased anti-HIV efficacy, sulfonated polyanions are thought to provide significantly greater antiviral potency due to a low pK_a. For instance, sulfonated polyanionic groups including polymers consisting of vinylsulfate,[23] vinylsulfonate,[22] styrenesulfonate,[22] or 2-acrylamido-2-methyl-1-propanesulfonate[22] demonstrated high anti-HIV efficacies. Furthermore, polysulfonates have also demonstrated inhibition potency against reverse transcriptase,[25,26] a feature that has not been enabled with (meth)acrylic acid polymers. Therefore, polysulfonate polymers with lamivudine drug releasing capability make excellent candidates as effective macromolecular prodrugs with triple activity, with potential in topical strategies that prevent mucosal transmission of HIV.

In this article, a library of polymers that possess three independent modes of antiviral activity is described. The copolymers consisting of sulfonated side chains, hydroxylated side chains, and self-immolative pendent chains capable of releasing lamivudine were created by reversible addition−fragmentation chain transfer (RAFT) polymerization.[27,28] Both the molar mass and the composition of these polymers was systematically varied, which permitted us to evaluate structure−anti-HIV efficacy relationships. As depicted in Figure 1, the modes of antiviral activity were assayed including their extracellular antiviral activity as (1) entry inhibitors, (2) kinase-independent inhibitors of reverse transcriptase, and (3) kinase-dependent inhibtiors of reverse transcriptase mediated via the release of lamivudine from the polymer. In addition, the kinetics of GSH mediated lamivudine release as well as cytotoxicity of these conjugates in fibroblasts, hepatocytes, and lymphocytes was assessed. Taken together, we envision that with careful design, polymer therapeutics that exhibit multimodal antiviral activity could prove to be useful, active agents that provide longer lasting, efficacious anti-HIV activity while maintaining low cytotoxicity profiles. In addition, the design laid out here could also pave the way to novel multimodal polymer therapeutics against other infectious viruses.

EXPERIMENTAL SECTION

Materials. Acetone (for analysis EMSURE ≥99.8%), acryloyl chloride (stabilized with phenothiazine for synthesis, ≥96%), ammonium chloride (≥99.8%), chloroform (for liquid chromatography LiChrosolv, ≥99.8%), 4-dimethylaminopyridine (DMAP, ≥99%), *N*,*N*-dimethylformamide (DMF, Seccosolv), ethyl acetate (EtOAc, ≥99.5%), and 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. 2-Acrylamido-2-methanepropane sodium sulfonate (AMPS, 50 wt % solution in H_2O), 1-butanethiol (99%), 4,4′-azobis(4-cyanovaleric acid) (Wako V-501, 98%), 2-bromopropionic acid (99%), *N*,*N*-diisopropylethylamine (DIPEA, 99.5%), hydroquinone (99.5%), *N*-hydroxyethyl acrylamide (HEAm, contains 1000 ppm monomethyl ether hydroquinone as stabilizer, 97%), 2-hydroxyethyl disulfide (technical grade, 96%), 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%) was obtained from APICHEM. Silica (0.020−0.045 mm) was purchased from Davisil. AMPS was diluted 4-fold in water and freeze-dried to yield a white powder prior for use in polymerizations. HEAm was passed over a column of inhibitor removers prior to use. All monomers, monomer precursors, chain transfer agent, and V-501 were stored in the freezer (−18 °C) prior to use. In-house deionized water was used for all reactions and chemical product washings. Phosphate buffered saline (10 mM PBS including 138 mM NaCl, 2.7 mM KCl) was made through dissolving one pouch of PBS powder (Sigma-Aldrich) in deionized water (1 L) at 25 °C. Similarly, to obtain 10 mM PBS (including 138 mM NaCl, 2.7 mM KCl) at pH 5.8, a pouch of PBS powder (Sigma-Aldrich) was dissolved in deionized water (950 mL), pH adjusted using an aqueous solution of HCl (5 M), and topped up with deionized water in a 1 L volumetric flask.

Synthesis Methods. *2-((2-Hydroxyethyl)disulfanyl)ethyl Acrylate (**1**).* Acryloyl chloride (5 g, 0.056 mol, 1.0 equiv) was diluted in CH_2Cl_2 (20 mL) and added dropwise over 30 min to a solution consisting of dithiodiethanol (19.0 g, 0.092 mol, 1.63 equiv) and triethylamine (11.2 g, 0.111 mol, 2.0 equiv) in CH_2Cl_2 (100 mL). The mixture was stirred at room temperature for 16 h. The reaction was quenched by vigorous stirring with a saturated NH_4Cl solution (100 mL). The CH_2Cl_2 phase was collected and washed with H_2O (2-fold, 200 mL) where the aqueous phase was re-extracted against CH_2Cl_2 (100 mL). The CH_2Cl_2 phase was collected and pooled with other collected CH_2Cl_2 phase. The CH_2Cl_2 phase is finally washed with brine, dried over $MgSO_4$, and filtered. 2-((2-Hydroxyethyl)disulfanyl)ethyl acrylate (**1**) was purified via flash silica chromatography using pentane/ethyl acetate (1/1 v/v) as the eluent. R_f: 0.38. Hydroquinone (3 mg) was added to prevent polymerization. A colorless viscous liquid was obtained upon concentration via rotary evaporation. The product was stored at −18 °C and can be stored for a week without transesterification occurring. Yield: 42−52% (based on different batches). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.38 (dd, J = 17.3, 1.4 Hz, 1H), 6.08 (dd, J = 17.3, 10.5 Hz, 1H), 5.82 (dd, J = 10.5, 1.4 Hz, 1H), 4.40 (t, J = 6.7 Hz, 2H), 3.65 (q, J = 5.6 Hz, 2H), 2.92 (t, J = 6.7 Hz, 2H), 2.83 (t, J = 5.9 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166, 131.6, 128.2, 62.6, 60.4, 41.7, 37.1. Refer to the Supporting Information for NMR spectra and assignments (Figure S1).

*2-((2-(((4-Nitrophenoxy)carbonyl)oxy)ethyl)disulfanyl)-ethyl Acrylate (**2**).* Compound **1** (3.0 g, 0.0144 mol, 1.0 equiv) was dissolved in CH_2Cl_2 (40 mL) and was followed by the addition of triethylamine (2.91 g, 0.0287 mol, 2.0 equiv). 4-Nitrophenyl chloroformate (3.18 g, 0.015 mol, 1.1 equiv) was dissolved in CH_2Cl_2 (30 mL) and added dropwise over 30 min to the solution containing **1** and triethylamine. The solution turned yellow as it was stirred at room temperature. After 4 h, the solution was quenched with saturated NH_4Cl solution (2-fold, 100 mL). The CH_2Cl_2 phase is collected and subsequently washed against brine (100 mL). A colorless viscous liquid was obtained after flash silica chromatography using CH_2Cl_2/pentane (8/2 v/v) as eluent. R_f: 0.28. Rotary evaporation yielded **2** as a colorless viscous liquid. The product is stored at −18 °C. Yield: 64−75% (based on different batches). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (d, J = 9.2 Hz, 2H), 7.35 (d, J = 9.2 Hz, 2H), 6.38 (dd, J = 17.3, 1.4 Hz, 1H), 6.08 (dd, J = 17.3, 10.5 Hz, 1H), 5.82 (dd, J = 10.5, 1.4 Hz, 1H), 4.50 (tr, J = 9.5 Hz, 2H), 4.40 (t, J = 6.6 Hz, 2H), 3.00 (t, J = 6.5 Hz, 2H), 2.96 (t, J = 6.6 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166, 155.5, 152.4, 145.6, 131.6, 128.1, 125.5, 121.9, 66.9, 62.5, 37.3, 36.9.

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Refer to the Supporting Information for NMR spectra and assignments (Figure S2).

