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Multivalent Benzoboroxole Functionalized Polymers as gp120 Glycan Targeted Microbicide Entry Inhibitors

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Abstract: Microbicides are women-controlled prophylactics for sexually transmitted infections. The most important class of microbicides target HIV-1 and contain antiviral agents formulated for topical vaginal delivery. Identification of new viral entry inhibitors that target the HIV-1 envelope is important because they can inactivate HIV-1 in the vaginal lumen before virions can come in contact with CD4+ cells in the vaginal mucosa. Carbohydrate binding agents (CBAs) demonstrate the ability to act as entry inhibitors due to their ability to bind to glycans and prevent gp120 binding to CD4+ cells. However, as proteins they present significant challenges in regard to economical production and formulation for resource-poor environments. We have synthesized water-soluble polymer CBAs that contain multiple benzoboroxole moieties. A benzoboroxole-functionalized monomer was synthesized and incorporated into linear oligomers with 2-hydroxypropylmethacrylamide (HPMAm) at different feed ratios using free radical polymerization. The benzoboroxole small molecule analogue demonstrated weak affinity for HIV-1BaL gp120 by SPR; however, the 25 mol % functionalized benzoboroxole oligomer demonstrated a 10-fold decrease in the K_D for gp120, suggesting an increased avidity for the multivalent polymer construct. High molecular weight polymers functionalized with 25, 50, and 75 mol % benzoboroxole were synthesized and tested for their ability to neutralize HIV-1 entry for two HIV-1 clades and both R5 and X4 coreceptor tropism. All three polymers demonstrated activity against all viral strains tested with EC_{50}s that decrease from 15000 nM (1500 μg mL^{-1}) for the 25 mol % functionalized polymers to 11 nM (1 μg mL^{-1}) for the 75 mol % benzoboroxole-functionalized polymers. These polymers exhibited minimal cytotoxicity after 24 h exposure to a human vaginal cell line.

Keywords: Synthetic carbohydrate binding agent; gp120; benzoboroxole; multivalency; entry inhibitor, HIV-1

Introduction

Rising rates of HIV infection among women, especially in the pandemic regions of sub-Saharan Africa,[1] combined with recent HIV-1 vaccine failures[2] have motivated the development of women-controlled prophylactics, or microbicides, for preventing HIV-1 infection.[3] Microbicides contain anti-HIV-1 or other antiviral agents formulated for topical delivery in the vaginal lumen. However, successful inactivation of HIV-1 in the vaginal lumen has been hindered by the limited number of microbicide candidates that target the HIV-1 envelope in comparison to antiretroviral agents that are delivered to the cervicovaginal tissue such as coreceptor antagonists or reverse transcriptase inhibitors.[4] The viral envelope protein plays a critical role in HIV-1 entry into cells, with the surface unit, gp120, initiating the binding

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events to susceptible cells containing CD4 receptors on immune system cells. Antiviral agents that act as entry inhibitors by binding to gp120 have the capability to inactivate HIV-1 virions in the vaginal lumen before they reach susceptible CD4+ cells and thus prevent the first step in the male-to-female heterosexual transmission of HIV-1.[5] The recent failure of sulfonated anionic polymer entry inhibitors to demonstrate efficacy in phase III clinical trials exemplifies the need for new HIV-1 entry inhibitors.[3]

Carbohydrate-binding agents (CBAs) capable of binding to the multiple glycans on the N-linked glycosylated regions of gp120 represent a promising class of entry inhibitors.[6] Of these, the CBA protein, cyanovirin-N (CV-N), which binds specifically to α(1,2)mannose glycans, has shown consistent suppression of HIV-1 and HIV-2 replication irrespective of the virus, the cell type, or the coreceptor tropism of the viral strain.[7] However, production, formulation and stability of protein-based lectins may economically impede their use by women in resource-poor pandemic regions like sub-Saharan Africa.[8] Investigations are therefore ongoing to identify non-peptide or protein based CBAs.[9−12] We are interested in developing multivalent polymer based synthetic-CBAs which may provide the broad-spectrum targeting capabilities of the CBAs without the cost of protein production, isolation, and subsequent chemical and structural stability issues.

Phenylboronic acid (PBA) forms reversible covalent complexes with diols,[13] a common chemical moiety in saccharides like fructose, glucose and mannose. Boronic acids undergoes a well-known condensation reaction with 1,2- or 1,3-diols to form five-or six-membered cyclic boronate esters. This condensation reaction is reversible and is highly influenced by the pH and chemical structure of the boronic acid and diols.[14,15] Conversion of the boronic acid to the charged boronate tetrahedral conformation yields a more stable complex that resists hydrolysis compared to its trigonal form of the complex which is more readily reversible. Investigations by Bérubé et al. reveal that the water-soluble, *o*-hydroxymethylphenylboronic acid (benzoboroxole, **1**), demonstrates binding at physiological pH to nonreducing sugars like methyl α-D-mannopyranoside and methyl β-D-galactopyranoside[16] which are structurally similar to the terminal sugar moieties found on the high mannose and complex-type N-linked glycans of gp120. It is hypothesized that the *o*-hydroxymethyl substituent is able to stabilize the arylboronic acid in its tetrahedral conformation at neutral pH thus preventing rapid hydrolysis of the arylboronic acid−diol complex at physiological pH.[16] Benzoboroxole-containing constructs are currently being exploited in glycoprotein detection.[17,18]

There are 24 N-linked glycosylation sites (HIV-1/III_B) on gp120 of which 30−50% are high mannose glycans with three terminal nonreducing mannose residues, and 50−63% are complex glycans terminating to some degree with