*2-((2-(((Lamivudine)carbonyl)oxy)ethyl)disulfanyl)ethyl Acrylate (**3**).* Lamivudine (10.1 g, 0.044 mol, 1.1 equiv), *N*,*N*-diisopropylethylamine (DIPEA, 10.4, 0.080 mol, 2 equiv), and 4-(dimethylamino)pyridine (DMAP, 0.492 g, 0.004 mol, 0.1 equiv) were dissolved in dimethylformamide (DMF, 50 mL). In a separate vessel, **2** (15 g, 0.04 mol, 1.0 equiv) was dissolved in DMF (50 mL) and added dropwise to the mixture containing lamivudine, DIPEA and DMAP. The reaction was allowed to stir at room temperature for 16 h, which yielded a yellow solution. This solution was dried down using a stream of nitrogen, which resulted in a yellow residue. Further drying was achieved by placing the flask under high vacuum or by precipitation in diethyl ether (100 mL). The yellow residue was redissolved in CH_2Cl_2/methanol (9/1 v/v) and purified via silica chromatography using CH_2Cl_2/methanol (9/1 v/v) as the eluent. A viscous, faint yellow product **3** was isolated after concentration via rotary evaporation. R_f: 0.41 (R_f 0.58 is 4-nitrophenol and R_f 0.12 is unreacted lamivudine). The product is stored at −18 °C. Yield 88%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (d, J = 7.6 Hz, 2H), 6.82, (br m, 2H), 6.43 (dd, J = 17.3, 1.4 Hz, 1H), 6.32 (tr, J = 5.2 Hz, 1H), 6.11 (dd, J = 17.3, 10.5 Hz, 1H), 6.00 (d, J = 7.4, Hz, 1H), 5.85 (dd, J = 10.4, 1.4 Hz, 1H), 5.36 (dd, J = 4.8, 3.3 Hz, 1H), 4.54 (m, 2H), 4.48−4.40 (overlapping m, 4H), 3.53 (dd, J = 11.9, 5.3 Hz, 1H), 3.12 (dd, J = 11.8, 5.0 Hz, 1H), 2.89 (overlapping m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.2, 164.7, 154.8, 154.7, 141.4, 131.8, 128.1, 95.0, 87.7, 83.4, 67.4, 66.3, 62.5, 38.5, 37.4, 37.2, 36.7. HRMS (APCI) $[M + H]^+$ $C_{16}H_{22}O_7N_3S_3$ calculated 464.0614, found 464.0618. Refer to the Supporting Information for NMR spectra and assignments (Figure S3 and Figure S4).

2-(((Butylthio)carbonothioyl)thio)propanoic Acid Chain Transfer Agent (CTA). The chain transfer agent (CTA) 2-(((butylthio)carbonothioyl)thio)propanoic acid was synthesized using butanethiol, carbon disulfide, and 2-bromopropionic acid, adapted as from Ferguson et al.[30] Briefly, butanethiol (24.5 g, 271 mmol) was added to a 1 L round-bottom flask containing water (34 mL) and NaOH solution (22 mL of 50 w/v% in H_2O, 275 mmol). Acetone (8 mL) was added followed by carbon disulfide (21.1 g, 277 mmol), and the flask was stirred on ice for 30 min. 2-Bromopropionic acid (42.3 g, 277 mmol) followed by NaOH solution (22 mL of 50% w/v in H_2O, 275 mmol) was added dropwise to the reaction solution at a rate such that the internal temperature of the reaction solution did not exceed 30 °C. After 1 h on ice, water (34 mL) was added to the reaction mixture, and the flask was stirred at room temperature for 16 h. Once again, the reaction flask was placed on ice and fuming HCl (60 mL) was added yielding an oil, which crystallizes into clumps of yellow product. The clumps are filtered by suction filtration and washed with cold water (500 mL). The product (52.1 g) was obtained by recrystallization from heptane (140 mL) to yield bright yellow crystals. Yield 80.3%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.84 (q, J = 7.4 Hz, 1H), 3.35, (t, J = 7.5 Hz, 2H), 1.67 (quin, J = 9.0 Hz, 2H), 1.61 (d, J = 7.4 Hz, 3H), 1.42 (sex, J = 7.6 Hz, 2H), 0.92 (t, J = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 222.1, 176.8, 47.6, 37.3, 30.0, 22.2, 16.8, 13.7. HRMS (ESI) $[M + Na]^+$ $C_8H_{14}O_2NaS_3$ calculated 261.0048, found 261.0048. Refer to the Supporting Information for NMR spectra and assignments (Figure S5).

General Polymerization Procedure. All polymerizations were performed in a Biotage Initiator Microwave apparatus equipped with a Robot Eight. The polymerization feed (Table S1) consisting of the 2-(((butylthio)carbonothioyl)thio)-propanoic acid (CTA) and 4,4′-azobis(4-cyanopentanoic acid) (V-501) as the azo-initiator as well as the monomers HEAm and/or AMPS and/or 3TC self-immolative acrylate (**3**) and solvent (DMF or DMF/H_2O mixtures (1/1 v/v or 1/9 v/v, respectively)) were added to a 5 mL microwave vial. In order to add accurate amounts of the CTA and V-501, stock solutions at 10 and 40 mg/mL, respectively, were prepared in DMF or DMF/H_2O mixtures (1/1 v/v or 1/9 v/v). The amount of the V-501 was adjusted for all polymerizations as described in Table S1 to minimize termination of the polymer chains as well as avoiding excessive amounts of initiator derived chains. The vial was capped and sealed, and the contents were degassed by nitrogen gas bubbling for 5 min. The vial was then placed in the Biotage microwave apparatus and programmed to maintain temperature at 75 °C with stirring at 600 rpm for the time of polymerization described in Table S1.

After completion, the polymerization solution was cooled to room temperature and aerated by removing the vial cap. A 30 μL sample was removed for ^{1}H NMR conversion analysis, and a 10 μL sample was removed for GPC analysis. The polymers were dissolved in methanol/H_2O (2/8 v/v) and purified by dialysis using regenerated cellulose snakeskin membranes (MWCO 3.5 kDa) or cellulose acetate Float-a-Lyzer membranes (MWCO 1 kDa) over 3 days. The polymers were freeze-dried yielding pale yellow powders. The freeze-dried polymers were then analyzed by NMR in DMSO-d_6 or D_2O and GPC analysis to confirm that no adverse changes to the polymers occurred during dialysis and freeze-drying process.

In Vitro Lamivudine Release from Polymers. The lamivudine functionalized polymers **P6** and **P9** were each dissolved in 10 mM PBS, pH 7.4 (or pH 5.8 in the control experiments, see Supporting Information). After dissolution, 5 mM GSH was added to the polymer solution to yield final polymer concentration of 1 mg mL^{-1}. This solution was then immediately transferred to a Spectra/Por Float-A-Lyzer dialysis tube (MWCO 3.5−5.0 kDa, volume = 1.0 mL). The dialysis tubes were incubated in 6 mL of 10 mM PBS, pH 7.4 + 5 mM GSH at 20 °C, and the release of lamivudine in the dialysate was monitored by UV−vis at 271 nm (ε_{271} = 9852 M^{-1} cm^{-1}). Concomitantly, control experiments were performed as described above where the polymers are incubated under the same conditions as described except that GSH was not added to the solution in the dialysis tube or to the dialysate. The amount of lamivudine was quantified spectrophotometrically for all samples using an extinction coefficient of lamivudine at 271 nm (ε_{271} = 9852 M^{-1} cm^{-1}), which was predetermined from a dilution series of lamivudine in 10 mM PBS, pH 7.4.

The percentage of lamivudine released from the polymer was calculated as follows:

$$\%3TC\ released = (N_{3TC,dialysate}/N_{3TC,polymer}) \times 100 \quad (1)$$

where $N_{3TC,dialysate}$ is the amount (in nmol) of lamivudine in the dialysate and $N_{3TC,polymer}$ is the amount (in nmol) of lamivudine present in the solution containing either polymer **P6** or **P9** prior to GSH-induced release.

Polymer Characterization. Gel permeation chromatography (GPC) for polymers **P1**−**P8**, **P10**−**P12**, and **P19**−**P22** was performed on Shimadzu system equipped with a CMB-20A controller system, a SIL20 HT autosampler, a LC-20AT tandem pump system operating at a combined flow rate of 1 mL·min^{-1}, a DGI-20A degasser, a CTO-20AC column oven

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Table 1. Composition and Properties of RAFT Polymers Synthesized

	conv., p (%)[a]			polymer composition[b]						
polymer	HEAm	AMPS	3TC	HEAm	AMPS	3TC	$M_{n,\,th}$[c] (g·mol^{-1})	$M_{n,\,NMR}$[d] (g·mol^{-1})	$M_{n,\,GPC}$[e]	M_w/M_n[e]
P1	24.0	0	35.0	110	0	13	16700	18900	25000	1.16
P2	22.0	0	42.0	63	0	7	10800	10700	19100	1.12
P3	22.0	0	38.0	25	0	3	4300	4500	10200	1.09
P4	16.0	0	11.0	9	0	1	1300	1700	6300	1.05
P5	17.4	0	5.3	107	0	10	11700	17200	22600	1.18
P6	78.0	0	31.4	327	0	15	40400	44800	57700	1.21
P7	44.3	0	16.0	104	0	13	22500	18200	16300	1.17
P8	50.9	59.4	54.4	91	105	8	42300	38500	44100	1.19
P9	38.0	55.6	53.3	121	130	17	40900	51800	24400[f]	1.28[f]
P10	27.5	45.2	34.5	54	50	7	19500	21200	20200	1.20
P11	21.3	39.3	26.9	16	18	2	6600	7100	11800	1.13
P12	10.6	36.9	21.3	5	5	1	2900	2400	7200	1.07
P13	86.3	93.5	0	182	217	0	73200	70900	68400[f]	1.20[f]
P14	90.0	86.7	0	171	209	0	70500	67800	73900[f]	1.09[f]
P15	93.5	94.4	0	98	113	0	32600	37400	37200[f]	1.07[f]
P16	0	98.2	0	0	369	0	90300	84820	129100[f]	1.11[f]
P17	0	97.1	0	0	206	0	44800	47500	51200[f]	1.11[f]
P18	0	88.8	0	0	43	0	10400	10100	12400[f]	1.11[f]
P19	86.0	0	0	483	0	0	60600	55800	137600	1.19
P20	91.0	0	0	169	0	0	21200	19700	47000	1.10
P21	94.0	0	0	111	0	0	11100	13000	27700	1.08
P22	96.0	0	0	59	0	0	5800	7000	15200	1.07

[a]Conversion, p relative to trioxane of the monomers *N*-hydroxyethyl acrylamide (HEAm), 2-acrylamido-2-methylpropane sodium sulfonate (AMPS), and the 3TC SIL acrylate. [b]The number of units of HEAm, AMPS, and 3TC SIL acrylate determined by using the ω-chain terminal $-CH_3$ group. [c]Theoretical molar mass, $M_{n,th} = (p_{HEAm}\cdot m_{HEAm}\cdot M_{HEAm}) + (p_{AMPS}\cdot m_{AMPS}\cdot M_{AMPS}) + (p_{AMPS}\cdot m_{AMPS}\cdot M_{AMPS}) + M_{CTA}$, where p is the monomer conversion (in %); m_{HEAm}, m_{AMPS}, and m_{3TC} are the mole feed ratio of the monomers relative to the CTA; M_{HEAm}, M_{AMPS}, M_{3TC} and M_{CTA} are the relative molar mass of the monomers and the CTA, respectively. [d]Molar mass determined by using the α-chain terminal $-CH_3$ group using equations S3−S7 (Supporting Information). [e]Molar mass (in polystyrene equivalents) and dispersity obtained by DMAc GPC using PS standards calibration set. [f]Molar mass and dispersity obtained by aqueous GPC via light scattering detection. The dn/dc for **P9** is 0.1305, **P13** is 0.108 mL mg^{-1}, **P14** is 0.1395 mL mg^{-1}, **P15** is 0.1239 mL mg^{-1}, **P16** is 0.1000 mL mg^{-1}, **P17** is 0.1191 mL mg^{-1}, and **P18** is 0.1226 mL mg^{-1}, assuming full mass recovery.

operating at 80 °C, and a RDI-10A refractive index detector. The samples were run in *N*,*N*-dimethylacetamide (DMAc) containing 0.1% LiBr over four Waters Styragel columns (HT5, HT4, HT3, and HT2 each 300 mm × 7.8 mm). Number-average molar mass (M_n) and weight-average molar mass (M_w) were determined against polystyrene standards ranging from 4430−3242000 g·mol^{-1} using the LabSolutions postrun analysis software (version 5.63). Chromatograms are presented in the Supporting Information (Figures S6−S8).

For polymers **P9**, **P13**−**P18**, a GPC 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 provided an effective molecular weight range of 1000−1000000. The solvent used for the analysis was 0.01 M phosphate buffer with 300 ppm sodium azide. The results of the GPC analysis are presented in Table 1. Chromatograms are presented in the Supporting Information (Figure S9).

NMR Spectroscopy. Both ^{1}H and ^{13}C NMR were recorded on a Bruker Avance 400 MHz spectrometer using a relaxation delay, d1 of 10 s. All spectra were measured baseline corrected and analyzed using TopSpin software (version 3.1).

Monomer to polymer conversions were based on the vinyl peaks corresponding to HEAm, AMPS, and **3** relative to trioxane as an internal standard. In all spectra the integration of trioxane at 5.17 ppm peak was set at 1 and the integrations of a vinyl peak of each monomer (e.g., HEAm at 5.57 ppm, AMPS at 5.50 ppm, and **3** at 5.92 ppm) was recorded at time zero (t_0) and at final polymerization time (t_F). The polymerization conversion for these monomers is as presented in Table 1. For full details of the calculation of conversion (p), theoretical number-average molar mass ($M_{n,th}$) and theoretical number-average molar mass determined by $-CH_3$ end group ($M_{n,NMR}$), please refer to the Supporting Information. Representative ^{1}H NMR spectra of the five classes of polymers in this contribution are shown in the Supporting Information (Figures S10−S14).

Inhibition of HIV-1 Replication Assay. HeLa-derived TZM-bl cells (obtained from NIH AIDS Reagent Program from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc., catalog no. 8129)[31−33] were used to evaluate HIV infectivity. TZM-bl cells express the HIV receptor CD4 and coreceptors CCR5 and CXCR4 and contain a luciferase/β-galactosidase reporter system under the control of the HIV-1 long terminal repeats (LTRs). 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 flasks (Nunc, Roskilde, Denmark) at 37 °C with 5% CO_2.

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Mol. Pharmaceutics 2016, 13, 2397−2410

HIV-1 strain BaL was generated by transfection of HEK293T cells using lipofectamine 3000 reagent (Life Technologies). Briefly, HEK293T cells were seeded at 7×10^4 per cm^2 on T75 bottle (Nunc, Roskilde, Denmark). After overnight culture, cells were transfected with 15 μg of HIV-1 plasmid (NIH AIDS Research and Reference Reagent Program, Bethesda, USA, pWT/BAL, catalog no. 11414)[34] according to the protocol provided by manufacturer. Twenty-four hours after transfection 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. Figure S15 shows the TCID50 obtained with a titration of 3TC on TZM-bl cells incubated with HIV-1 BaL.

Analyzed polymers were dissolved by adding DMSO and then PBS to final concentration of 10 g/L. Volume of DMSO was equivalent of 5% of total volume of PBS added. Polymer solutions were stored in aliquots at −20 °C.

For the assay TZM-bl cells were seeded in 96-well flat-bottomed culture plates (Sarstedt) at a density of 1×10^4 cells per well and cultured overnight. Cells were preincubated with polymers at indicated concentrations for 24 h and then infected with HIV-1 BaL (12 × TCID50). After 48 h, media was removed, and cells were incubated with 90 μL of 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 using 90 μL of britelite plus reagent (PerkinElmer, Skovlunde, Denmark) per well. After mixing, 150 μL of the solution was transferred to the white 96-well plates (PerkinElmer, Skovlunde, Denmark). Luciferase activity was quantified by measuring luminescent signal with BritelitePlus on a FLUOstar Omega plate reader (BMG Labtech, Ortenberg, Germany). A negative control (PBS) was included in the assays to ensure infectivity was achieved.

Statistical analysis was performed in Graphpad Prism for Mac OS X v. 6.0. Multiple unpaired *t* tests were performed between the concentrations for each of the polymers. Statistical significance determined was without correction for multiple comparisons, with $\alpha = 5.000\%$, meaning significance $p < 0.05$. Each row was analyzed individually, without assuming consistent SD of the groups.

Inhibition of DNA−DNA Polymerase. To analyze inhibitory effect of polymers on DNA−DNA polymerase activity, real time quantitative PCR (qPCR) reaction was performed using Power SYBR Green PCR Master Mix (Life Technologies) according to the protocol provided by the manufacturer. For one sample 10 μL of Green PCR Master Mix was mixed with forward 5′-GGTCTCTCTGGTTAGACC-AGAT-3′ and reversed primer 5′-CTGCTAGAGATTTCCAC-ACTG-3′, 1.8×10^6 copies of pHXB2-env plasmid (NIBSC, Programme EVA Centre for AIDS Reagents, reference number: ARP206), and a polymer diluted in PBS at concentration 100 $mg \cdot L^{-1}$. Primers were complementary to LTR upstream element of gag. The program used was 95 °C for 5 min followed by 45 cycles with 95 °C for 12 s and 62 °C for 26 s, and during the last cycle, a melting curve was made. A level of inhibition of Taq polymerase was calculated by comparing to a positive control without polymer as 100% of enzyme activity.