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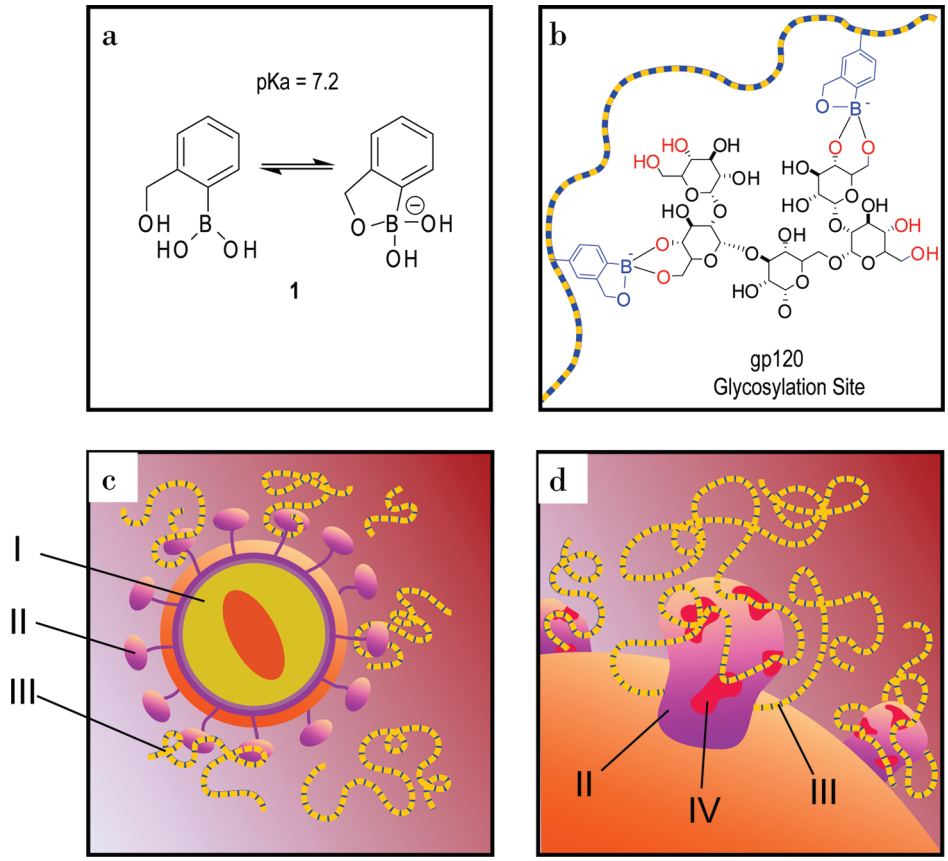
Scheme 1[a]



[a] (a) *o*-Hydroxymethylphenylboronic acid (benzoboroxole, **1**). (b) Hypothetical scheme of the binding chemistry: multivalent polymer-bound **1** binding due to complexation with a high mannose region of gp120 through interaction with the 4,6-diols of mannopyranose residues.[16] (c) Graphical depiction of the multivalent benzoboroxole-functionalized polymer (III) interacting with the gp120 complex (II) of HIV-1 (I). The polymer−gp120 interaction creates a sterically constrained surface. (d) The interaction drawn to scale with multivalent polymers (III) interacting with the gp120 heterotrimer's (II) many glycosylated regions (IV).

nonreducing galactose moieties.[19,20] Considering that up to 14 gp120-trimers have been identified on the envelope of HIV-1,[21] the HIV-1 envelope presents multiple binding sites for benzoboroxole-containing ligands. Incorporating the benzoboroxole ligand into a flexible and water-soluble polymer backbone would provide multivalent interaction with abundant sites on the HIV-1 envelope, thus reducing the impact of hydrolysis for a single benzoboroxole−diol complex. Furthermore, binding of the polymer could create a sterically hindered polymer layer on HIV-1 that prevents interaction of gp120 with CD4 and/or CCR5 receptors (Scheme 1). Multivalent conjugate approaches have been utilized to improve the affinity for ligand binding, especially in cases of binding to surfaces that have repeated epitopes.[22] Numerous studies have demonstrated the improvement of multivalent affinity over monovalent interactions by conjugating small molecules,[23,24] Fab,[25] and peptides[26] into polymeric architectures.

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The aim of this study is to investigate the binding of **1** to gp120 and to determine the impact that its incorporation into a polymer backbone at different mole ratios has on binding affinity and antiviral activity. To this end, we synthesized a benzoboroxole-functionalized monomer and incorporated it at different feed ratios into a linear water-soluble, biocompatible backbone poly[*N*-2-hydroxypropyl]methacrylamide] (pHPMAm).[27] In this study binding affinity of benzoboroxole (**1**) and benzoboroxole-functionalized pHPMAm oligomers to gp120 were assessed by SPR. Higher molecular weight polymers were then tested for antiviral activity against three strains of HIV-1. Because these polymers are intended to be used in the vaginal lumen as an inhibitor of HIV-1 entry, the polymers' cytotoxicity was tested in the Vk2/E6E7 human vaginal cell line.[28]

Experimental Section

Materials. Methacrylic acid (MAA) (stabilized with 250 ppm MeHQ) was purchased from Acros. *O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU) was purchased from AK Scientific, Inc. (Mountain View, CA). 5-Amino-2-hydroxymethylphenylboronic acid, HCl, dehydrate and *N*-acetylneuraminic acid (sialic acid) were purchased from Combi-Blocks, Inc. (Chester, PA). Methyl β-D-galactopyranoside was purchased from Alfa Aaesar. All other solvents and reagents were purchased from Sigma-Aldrich (St. Louis, MO) including 2,2′-azobisisobutyronitrile (AIBN), which was then recrystallized from chloroform in a chloroform$-N_2$(l) bath. All other reagents were used as purchased unless noted. When necessary, solvents such as tetrahydrofuran (THF) and ethyl acetate were dried over activated 4Å sieves, as indicated by the solvent name followed by 4Å. Thin layer chromatography plates were purchased from Whatman (aluminum backing, UV fluorescence 254 nm, silica gel, Kent, U.K.). Flash chromatography was performed using silica gel (200−400 mesh, 60 Å, Aldrich, St. Louis, MO). All ^{1}H and ^{13}C NMR spectra were acquired on a Varian Mercury 400 MHz spectrometer. ^{1}H chemical shifts are reported as δ referenced to solvent, and coupling constants (*J*) are reported in Hz. Polymer molecular distributions were determined in HPLC grade DMF using GPC (GPC 1100, Agilent Technologies, Santa Clara, CA) equipped with an organic column (PLgel mixed-B, Polymer Laboratories, Amherst, MA), a differential refractive index detector (BI-DNDC, Brookhaven Instruments, Holtsville, NY) and a multiangle light scattering detector (BI-MwA, Brookhaven Instruments, Holtsville, NY). A standard polymer (PEO, Varian Inc., Palo Alto, CA) was prepared at 1 mg mL^{-1} in DMF and used for calibration.

SPR Benzoboroxole (1) Affinity to gp120. 2-Hydroxymethylphenylboronic acid (Frontier Scientific, Logan, UT) was prepared at 50 mM in pH 7.5 25 mM phosphate buffer or a 25 mM carbonate buffer. HIV-1_{BaL} gp120, recombinant, produced in human embryonic kidney 293 cells (HEK), was obtained from the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIH). HIV-1_{BaL} gp120 was attached to a CM4 or C1 sensor chip using standard amine coupling chemistry at two to three densities (9,000 and 3,000 or 1600, 1400, and 900 RU respectively for the two types of chips). Briefly, using HBS-P as the running buffer the carboxy surface of a surface plasmon resonance (SPR) chip (CM4 (carboxydextran) or C1 (carboxymethyl) sensor chip, Biacore Life Sciences, Piscataway, NJ) was activated with an injection of a 1:1 ratio of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC): 0.1 M *N*-hydroxysuccinimide. HIV-1_{BaL} gp120 in 10 mM sodium acetate (pH 5.5) was then coupled to the surface. The remaining activated groups were then blocked by an injection of 1 M ethanolamine (pH 8.5). All coupling and affinity analysis were performed at 25 °C using the Biacore 3000 (Biacore Life Sciences), which has improved signal-to-noise ratio for samples that exhibit large bulk index of refraction changes. Affinity for the gp120 functionalized surface was determined by comparing raw data to three reference cells: two of the unactivated CM4 or C1 surfaces, and an activated CM4 or C1 surface capped completely by ethanolamine. The small molecule ligand was injected at the highest concentration prepared, followed by a 2-fold dilution series in the appropriate buffer to produce a concentration series. The association and dissociation phases were monitored for 40−60 s and 3−10 min respectively. All conditions were repeated at least twice. The reference surface was used to subtract out any bulk refractive index change. Data was fitted to a one-to-one model using local maxima using Scrubber2 software (BioLogic Software, Cambell, Australia) to determine K_D and k_D.

Chemical Synthesis of 5-Methacrylamido-2-hydroxymethylphenylboronic Acid (MAAm-OHMPBA, 4). MAAm-OHMPBA (**4**) was synthesized as follows. First the HBTU activation of methacrylic acid (MAAm-HBTU, i, Scheme 2) was synthesized as follows. In the first step, i, methacrylic acid (**2**, MAAm, 0.5256 mL, 6.20 mmol) was dissolved in 25 mL of tetrahydrofuran (THF) 4Å. HBTU (2.9456 g, 7.78 mmol) and diisopropylethylamine (DIPEA, 1.127 mL, 6.47 mmol) were added to the solution. The reaction was flushed with N_2(g) and ran for 4 h at room temperature. TLC showed that reaction had gone to completion (MAAm, $R_f = 0.7$; MAAm-HBTU, $R_f = 0.5$; HBTU, $R_f = 0$; 2:1 hexane:ethyl acetate 1% acetic acid). Reaction material was moved forward without any additional workup. In a second round-

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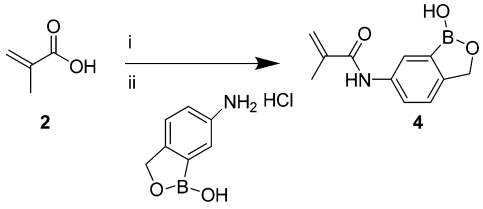
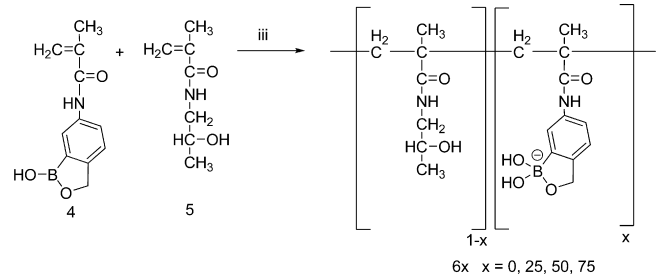
Scheme 2. Synthetic Conditions for Synthesis of 5-Methacrylamido-2-hydroxymethylphenylboronic Acid (**4**, MAAm-OHMPBA)[a]



[a] (i) Methacrylic acid (**2**, MAAm), HBTU, DIPEA, THF 4Å, N_2(g), 4 h, RT. (ii) 5-amino-2-hydroxymethylphenylboronic acid, HCl, dehydrate (**3**), DIPEA, N_2(g), 0 °C during addition of i, warmed to RT, overnight.

Scheme 3. Free Radical Polymer Synthesis of 5-Methacrylamido-2-hydroxymethylphenylboronic Acid (**4**) with 2-Hydroxypropylmethacrylamide (**5**)[a]



[a] (iii) MAAm-OHMPBA (**4**, 0 M, 0.125 M, 0.25 M, or 0.375 M) and HPMAm (**5**, 0.5 M, 0.375 M, 0.25, or 0.125 M) respectively, AIBN (0.025 M), DMF, 65 °C, 24 h, N_2(g). Polymer is drawn in the hemiboronic ester tetrahedral conformation (1, pK_a = 7.2) creating the stable form for hexopyranoside binding.

bottom flask, 5-amino-2-hydroxymethylphenylboronic acid HCl dehydrate (**3**, 1.00 g, 5.39 mmol) was combined with THF 4Å (15 mL). DIPEA (1.88 mL, 10.79 mmol) was added and the solution flushed with N_2(g) while being chilled in an ice bath. To this chilled solution, the reaction solution, i, was added dropwise via syringe while vigorously stirring. After addition **3** dissolved. The reaction was allowed to warm to ambient temperature and reacted overnight (ii, Scheme 2). TLC confirmed completion (MAAm-OHMPBA, $R_f = 0.5$, HBTU, $R_f = 0$, MAAm-HBTU, $R_f = 0.7$, 90:8:2 chloroform: methanol:acetic acid). Reaction was condensed to a brown oil and redissolved in 4 mL of $CHCl_3$ containing 200 μL of acetic acid and loaded onto a silica column slurry packed in 95:5 $CHCl_3$:MeOH 1% acetic acid, with an initial 20 mL of $CHCl_3$ eluted through (dry silica, 150 g; column dimensions, inner diameter 4.5 cm, height, 23 cm). An additional 20 mL of $CHCl_3$ was eluted through after loading, followed by elution in 95:5 $CHCl_3$:MeOH 1% acetic acid. Five milliliter fractions were collected. Compound began eluting in fraction 17. Fractions 17−30 were collected and stripped to yield **4** as a brown solid (1.484 g, 98% yield): ^{1}H NMR (400 MHz, MeOD with NaOD to aid in dissolution) 7.4 (m, 2H), 7.0 (d, 1H, $J = 9$ Hz), 5.77 (s, 1H), 5.45 (s, 1H), 4.82 (s, 1H), 2.02 (s, 3H); ^{13}C NMR (400 MHz, MeOD with NaOD to aid in dissolution) δ 168.8, 150.1, 140.9, 137.5, 124.3, 122.5, 121,3, 119.5, 71.0, 37.7, 17.2.