Inhibition of RNA−DNA Polymerase. Influence of polymers on activity of reverse transcriptase was determined using EnzChek Reverse Transcriptase Assay Kit (Life Technologies). The reaction mixture was prepared according to the protocol provided by the manufacturer. The polymers diluted in PBS were subsequently added at concentration 10 $mg \cdot L^{-1}$, and then 5 units of MuLV Reverse Transcriptase (Life Technologies, Cat. Nr. N8080018) was added to each sample. The reaction was performed at 37 °C for 1 h. Nucleic acids were stained with PicoGreen dye, and fluorescence was measured on a plate reader (BMG Labtech, Ortenberg, Germany). A level of inhibition of reverse transcriptase was calculated by comparing each sample to a positive control without polymer as 100% of enzyme activity.

RESULTS AND DISCUSSION

Polymer Design and Synthesis. Inhibition of HIV by polyanionic compounds is a well-documented phenomenon.[4,22,25,35−37] Compounds such as sulfated polysaccharides undergo electrostatic interactions with the positively charged V3 loop located on the HIV envelope glycoprotein gp120. These electrostatic interactions with the envelope glycoprotein in turn restricts binding between the virus and host cell and ultimately reduces the infectivity of HIV.[38] In addition, polyanionic compounds exhibit precedence for kinase-independent inhibition of HIV reverse transcriptase. Mitsuya et al. demonstrated that dextran sulfate completely suppressed reverse transcriptase activity at >1 μM.[25] Similarly Witvrouw et al. showed that dendrimers functionalized with sulfonate and carboxylate groups exhibited inhibitory activity against reverse transcriptase with IC_{50} values at 0.2 μg/mL and 17.1 μg/mL, respectively.[26] Moreover, with the polymers consisting of pendent sulfonate moieties and pendent lamivudine (3TC) self-immolative moieties we hypothesized that three modes of anti-HIV activity could be exhibited including (1) entry inhibition, (2) kinase-independent inhibition of the HIV reverse transcriptase moieties, and (3) kinase-dependent inhibition of the HIV reverse transcriptase mediated by the release of 3TC (Figure 1).

The 3TC has proven to be an effective reverse transcriptase inhibitor against HIV. However, several severe adverse side effects such as lactic acidosis and hepatomegaly including fatal cases have been reported as a consequence of daily 300 mg 3TC administration.[39] Therefore, the polymer bound 3TC was envisioned to lead to a decreased 3TC elimination rate, making less frequent dose administrations possible and hence lowering occurrence of adverse effects associated with administration. Although the polymers designed here are envisioned for topical administration as virustats, a gradual 3TC prodrug release would also lead to a slow uptake into the bloodstream and therefore should also lead to a decreased occurrence of adverse effects.

The synthesized 3TC self-immolative acrylate (**3**) consists of a disulfanylethyl carbonate moiety (also known as the disulfide self-immolative linker (SIL)), which can undergo self-immolation under reductive conditions.[15,40,41] In addition, an acrylate based monomer was judiciously synthesized as it facilitated the copolymerization compatibility with the 2-acrylamido-2-methylpropane sodium sulfonate (AMPS) and *N*-hydroxyethyl acrylamide (HEAm) monomers used in this work. Furthermore, acrylates and acrylamides exhibit higher polymerization rate constants than methacrylate, methacrylamide, or styrenic monomers. Thus, the acrylate/acrylamide system devised here leads to short polymerization times. As shown in Scheme 1, the self-immolative 3TC acrylate (**3**) was

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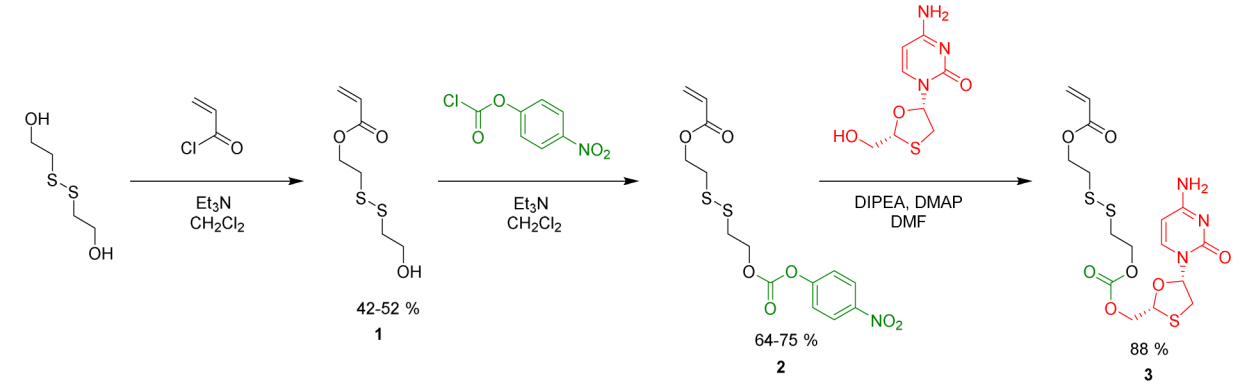
Mol. Pharmaceutics 2016, 13, 2397−2410

Scheme 1. Synthesis of the Self-Immolative Lamivudine (3TC) Acrylate



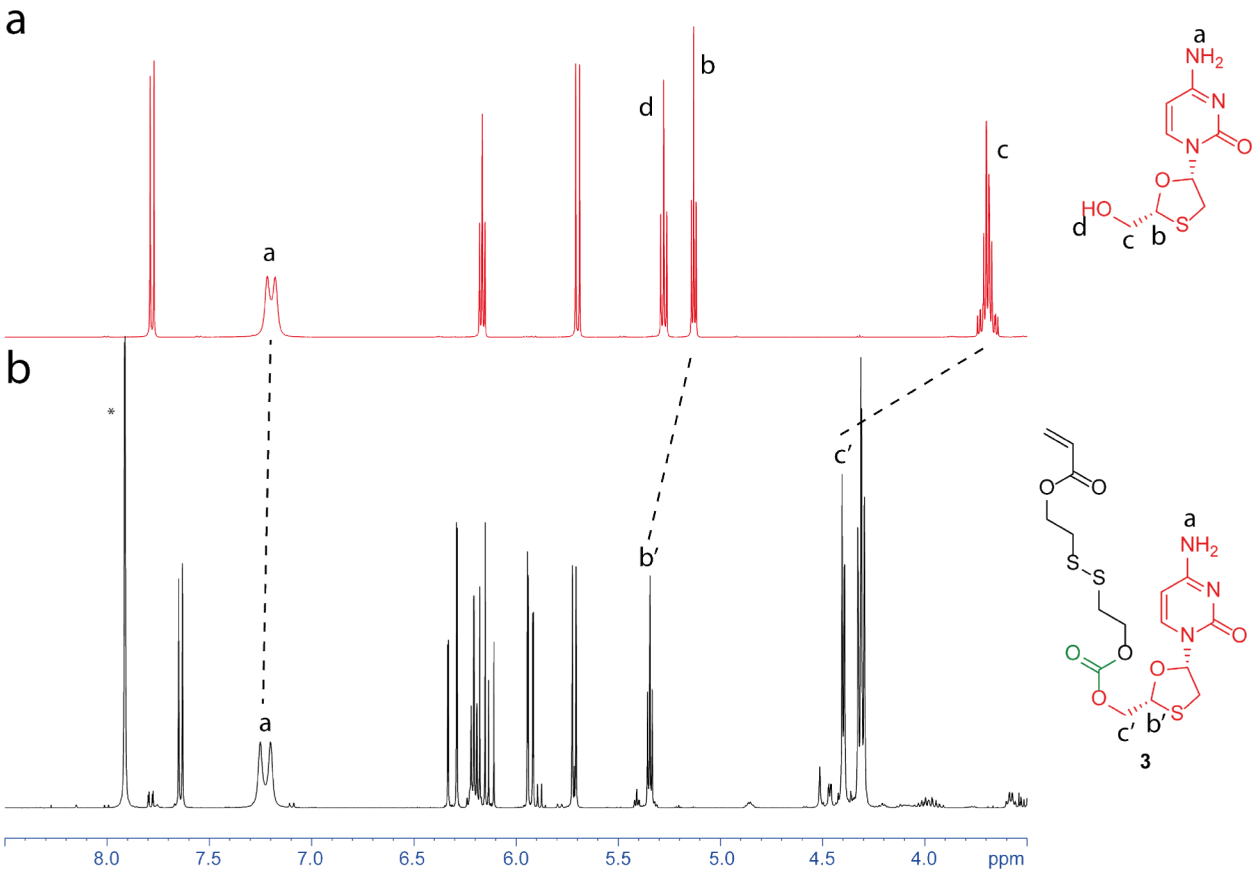


Figure 2. ^{1}H NMR of the (a) 3TC and (b) self-immolative 3TC acrylate monomer in DMSO-d_6. Verification of the product **3** was established through the disappearance of the hydroxyl group (peak **d**) and through the shift of the peaks corresponding to the $-CH_2-$ and $-CH<$ groups (peaks **c′** and **b′**, respectively). * indicates residual DMF solvent.