Synthesis of 2-Hydroxypropylmethacrylamide (HPMAm, 5). The synthesis was modified from a previously published procedure.[29] In short 1-amino-2-propanol (73.8 mL, 957 mmol) was added to a 1 L straight three neck round-bottom flask followed by 500 mL of ethyl acetate 4Å. The flask was equipped with an overhead stirrer (Caframo RZR 2000, Digital 2000), thermometer and a 250 mL constant addition funnel. To the addition funnel was added methacryloyl chloride (Fluka) from a new, unopened container (48 mL, 478 mmol). Dry ethyl acetate (70 mL) was added to the addition funnel to dilute the methacryloyl chloride and mixed to create a homogeneous solution. The reaction chamber was flushed with N_2(g) while the bulk reaction was cooled to −20 °C in an ethanol−dry ice bath. The methacryloyl chloride solution was added dropwise at a rate of 1 drop s^{-1}. After addition, the reaction was warmed to room temperature and stirred for an additional 3 h. Upon completion of reaction, the white solid (1-amino-2-propanol hydrochloride) was filtered off and rinsed with ethyl acetate (100 mL). The combined reaction supernatant and rinse were placed in a −20 °C freezer. Crystals were collected after 24 h via vacuum filtration and rinsed with chilled (4 °C) ethyl acetate and dried under high vacuum. The remaining white solid which still showed HPMAm by TLC was rinsed with acetone (3 times 100 mL) and concentrated. The white solid was redissolved in ethyl acetate (500 mL) and recrystallized for 24 h at −20 °C. Crystals were filtered and dried under hi-vac for a combined yield of 44 g, 307 mmol, 65% yield and characterized by ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.51 (br, 1H), 5.69 (s, 1H), 5.30 (s, 1H), 3.90 (m, 1H), 3.45 (m, 1H), 3.13 (m, 1H), 1.92 (s, 3H), 1.15 (d, 3H, $J = 6$).

Oligomer Synthesis. Oligomers were synthesized by free radical polymerization (Scheme 3) in the presence of a chain transfer agent, 2-aminoethanethiol, with feed ratios of 100:0, 75:25, and 50:50 of either HPMAm:MAAm-OHMPBA or HPMAm:acrylic acid (AA, distilled), which was used as a control to determine if nonspecific electrostatic interactions occurred with the protein due to a negatively charged backbone. Polymerizations were performed using 2.5 M solution of monomer at appropriate molar feed ratios with 2,2′-azobisisobutyronitrile (AIBN, 1 mol %) and 2-aminoethanethiol (8 mol %) in MeOH 4Å at 60 °C for 20 h under nitrogen atmosphere.[30] All oligomer reactions were triturated 3 times in 40 mL of acetone, centrifuged at 500 rpm for 5−15 min and supernatant decanted. HPMAm:acrylic acid oligomers were further purified by dialysis at 50 mg mL^{-1} using 500 MWCO membrane (Spectrum) against DDI water

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Table 1. Oligomer and Polymer Compositions and Molecular Weight Distributions[a]

	actual mole ratio[b] (mol %)			molecular weight[c] (kDa)	
oligomer/polymer	HPMAm	MAAm-OHMPBA	AA	M_w	M_n
oligo($HPMAm_{100}$) (oligo-$\mathbf{6}_0$)	100			1.5	
oligo($HPMAm_{75}$-($MAAm\text{-}OHMPBA_{25}$)) (oligo-$\mathbf{6}_{25}$)	74	26		0.75	
oligo($HPMAm_{50}$-($MAAm\text{-}OHMPBA_{50}$)) (oligo-$\mathbf{6}_{50}$)	57	43		1.24	
oligo($HPMAm_{75}$-(AA_{25})) (oligo-AA_{25})	65		35	1.9	
oligo($HPMAm_{50}$-(AA_{50})) (oligo-AA_{50})	49		51	1.8	
p($HPMAm_{100}$) ($\mathbf{6}_0$)	100			118.1	65.9
p($HPMAm_{75}$-($MAAm\text{-}OHMPBA_{25}$)) ($\mathbf{6}_{25}$)	71	29		103.9	88.4
p($HPMAm_{50}$-($MAAm\text{-}OHMPBA_{50}$)) ($\mathbf{6}_{50}$)	48	52		113.9	73.6
p($HPMAm_{25}$-($MAAm\text{-}OHMPBA_{75}$)) ($\mathbf{6}_{75}$)	30	70		74.5	49.6

[a] HPMAm: 2-hydroxypropylmethacrylamide. MAAm-OHMPBA: 5-methacrylamido-2-hydroxymethylphenylboronic acid. AA: acrylic acid. [b] Actual molar ratio was determined by ^{1}H NMR. [c] M_w for oligomers was determined by MALDI-TOF, and M_w and M_n for polymers were determined by GPC equipped with multiangle light scattering and differential refractive index detectors and are represented as means of at least triplicate measurements.

followed by lyophilization. Purity and actual molar feed ratios of polymers were determined in DMSO by ^{1}H NMR (Mercury 400 MHz spectrometer, Varian) (Table 1). Molecular weight distributions were determined using MALDI-TOF in the presence of NaCl under alkaline conditions (Table 1).