prepared through a three-step reaction, which involved the activation of 2-((2-hydroxyethyl)disulfanyl)ethyl acrylate (**1**) with 4-nitrophenyl chloroformate to yield the active 4-nitrophenyl carbonate (**2**) followed by a transesterification of this activated monomer with the 5′-hydroxyl on 3TC. Alternatively, the self-immolative 3TC acrylate (**3**) could be produced via 4-nitrophenyl carbonate activation at the 5′-hydroxyl of 3TC followed by transesterification with the 2-((2-hydroxyethyl)disulfanyl)ethyl acrylate (**1**). However, the synthesis route provided in Scheme 1 provided higher yields and was therefore preferred over the alternative synthesis route (Scheme S1). NMR and mass spectrometry confirmed the structure of the product, where the presence of a broad peak observed by ^{1}H NMR at 7.25 ppm indicated that the amine present on the pyrimidine ring (peak **a** in Figure 2) was untouched upon functionalization with the 4-nitrophenyl carbonate (**2**). Corroboration for the structure of the 3TC self-immolative monomer **3** was confirmed by the disappearance of the peak attributed to the hydroxyl group of 3TC (peak **d** in Figure 2a). A peak shift attributed to the methylene ($-CH_2-$) group adjacent to the hydroxyl from 3.65 to 4.40 ppm (peak **c′** in Figure 2b) as well as a peak shift from 5.15 to 5.33 ppm attributed to the $-CH<$ group (peak **b′** in Figure 2b) further confirmed attachment of the hydroxyl group to the disulfanylethyl carbonate linker.

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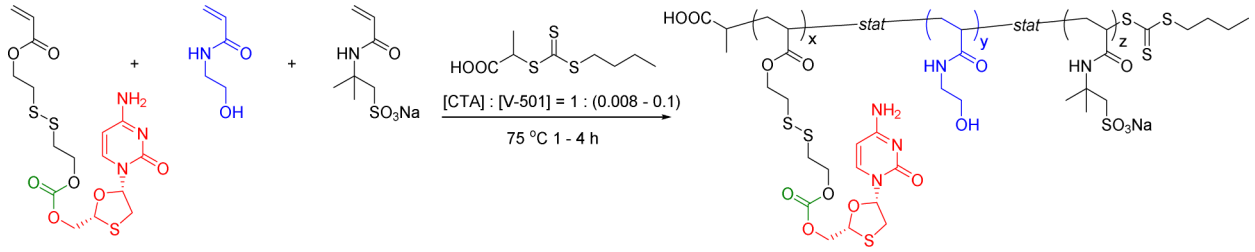
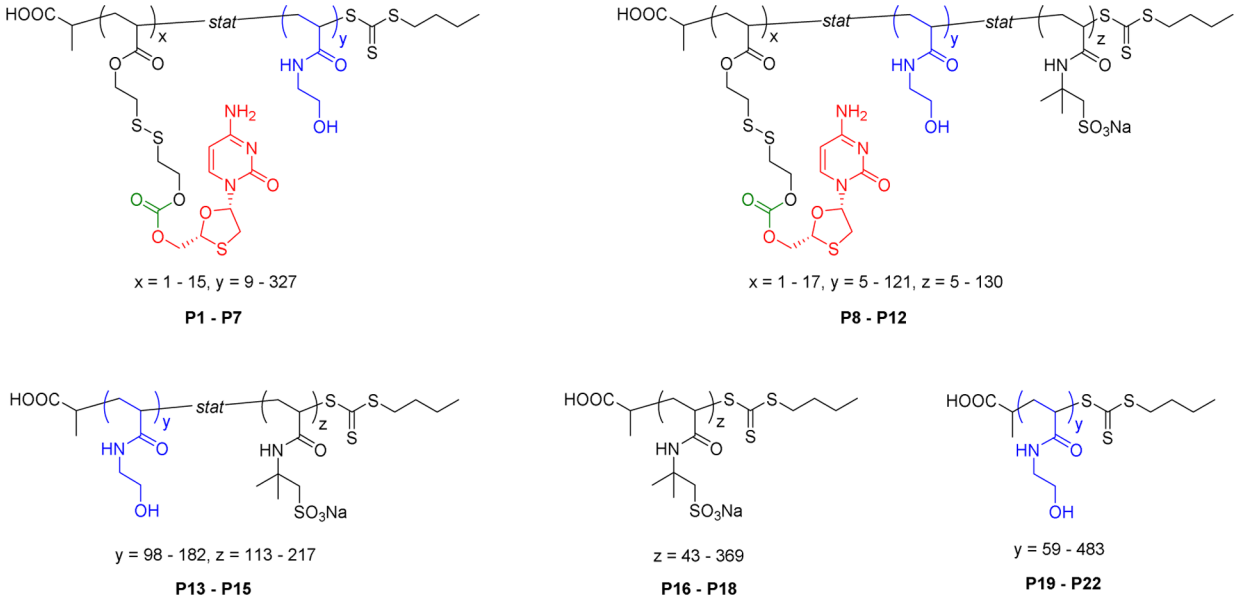
Mol. Pharmaceutics 2016, 13, 2397−2410

Scheme 2. General RAFT Polymerization Procedure



Scheme 3. Structures of the Polymers Synthesized and Assessed in This Study



As shown in Scheme 2, RAFT polymerization[27,28] was employed to create a library of polymers consisting of the pendent self-immolative 3TC linker, AMPS, and HEAm. The HEAm was incorporated into the polymer to aid water solubility as well as to provide a means to vary the degree of polymer sulfonation. We opted to use a commercially available sulfonated acrylamide monomer AMPS as it has a $pK_a \approx 2$, which is much lower than the pK_a of the carboxylic acid monomers such as acrylic acid ($pK_a \approx 4.5$) and methacrylic acid ($pK_a \approx 6.5$). Incorporation of the AMPS in the polymer would provide strong interactions with the positively charged V3 of the envelope glycoprotein gp120. Table 1 and Scheme 3 present and summarize the classes of polymers synthesized, including polymers consisting of HEAm and the self-immolative 3TC linker (**P1**−**P7**), HEAm, AMPS, and self-immolative 3TC linker (**P8**−**P12**), HEAm and AMPS (**P13**−**P15**), the poly(AMPS) controls (**P16**−**P18**), and the poly(HEAm) controls (**P19**−**P22**). We utilized the 2-(((butylthio)carbonothioyl)thio)propanoic acid chain transfer agent (CTA), which controls the polymerizations of acrylates and acrylamides very well and also provides an internal reference peak, which can be used to calculate polymerization conversions, polymer composition, and degree of polymerization.[42,43]

It must be noted that an increased degree of polymer sulfonation (i.e., incorporation of AMPS monomer) leads to an increased anticoagulant activity of blood.[23,44−46] In order to minimize this effect, polymers consisting of the pendent self-immolative 3TC linker with ∼50 mol % as opposed to copolymers with >90 mol % AMPS were synthesized. The polymers synthesized with pendent self-immolative 3TC linkers had a molar mass that ranged from 1700 to 51800 g·mol^{-1} and had exhibited low dispersities. Furthermore, all polymers were made within 1 to 4 h and employing down to 0.8 mol % of the azo-initiator V-501 to ensure a high (∼95%) fraction of living chains at the ω-chain end (Table S1). The polymer library devised permitted access to study the effects of polymer molar mass, varied degrees of pendent self-immolative 3TC, and degree of sulfonation upon anti-HIV activity.