Oligomer SPR. Experiments were run identically to the small molecule SPR but on a C1 flat carboxylated sensor chip. Oligo($HPMAm_{100}$) (oligo-$\mathbf{6}_0$), oligo($HPMAm_{75}$-($MAAm\text{-}OHMPBA_{25}$)) (oligo-$\mathbf{6}_{25}$), and oligo($HPMAm_{75}$-(AA_{25})) (oligo-AA_{25}) were prepared at 15 mg mL^{-1} in 25 mM phosphate buffer, while the oligo($HPMAm_{50}$-($MAAm\text{-}OHMPBA_{50}$)) (oligo-$\mathbf{6}_{50}$) and oligo($HPMAm_{50}$-(AA_{50})) (oligo-AA_{50}) were prepared at 1 mg mL^{-1} in 2% DMSO (v/v) in 25 mM phosphate buffer. SPR was also performed in the presence of fructose and glucose to determine how incubation with physiological sugar concentration impacts the benzoboroxole-functionalized oligomers binding to gp120. For these experiments oligo-$\mathbf{6}_{25}$ and oligo-$\mathbf{6}_{50}$ were prepared at 10 or 1 mg mL^{-1} respectively in 2% DMSO, 25 mM phosphate buffer containing 16 mM fructose and 6 mM glucose, the physiological sugar concentrations found in seminal plasma.[31] To more specifically determine how the type of sugar impacted the binding of oligo-$\mathbf{6}_{25}$ to the gp120-functionalized surface a solution of 7.5 mg mL^{-1} oligo-$\mathbf{6}_{25}$ was preincubated with fructose, *N*-acetylneuraminic acid (sialic acid), methyl α-D-mannopyranoside or methyl β-D-galactopyranoside at 0.5, 1, or 10 times the ratio of sugar to oligomer-bound benzoboroxole. Solutions were prepared in a pH 7.5 25 mM phosphate buffer. The pH 7.5 25 mM phosphate buffer was also used as the running buffer. The concentration of benzoboroxole in the oligomer (oligomer-bound **1**) solutions in DMF was determined by UV/vis spectroscopy (USB-ISS-UV/vis USB4000 spectrometer, Ocean Optics, Dunedin, FL) at 320 nm using a calibration curve created from the MAAm-OHMPBA monomer ensuring to not measure at a wavelength where the alkene absorbed. For all SPR experiments the reference surface was used to subtract out any bulk refractive index change. The response units were normalized by molecular weight of the analyte, 152 and 750 Da for **1** and oligo-$\mathbf{6}_{25}$ respectively. Data were fit to a one-to-one model using a global maximum in Scrubber2 software to determine the K_D.

Polymer Synthesis. Polymers were synthesized by free radical polymerizations using 100:0 ($\mathbf{6}_0$), 75:25 ($\mathbf{6}_{25}$), 50:50 ($\mathbf{6}_{50}$) or 25:75 ($\mathbf{6}_{75}$) mole ratios of HPMAm (**5**) with MAAm-OHMPBA (**4**). Polymerizations were performed using 0.5 M solution of monomer with 2,2′-azobisisobutyronitrile (AIBN, 5 mol %) in DMF 4Å at 65 °C for 24 h under nitrogen atmosphere. All polymers reactions were triturated into 40 mL of ether, centrifuged at 500 rpm for 5−15 min and supernatant decanted. Polymers were placed under high vacuum overnight to remove residual DMF. Polymers were further purified by dissolving in 100 mM PBS buffer (50 mM NaCl, iso-osmolar) to a concentration of 10 mg mL^{-1} and centrifuging through a 3000 MWCO membrane (Amicon Ultra, 4 mL, 3000 MWCO, Millipore, Bellerica, MA). The 75 mol % sample required adjusting the buffer to pH 11 in order for complete dissolution to occur. Samples were centrifuged at 3000 rpm for 45 min, filtrate removed and retentate diluted up to initial volume in buffer. This was repeated three times with buffer, followed by three times with DDI H_2O. Retentate was lyophilized. The resulting yields after purification were around 60% for each polymer. Actual molar feed ratios of polymers were determined in D_2O by ^{1}H NMR (Mercury 400 MHz spectrometer, Varian) (Table 1). Polymer molecular distributions were determined in DMF using GPC (GPC 1100, Agilent Technologies, Santa Clara, CA) equipped with an organic column (PLgel mixed-B, Polymer Laboratories, Amherst, MA), a differential refractive index detector (BI-DNDC, Brookhaven Instruments, Holtsville, NY) and a multiangle light scattering detector (BI-MwA, Brookhaven Instruments, Holtsville, NY) (Table 1). The concentration of polymer-bound **1** in each polymer solution was determined in the same manner as that described for the oligomers.

Neutralization Assay. We used two R5-tropic, well-characterized, reference strains of HIV-1 isolated from acute, sexually transmitted infections and grown in peripheral blood

(31) Owen, D. H.; Katz, D. F. A Review of the Physical and Chemical Properties of Human Semen and the Formulation of a Semen Simulant. *J. Androl.* **2005**, *26* (4), 459–469.

mononuclear cells (PBMCs): HIV-1 DU156 (Clade C)[32] and TORNO (Clade B)[33] as well as a pseudotyped X4-tropic strain, WEAU (Clade B).[34] Polymers were tested for neutralization activity using a previously established and validated assay for testing neutralizing antibodies.[35]

Polymers and Chicago Sky Blue, a sulfate antiviral control, were prepared in DMEM (4.5 g L^{-1} D-glucose, 110 mg mL^{-1} sodium pyruvate and L-glutamine, Invitrogen, Carlsbad, CA) alone, with the pH adjusted to 7.5 as necessary, and pasteurized for 5 min at 70 °C, followed by 2-fold dilution with 2X growth media in the first row of the 96-well plate: DMEM supplemented with 20% fetal bovine serum (FBS, heat activated, Hyclone, Logan, UT), 50 mM HEPES (Gibco/Invitrogen, Carlsbad, CA) and 100 μg mL^{-1} gentamicin (Sigma, St. Louis, MO), referred to as 2xDMEM. Preparation was designed to pasteurize the polymers without proteins being present in the media. The highest concentration for each polymer was $\mathbf{6}_0$ at 20 mg mL^{-1}, $\mathbf{6}_{25}$ at 20 mg mL^{-1}, $\mathbf{6}_{50}$ at 10 mg mL^{-1}, and $\mathbf{6}_{75}$ at 3 mg mL^{-1}, and Chicago Sky Blue at 0.08 mg mL^{-1} in DMEM with 10% FBS, 25 mM HEPES, and 50 μg mL^{-1} gentamicin.