Lamivudine Release from Polymers. GSH is a naturally occurring peptide present in the cell cytosol at concentrations ranging between 1 and 10 mM.[47] To mimic these conditions, the release of the 3TC prodrug from the polymer in response to 5 mM GSH was assessed. GSH undergoes a thiol exchange with the disulfide SIL, which prompts intermolecular cyclization to yield a cyclic thiocarbonate and concomitant release of the 3TC prodrug (Figure 3a). The release of 3TC was probed with a dialysis assay where the polymer solution in a pH 7.4 phosphate buffer (in presence or absence of 5 mM GSH) was incubated in the dialysis membrane. The dialysate was then analyzed at regular intervals for the presence of 3TC using UV−vis spectrometry at 271 nm. As Figure 3b shows, experiments with free 3TC determined that 50% of 3TC diffused into the dialysate within 160 min at pH 7.4. Accounting for this diffusion lag time yielded corrected GSH-triggered release half-lives of 340 and 640 min for non-sulfonated polymer **P6** and sulfonated polymer **P9**, respectively. The higher half-life determined for **P9** was attributed to the repulsive forces that can reduce the rate of thiol-exchange

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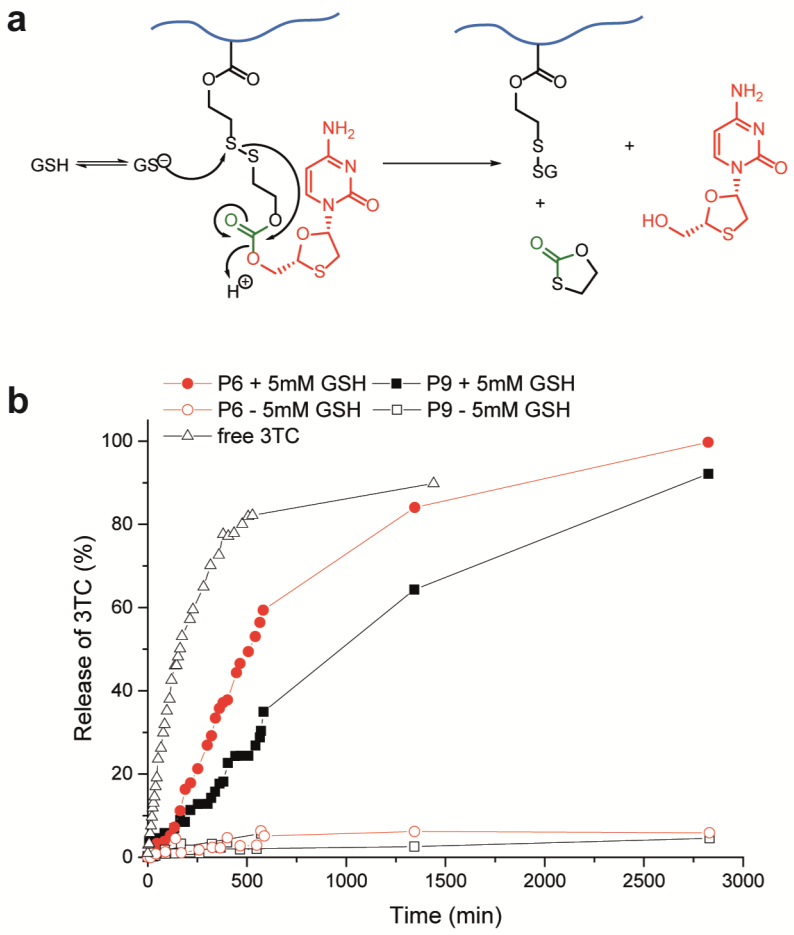

Figure 3. (a) Proposed mechanism for GSH induced cleavage of the cyclic thiocarbonate linker ultimately causing release of 3TC from polymer. (b) 3TC release from a copolymer **P6** consisting of HEAm and pendent self-immolative 3TC linker (red symbols) and terpolymer **P9** consisting of HEAm, AMPS, and pendent self-immolative 3TC linker (black symbols) in the presence (closed symbols) and absence (open symbols) of 5 mM GSH at 20 °C.

between the net negatively charged GSH and the negatively charged sulfonated side chains.

Conversely in the absence of GSH, less than 10% 3TC was released, which corroborates that the GSH trigger was required for the release of 3TC from the polymer. The amount of 3TC prodrug released in the absence of a thiol trigger was very similar to values reported for polymers consisting of ribavirin when incubated in phosphate buffer for 48 h.[48] Furthermore, control experiments performed at pH 5.8 in the presence and absence of GSH showed that only ∼10% of 3TC was released. These results indicated that the 3TC conjugated to the polymer via the disulfide SIL was stable at pH 7.4 in the absence of GSH as well as at pH 5.8 even in the presence of 5 mM GSH (Figure S16). These results indicate that the release of 3TC from the polymer takes place at physiological conditions when the thiol of GSH is slightly deprotonated. This, in turn, suggests that GSH-triggered 3TC release will take place exclusively when the polymer is present in the cell cytosol and not when taken up into the endosomes or lysosomes. Nevertheless, the presence of reductases in the endosomes and lysosomes may result in release of 3TC from the polymer. Although other studies indicated that similar disulfide SIL systems released the drug with a half-life of between 30−60 min,[15,49] we attribute the higher half-life as shown in Figure 3 to our dialysis set up to measure 3TC release. With the assay used in this contribution, the lag time requires the drug to diffuse through the pores of the dialysis membrane into the dialysate, thereby resulting in a delay before it is detected by UV−vis spectrometry. Furthermore, the assays in this article were carried out at 20 °C. The rate of 3TC release is envisioned to increase upon incubation at average body temperature (37 °C).

Antiviral Activity. The inhibitory potency of the polymers against HIV was evaluated using a TZM-bl cell assay. These TZM-bl cells were incubated with the polymers at 1, 10, and 100 mg·L^{-1} for 24 h prior to the addition of the HIV-1 BaL virus. After a further 48 h, the infectivity of HIV was measured via the TZM-bl luciferase reporter gene system, which was activated upon infectivity with HIV in the presence of luciferin and adenosine triphosphate.

As shown in Figure 4, the statistical copolymers consisting of HEAm and pendent self-immolative 3TC (**P1**−**P4**) ranging from 18900 to 1700 g·mol^{-1} with ∼10 mol % 3TC exhibited no significant difference in inhibitory potency against HIV infectivity. Furthermore, as shown by copolymers **P5**−**P7**, a decrease in 3TC mol % on the copolymer did not lead to a significant difference in inhibitory potency. This suggests that a high antiviral potency is already achieved with as little as 10 mg·L^{-1} **P6** (3.3 μM 3TC). Similarly, the sulfonated terpolymers **P8**−**P12** demonstrated an analogous trend, where the molar mass of these polymers had no significant influence on the

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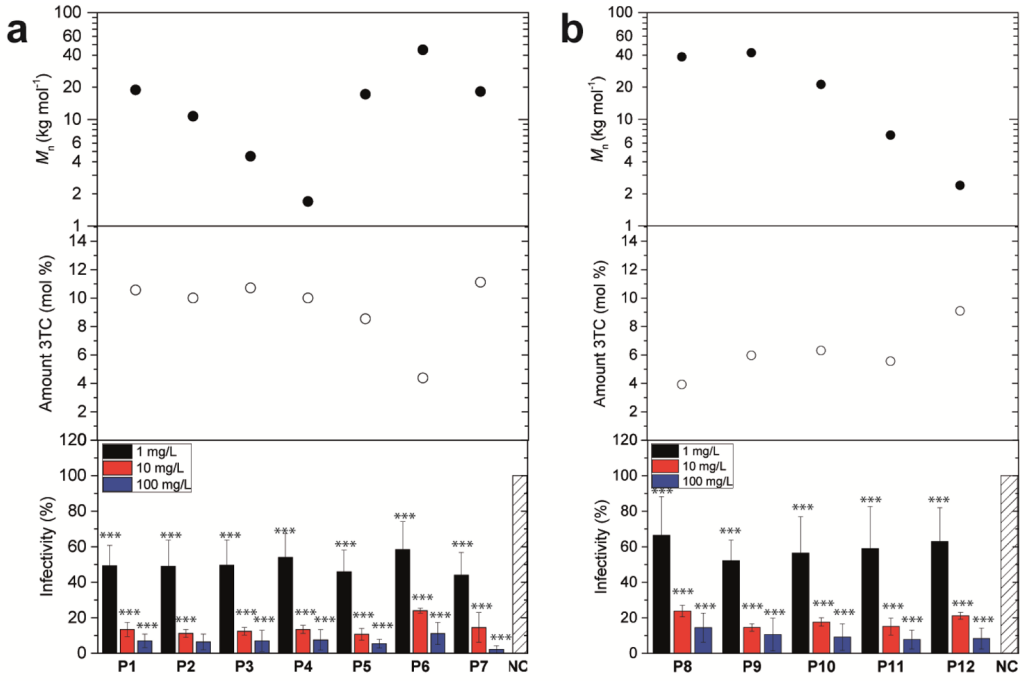

Figure 4. (a) Properties of the nonsulfonated copolymers **P1**−**P7** and their potency against HIV-1 infectivity upon incubation with 1, 10, and 100 mg·L^{-1} copolymer. (b) Properties of sulfonated terpolymers **P8**−**P12** and their potency against HIV-1 infectivity upon incubation with 1, 10, and 100 mg·L^{-1} terpolymer. n = 3 independent experiments for 100 mg·L^{-1} polymer and 10 mg·L^{-1} polymer; and n = 5 independent experiments for 1 mg·L^{-1} polymer. Statistical significance is given in relation to negative control, ***p < 0.01. NC, negative control (PBS).

inhibitory potency against HIV infectivity. For example, 10 mg·L^{-1} **P8** (2.0 μM 3TC) exhibited only 23.8 ± 3.3% HIV infectivity.