A 7.5-fold dilution series was prepared using normally prepared DMEM with 10% FBS, 25 mM HEPES, and 50 μg mL^{-1} gentamicin (referred to as normal media). The normal media alone was also used as a control. For the cell control wells, 150 μL of normal DMEM media was added. The viral control wells contained 100 μL of normal DMEM, and for the neutralization assay the wells contained 100 μL of the appropriate polymer concentration from the dilution series. Each solution was incubated with 50 μL of 350-650 TCID virus for 60 min. Then 10,000 TZM-bl cells (100 μL of 1×10^5 cells mL^{-1} in normal media with 10 μg mL^{-1} DEAE dextran (~500,000 Da, Sigma, St. Louis, MO) were added to each well. Cytotoxicity was also studied. These experiments were set up identically to the neutralization plates except 50 μL of normal media was added rather than the viral solution. Additionally a dilution of DMEM diluted with 2xDMEM was performed, followed by a similar dilution series as the polymers with normal DMEM to determine if this sample preparation impacted cytotoxicity or viral activity. Care was taken to ensure that all RLU values were within the linear range of the TZM-bl assay. After 48 h incubation at 37 °C, 150 μL was removed from each well and 100 μL of Britelite Reagent (PerkinElmer, Waltham, MA) was added. After two minute room temperature incubation, the wells were mixed by pipet action and 150 μL was transferred to a 96-well black plate and read in a luminometer (Wallac 1420 Victor, Perkin-Elmer, Waltham, MA). Relative luminescence values were compared to negative (cells-only, no virus) and positive (cells with virus) controls. Both polymer solutions and all media solutions alone were also analyzed for relative cytotoxicity using the same assay without any virus. In these cytotoxicity assays, the cellular luminescence from each well was compared to cell controls. A drop in well luminescence suggests a sample-mediated reduction in cell number. Concentrations showing a drop in cellular luminescence are not reported in the neutralization dose response plots due to potential cytotoxic effects on the cells used during antiviral neutralization. No effect was noted for the dilution of DMEM with 2xDMEM either in the cytotoxicity or viral samples. The positive control, Chicago Sky Blue successfully inactivated HIV-1 while the negative control $\mathbf{6}_0$ showed activity only at the highest concentration tested, 20 mg mL^{-1}. Antiviral activity is reported as the sample concentration at which the RLUs were reduced by 50% compared to virus control wells after subtraction of background RLUs. These EC_{50} values were determined using GraphPad Prism software (Graph Pad, LaJolla, CA).

Cell Toxicity. Polymer samples were prepared for cytotoxicity evaluation in keratintocyte serum-free media (KSF, Invitrogen, Carlsbad, CA) and pasteurized at 70 °C for five minutes. A 2-fold dilution was made with 2X growth media (KSF supplemented with 2-fold nutrients and pen-strep (P/S): 0.2 ng mL^{-1} EGF (epidermal growth factor, Invitrogen), 100 μg mL^{-1} BPE (bovinepituitary extract, Invitrogen), 2% v v^{-1} P/S (Invitrogen), 0.8 mM $CaCl_2$, referred to as 2xKSF) resulting in the same polymer concentrations as those used in the neutralization assay. Immortalized Vk2/E6E7 human vaginal cells[28] were seeded in 96-well plates, 5000 cells/well, in 250 μL of normal cell growth medium. The cells were cultured for 24 h; the growth medium was then removed and replaced with growth medium containing the polymers, and the cells were grown for an additional 24 h. A solution of N-9 (1 mg mL^{-1}) and a solution of $\mathbf{6}_0$, prepared identically as the polymers, served as toxic and nontoxic controls respectively. Metabolic activity of each well was determined relative to no-treatment control wells (dilution of the KSF) using the MTS cell proliferation assay (Promega, Madison, WI). Briefly, 30 μL of the MTS reagent was added to each well and the plate was incubated at 37 °C for three hours. After incubation, the absorbance was measured at 490 nm in a plate reader (Spectramax M2, Molecular Devices, Sunnyvale, CA). Absorbance of each well was subtracted

(32) Bures, R.; Morris, L.; Williamson, C.; Ramjee, G.; Deers, M.; Fiscus, S. A.; Abdool-Karim, S.; Montefiori, D. C. Regional Clustering of Shared Neutralization Determinants on Primary Isolates of Clade C Human Immunodeficiency Virus Type 1 from South Africa. *J. Virol.* **2002**, *76* (5), 2233–2244.

(33) Bures, R.; Gaitan, A.; Zhu, T.; Graziosi, C.; McGrath, K. M.; Tartaglia, J.; Caudrelier, P.; El Habib, R.; Klein, M.; Lazzarin, A.; Stablein, D. M.; Deers, M.; Corey, L.; Greenberg, M. L.; Schwartz, D. H.; Montefiori, D. C. Immunization with Recombinant Canarypox Vectors Expressing Membrane-Anchored Glycoprotein 120 Followed by Glycoprotein 160 Boosting Fails to Generate Antibodies That Neutralize R5 Primary Isolates of Human Immunodeficiency Virus Type 1. *AIDS Res. Hum. Retroviruses* **2000**, *16* (18), 2019–2035.

(34) Wei, X.; Decker, J. M.; Wang, S.; Hui, H.; Kappes, J. C.; Wu, X.; Salazar-Gonzalez, J. F.; Salazar, M. G.; Kilby, J. M.; Saag, M. S.; Komarova, N. L.; Nowak, M. A.; Hahn, B. H.; Kwong, P. D.; Shaw, G. M. Antibody Neutralization and Escape by HIV-1. *Nature* **2003**, *422* (6929), 307–312.

(35) Hammonds, J.; Chen, X.; Fouts, T.; DeVico, A.; Montefiori, D.; Spearman, P. Induction of Neutralizing Antibodies against Human Immunodeficiency Virus Type 1 Primary Isolates by Gag-Env Pseudovirion Immunization. *J. Virol.* **2005**, *79* (23), 14804–14814.

from the background (blank KSF media with MTS reagent) and percentage viability was calculated as: % viability = absorbance of sample well at 490 nm/absorbance of the corresponding media at 490 nm × 100.

Statistical Analysis. Statistical analysis was performed on the EC_{50} of the polymer concentration. A one-way ANOVA was performed to evaluate the significance the EC_{50} on each strain of HIV-1 tested. For comparisons of two data point sets a two-tailed *t* test was performed. A *p*-value of 0.05 was used.

Results

SPR Assays. We initially determined whether the weak binding of benzoboroxole, **1**, for hexopyranoside as determined by Bérubé et al. correlated to an affinity for gp120 by performing surface plasmon resonance (SPR). HIV-1_{BaL} gp120, recombinant, produced in HEK cells, was conjugated to a carboxydextran sensor chip. The binding affinity at pH 9.5 and pH 7.5 was assessed at two densities of gp120 (9,000 and 3,000 RU) (Figure 1). The response corresponded with the density of gp120 conjugated to the sensor chip with the higher density yielding a greater response at both pHs (see SFigure 1 in the Supporting Information). At pH 7.5 there was a higher response upon binding of **1** but more rapid dissociation compared to the pH 9.5 condition where a lower response was observed, but the dissociation was slower. The raw SPR suggests that at pH 7.5 there may be more binding sites for **1** on gp120, but the affinity is lower; at pH 9.5 there are correspondingly fewer binding sites but **1** appears to exhibit a stronger affinity for them. The kinetics observed during the washout phase exhibited dissociation rates with k_D's of 4.7 s^{-1} at pH 7.5 compared to 0.32 s^{-1} at pH 9.5. Using a one-to-one binding model, the average affinity determined from binding to the two densities of gp120 revealed a 4-fold increase in affinity at pH 9.5 compared to pH 7.5 with respective K_D's of 46.5 mM and 187.5 mM. No nonspecific binding to the carboxydextran reference sensor chip was detected.

Based on weak affinity of the benzoboroxole **1** for diols on gp120 as measured by SPR we synthesized linear benzoboroxole-functionalized oligomers to determine if multivalency could improve affinity. Oligomers were chosen to reduce the bulk refractive index changes in the SPR assay as compared to large molecular weight polymers used later in our studies. We first synthesized a benzoboroxole-functionalized monomer (MAAm-OHMPBA, **4**) by reacting methacrylic acid (**2**) with the commercially available precursor, 5-amino-2-hydroxymethylphenylboronic acid HCl dehydrate (**3**) using standard amidation chemistry. Oligomers were then synthesized by reacting **4** with HPMAm (**5**) using free radical polymerization in the presence of the chain transfer agent 2-aminoethanethiol at feed ratios of 0:100, 25:75 and 50:50 respectively. Acrylic acid based HPMAm oligomers were similarly prepared to determine if nonspecific electrostatic interactions affected binding affinity. The degree of incorporation was determined from ^{1}H NMR and found

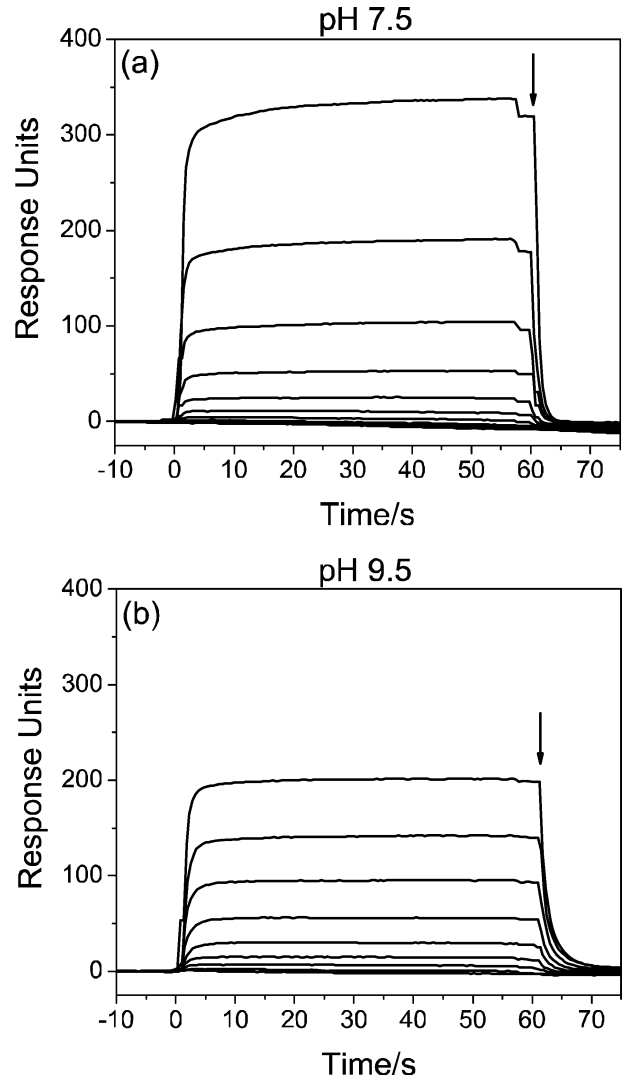

Figure 1. Responses for benzoboroxole (**1**, 75, 37.5, 18.75, 9.4, 4.7, 2.3, 1.2, 0.6, 0.3, 0.15 mM) binding to HIV_{BAL} gp120 captured on a carboxydextran surface at the highest density conjugated (9,000 RU) at pH 7.5 (a) and pH 9.5 (b) in a 25 mM phosphate or carbonate buffer, respectively. The arrow indicates the start of the dissociation phase.

to correlate with feed ratio (Table 1). The molecular weight was determined by MALDI-TOF and ranged from 750 to 1,900 Da (Table 1).

A flat carboxymethyl sensor chip was used to assess benzoboroxole-containing-oligomer binding to HIV_{BaL} gp120 as well as to prevent any nonspecific interactions of the benzoboroxole-functionalized oligomers with the dextran surface used for initial assessment of the small molecule **1**. The flat sensor chip reduces the overall density of gp120 conjugated to the surface, decreases the assay sensitivity, and modifies the surface presentation of gp120. These factors likely impact the K_D determined for benzoboroxole **1** on this sensor chip compared to that determined on the carboxydextran sensor chip (see SFigure 2 in the Supporting Information). The concentration of the oligomer-bound **1** present in the oligomer solutions tested in the SPR assay

was determined by UV/vis spectroscopy at 320 nm to allow direct comparison to the results for the small molecule **1**. A set of benzoboroxole-functionalized oligomers was prepared, but insolubility of oligo-$\mathbf{6}_{50}$ prevented quantitative analysis for binding affinity by SPR. Figure 2 displays the raw response units of the control oligo-AA_{25}, the small molecule benzoboroxole **1**, and the oligo-$\mathbf{6}_{25}$ at approximately 6 mM free or oligomer-bound benzoboroxole concentrations, as well as the SPR response units normalized for the differences in molecular weight for each molecule as a function of **1** or the oligomer-bound **1** concentration. The oligo-$\mathbf{6}_0$ and oligo-AA_{25} showed no affinity for the gp120 surface at any concentration (see SFigures 3−5 in the Supporting Information).