Furthermore, poly(HEAm) homopolymers exhibited no HIV inhibition (Figure S17). Comparison of the sulfonated terpolymers (**P8**−**P12**) with the nonsulfonated copolymers (**P1**−**P7**) revealed several statistical significances when analyzed at 1 mg·L^{-1} but no statistical differences in HIV inhibition for a polymer concentration at 1 mg·L^{-1} or at 100 mg·L^{-1} (Figure S18). While this is contrary to the hypothesis that polymer sulfonation increases potency against HIV infectivity, these results nonetheless suggest that the degree of sulfonation of the polymer plays an important role in HIV infectivity inhibition. Further corroboration of the lack of potency exhibited by the partial polymer sulfonation against HIV infectivity was evident from copolymers **P13**−**P15**. For these copolymers even highest polymer concentration of 100 mg·L^{-1} assayed did not exhibit significant HIV infectivity inhibition (Figure S19). Conversely, the poly(AMPS) homopolymers (**P16**−**P18**) did exhibit inhibitory potency against HIV infectivity and therefore suggests that the degree of polymer sulfonation plays a vital role in inhibitory potency against HIV. Although the higher molar mass polymers (**P16** and **P17**) displayed lower potencies against HIV infectivity compared with poly(AMPS) 12400 g·mol^{-1} (**P18**) or with copolymers containing pendent self-immolative 3TC (**P1**−**P12**) at 1 mg·L^{-1}, near complete HIV infectivity inhibition was observed for copolymers **P17** and **P18** for concentrations above 10 mg·L^{-1} (Figure S20). The lack of activity against HIV infectivity exhibited by copolymers **P13**−**P15** and the absence of increased potency expected from the sulfonated terpolymers **P8**−**P12** was attributed to the partial (~50%) degree of sulfonation. By contrast, Baba et al. found that 50% sulfation of poly(vinyl alcohol) polymers of ~20000 g·mol^{-1} exhibited an IC_{50} value of 0.18 μg·mL^{-1} against HIV-1 when assayed with MT4 cells.[23] Similarly, partially sulfated poly(maltoheptaose methacrylate) exhibited HIV inhibitory potencies ranging from 66 to 0.3 μg·mL^{-1} for polymers ranging from 276000−20000 g·mol^{-1}, respectively.[50] Interestingly, Kiser and co-workers described synergistic effects with a more than 2 log decrease in IC_{50} with copolymers consisting of AMPS, benzoboroxole methacrylamide, and *N*-2-hydroxypropyl methacrylamide.[21] While structural differences exist between the polymers made here compared to polymers assayed elsewhere, the main reason for the absence of inhibition potencies of the partially sulfonated polymers **P13**−**P15** and the lack of synergy of the sulfonated polymers with 3TC self-immolative linker (**P8**−**P12**) up to 100 mg·L^{-1} against HIV remains to be investigated. Nevertheless, the partially and fully sulfonated copolymers presented here make excellent antiviral candidates, which could be formulated as virustats that prevent the mucosal transmission of HIV.

Prior art has suggested that polyanionic compounds exhibit inhibitory activity against polymerases,[25,26] including DNA−DNA polymerases and RNA−DNA polymerase (also known as reverse transcriptase, RT). Identification of the polymers described here that display activity against either of these types polymerases would not only permit access to polymer therapeutics against HIV RT only but also open avenues to devising new sulfonated polymer therapeutics against other DNA and RNA viral polymerases and thus against other viruses as well. Furthermore, these assays have important implications because, unlike reverse transcriptase inhibitors such as 3TC, inhibition exhibited by the sulfonated polymers against polymerase activity are kinase independent and do not require (3×) phosphorylation to become active. This implies that any toxicity due to ATP depletion as a result of red blood cell uptake associated with antiviral nucleoside-type drug admin-

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istration (e.g., with ribavirin)[51] may be evaded with the use of sulfonated polymer therapeutics described herein.

Efficacy of the polymers against the activity of DNA−DNA polymerase was assessed with the quantitative PCR (qPCR) reaction of the HIV-1_{HXB2} plasmid using *Thermus aquaticus* (Taq) polymerase. As shown in Figure 5a, only the sulfonated

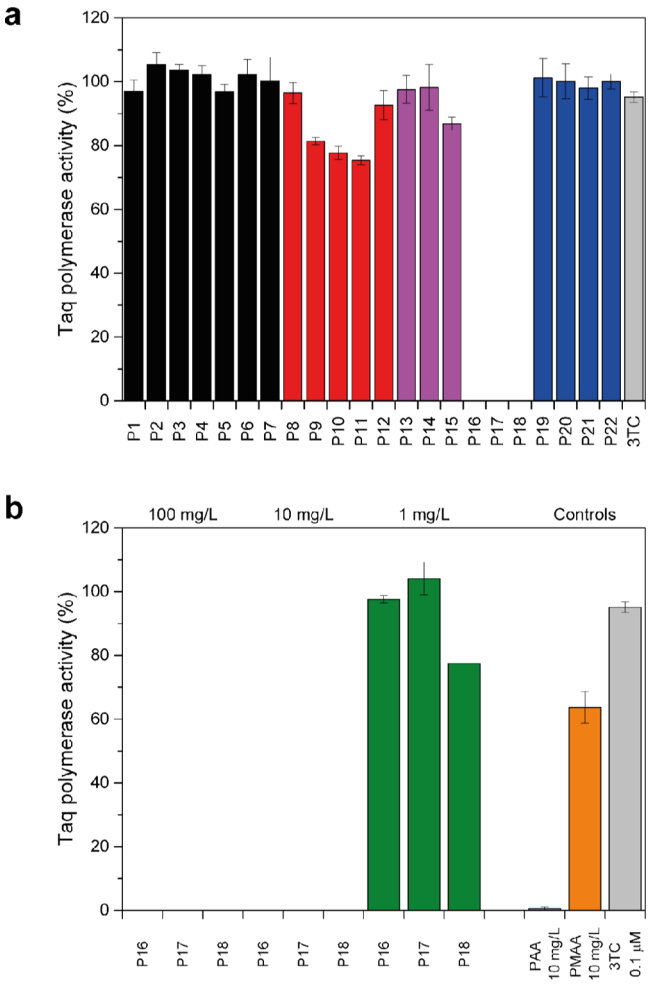

Figure 5. (a) Influence of DNA−DNA polymerase activity upon incubation with 100 mg·L^{-1} polymers. (b) Inhibition of polymerase activity at 100, 10, and 1 mg·L^{-1} of sulfonated polymers **P16−P18** compared to 10 mg·L^{-1} poly(acrylic acid) (PAA), 10 mg·L^{-1} poly(methacrylic acid) (PMAA), and 0.1 mM 3TC. Color coding: black, copolymers consisting of HEAm and self-immolative 3TC acrylate (**P1−P7**); red, terpolymers consisting of self-immolative 3TC acrylate, HEAm, and AMPS (**P8−P12**); purple, copolymers consisting of HEAm and AMPS (**P13−P15**); green, AMPS homopolymers (**P16−P18**); blue, homopolymers of HEAm (**P19−P22**); cyan, PAA homopolymer; orange, PMAA homopolymer; gray, 3TC.

homopolymers (**P16−P18**) exhibited complete inhibition of the DNA−DNA polymerase. Although the partially sulfonated ter- and copolymers (**P8−P15**) reduced the DNA−DNA polymerase activity to ∼80% at 100 mg·L^{-1}, the inhibitory effect was not as profound as with the sulfonated homopolymers (**P16−P18**). Figure 5b confirms that the inhibition of polymerase activity at 10 mg·L^{-1} of the sulfonated homopolymers (**P16−P18**) was comparable to poly(acrylic acid) (PAA) at 10 mg·L^{-1}. By contrast, the DNA−DNA polymerase only exhibited ∼60% activity in the presence of 10 mg·L^{-1} poly(methacrylic acid) (PMAA), which thereby confirms that polymer pK_a plays a significant role in DNA−DNA polymerase inhibition. It must be noted that 3TC does not exhibit any activity against the DNA−DNA polymerase (Figure 5) due to the absence of a kinase enzyme, which catalyzes the (3×) phosphorylation of the 3TC prodrug.

Next, the inhibitory activity of the polymers against the RNA−DNA polymerase (RT) was tested. As shown in Figure 6, the copolymers consisting of HEAm and pendent self-

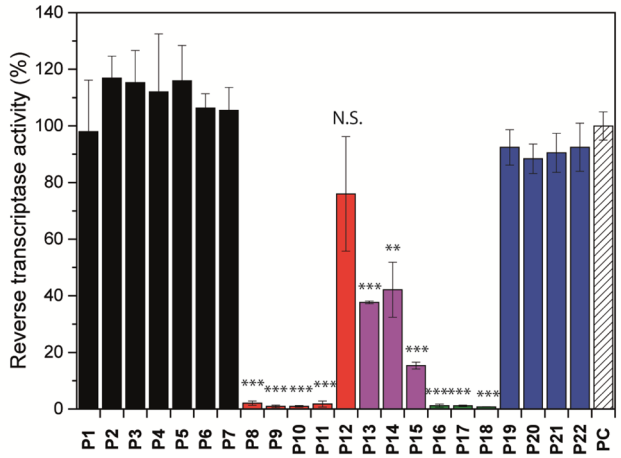

Figure 6. Reverse transcriptase inhibition induced by 10 mg·L^{-1} polymers. Color coding: black, copolymers consisting of HEAm and self-immolative 3TC acrylate (**P1−P7**); red, terpolymers consisting of 3TC self-immolative 3TC acrylate, HEAm, and AMPS (**P8−P12**); purple, copolymers consisting of HEAm and AMPS (**P13−P15**); green, AMPS homopolymers (**P16−P18**); blue, homopolymers of HEAm (**P19−P22**). 3TC is omitted from this graph as it does not exhibit antireverse transcriptase activity in the absence of a kinase. n = 3 independent experiments. Statistical significance is given in relation to positive control (no added polymer). N.S. not significant, $^{**}p < 0.02$, $^{***}p < 0.01$.

immolative 3TC exhibited no inhibition activity against RT. The polymers consisting of sulfonated ter- and copolymers (**P8−P18**) did, however, exhibit inhibitory RT activity at 10 mg·L^{-1}. Interestingly, terpolymer **P12** exhibited a lower inhibitory activity against RT than the larger terpolymers (**P8−P11**) suggesting competitive inhibition between the pendent sulfonated moieties, and the viral RNA is only achieved with polymers that are larger than $M_n \approx 2400$ g·mol^{-1}. Figure 6 also shows that the copolymers consisting of HEAm and AMPS (**P13−P15**) did show reduced inhibitory activity against the RT upon comparison to their terpolymer counterparts (**P8−P11**). While the reasons for differences in antireverse transcriptase activity are still unclear, we propose that the polymer conformation, due to the presence of pendent self-immolative 3TC linkers, may be different than the polymer conformation in the copolymers that only consist of HEAm and AMPS. The differences in polymer conformation may lead to differences in the ability of these polymers to competitively bind the polymerase. Similar to the DNA−DNA polymerase activity assay, the RT activity assay was kinase deficient and thus does not reflect the activity of 3TC released from the ter- or copolymers. The inhibitory activity demonstrated is therefore entirely attributable to the presence of pendent sulfonated moieties.

Cytotoxicity. Viability assays were performed on TZM-bl cells (HeLa derived cell line), which was used for the HIV

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Mol. Pharmaceutics 2016, 13, 2397−2410

assays. While none of the polymers in this contribution exhibited any cytotoxicity when applied to TZM-bl cells (Figure S21), the polymers were also tested on other cell lines including fibroblasts, hepatocytes, and T-cells. These cells serve as model cell lines, which should indicate any adverse effects caused by the polymers as well as by the self-immolation side products such as the cyclic thiocarbonate. Despite some outliers (**P3**, **P4**, and **P7**), the live−dead assays performed on SupT1 cells in the presence of the polymers indicated that most polymers nor 3TC had any profound effect on viability after 24 h incubation, which demonstrates that any released 3TC or cyclic thiocarbonate had no effect on the SupT1 cells (Figure 7). Similarly, live−dead assays of the mouse L929 fibroblasts

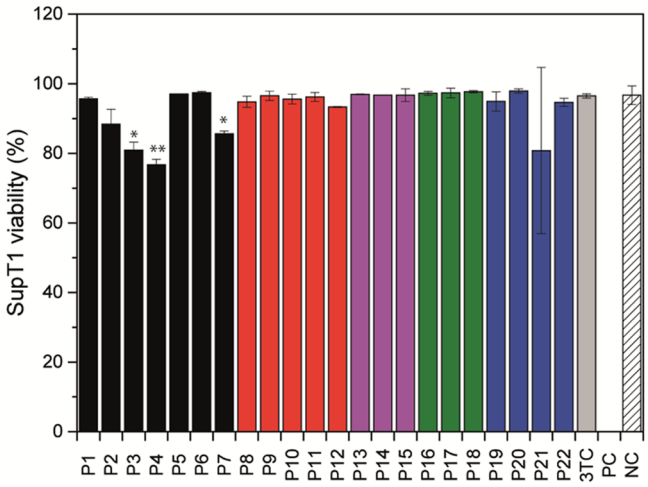

Figure 7. Live−dead viability of SupT1 cells following a 24 h incubation with 100 mg·L^{-1} polymer **P1**−**P22**. Color coding: black, copolymers consisting of HEAm and self-immolative 3TC acrylate (**P1**−**P7**); red, terpolymers consisting of 3TC self-immolative 3TC acrylate, HEAm, and AMPS (**P8**−**P12**); purple, copolymers consisting of HEAm and AMPS (**P13**−**P15**); green, AMPS homopolymers (**P16**−**P18**); blue, homopolymers of HEAm (**P19**−**P22**). n = 3 independent experiments. Statistical significance is given in relation to negative control (PBS). *p < 0.05, **p < 0.02.

(Figure S22) and HepG2 hepatocytes (Figure S23) exhibited little to no cell death upon incubation of the polymers up to 24 h. Thus, all these results indicate that the polymers made do not produce cytotoxic side effects in T-cells, fibroblasts, hepatocytes, and HeLa derived cells.

CONCLUSIONS

In this contribution, a high yielding synthetic route is described that provides access to well-defined homo-, co-, and terpolymers, which could be used as therapeutic agents against HIV. The assays performed here indicated that the polymers with sulfonated side chains mediate anti-HIV activity including entry inhibition and kinase-independent RT inhibition. Furthermore, kinase-dependent RT inhibition mediated by the released 3TC is envisioned with polymers that have pendent self-immolative 3TC moieties, which are released almost quantitatively within 48 h. The polymers therefore provide a route to prolong half-life (in vitro) and allow quantitative release within a time frame that is required for effective anti-HIV activity. Taken together, the polymers presented here exhibited anti-HIV activity via three independent mechanisms and thus permit access to polymer therapeutics that have potential to reduce the development of drug resistant HIV strains upon prolonged use, but further studies are required. In addition, the sulfonated polymers presented here also demonstrated kinase-independent activity against DNA−DNA polymerase and therefore provide a premise to encompass activity against other viruses, e.g., hepatitis B. The design of the polymers here could also pave the way for a new generation of polymer therapeutics especially as virustats that prevent mucosal transmission and that could target a broad spectrum of viruses including DNA and RNA viruses.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.6b00156.

Additional protocols and characterization including NMR of the self-immolative 3TC acrylate monomer and polymers as well as 3TC release data, viability, and HIV infectivity assays (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. M.D., A.H.F.A., K.Z., and C.F.R. performed the experiments. K.Z., C.F.R., A.A.A.S., M.T., A.N.Z., M.D., G.M., and A.P. designed the experiments. All authors have analyzed the data and given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

3TC, lamivudine; AMPS, 2-acrylamido-2-methylpropane sodium sulfonate; CTA, 2-(((butylthio)carbonothioyl)thio)-propanoic acid chain transfer agent; DMAc, dimethylacetamide; GSH, glutathione; GPC, gel permeation chromatography; HEAm, *N*-hydroxyethyl acrylamide; HIV, human immunodeficiency virus; RAFT, reversible addition−fragmentation chain transfer; SIL, self-immolative linker; Taq, *Thermus aquaticus*; V-501, 4,4′-azobis(cyanovaleric acid)

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