In comparing the dissociation phase in the raw SPR data for both molecules, it was observed that **1** returned rapidly to the baseline (see SFigure 2 in the Supporting Information), while oligo-$\mathbf{6}_{25}$ returns to baseline slowly (see SFigure 4 in the Supporting Information). The formation of multiple cross-links due to the multivalent interactions between the benzoboroxole ligands on the oligomer and terminal glycosylated residues on single or multiple gp120 units on the sensor chip may contribute to the slow dissociation. The normalized response curves yielded K_D values of 70 mM and 8.5 mM for the small molecule **1** and the oligo-$\mathbf{6}_{25}$-bound **1** respectively when fitted to a one-to-one model with a global maximum.

To understand how exposure to physiological levels of sugars affects affinity of oligo-$\mathbf{6}_{25}$ we studied the binding response in the presence of fructose and glucose which are present in seminal plasma at a concentration of approximately 16 mM and 6 mM respectively.[31] Bérubé et al. determined the K_D's of benzoboroxole **1** to be 1.65 mM and 32 mM for fructose and glucose respectively compared to a K_D of 42 mM for the methyl α-D-mannopyranoside. Binding of the oligo-$\mathbf{6}_{25}$ to the gp120 surface was efficiently disrupted after preincubation with physiological amounts of sugars. The fructose concentration was over four times higher than the highest oligomer-bound **1** concentration and outcompeted interaction for hexopyranoside residues on gp120. The dissociation phase correspondingly revealed a much more rapid return to baseline similar to that occurring in the reference cell for oligo-$\mathbf{6}_{25}$ without sugar present (see SFigures 4 and 5 in the Supporting Information).

We were also interested in elucidating how the type of terminal N-linked glycan moiety impacted the binding specificity of oligo-$\mathbf{6}_{25}$ for gp120. The oligo-$\mathbf{6}_{25}$ at 7.5 mg mL^{-1} was preincubated with increasing ratio of either fructose, sialic acid, methyl α-D-mannopyranoside or methyl β-D-galactopyranoside to the oligomer-bound **1** concentration (Figure 3). The binding affinity was then tested by SPR As expected, due to the high affinity of benzoboroxole for fructose,[16] the increasing concentrations of fructose resulted in a more rapid decrease in binding affinity to gp120. At 1 times the oligomer-bound **1** concentration, fructose reduced binding to gp120 to 24%. Complete inhibition of binding occurred at a fructose concentration of 10 times the oliogmer-bound **1** concentration. The negative SPR response signal at this concentration of fructose is due to the large mismatch between the injected oligomer-plus-fructose solution compared to the running buffer. The presence of methyl α-D-mannopyranoside reduced binding affinity to 88%, 85% and

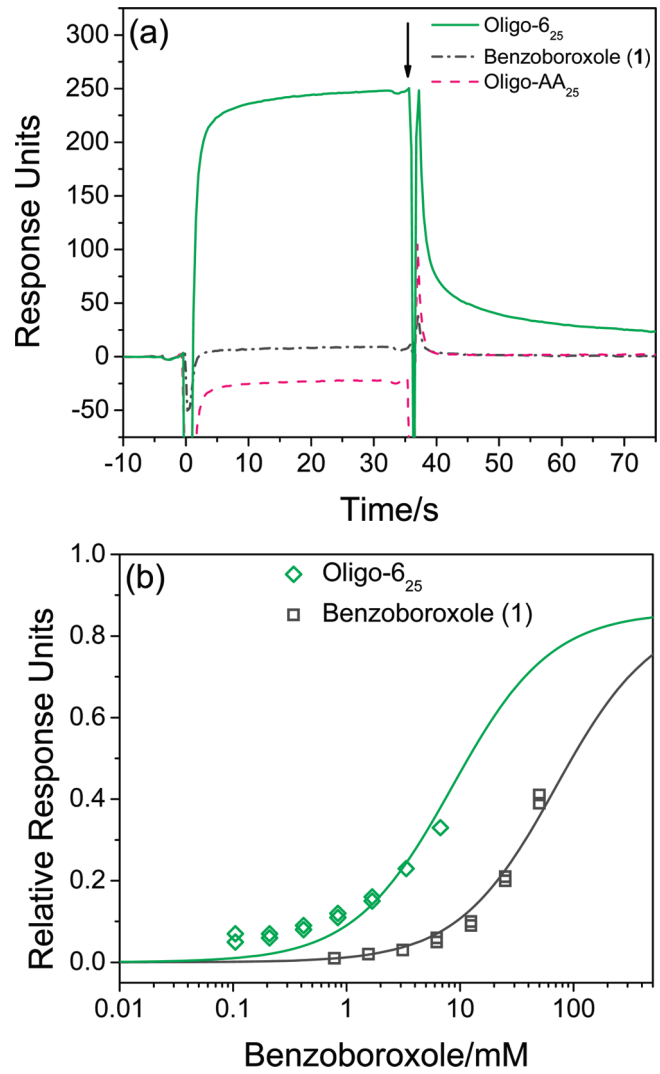

Figure 2. (a) Raw SPR analysis of interaction between the small molecule benzoboroxole (**1**, 6.25 mM), oligo-$\mathbf{6}_{25}$ (oligomer-bound **1** concentration 6.7 mM; oligomer concentration 15 mg mL^{-1}), and the control oligo-AA_{25} (oligomer concentration 15 mg mL^{-1}) and immobilized HIV_{BAL} gp120 grown in HEK cells (1600 RU) on a flat carboxymethyl sensor chip at approximately equivalent functional group concentrations. The arrow indicates the start of the dissociation phase. (b) Representative SPR equilibrium binding curves for **1** and oligo-$\mathbf{6}_{25}$ as a function of benzoboroxole concentration. Oligomer-bound **1** concentration was determined using UV/vis at 320 nm. The data was normalized by the molecular weight of the analytes, 152 and 750 Da respectively. The K_D was determined using a one-to-one model fit to a global maximum, yielding values of 70 mM and 8.5 mM for **1** and oligo-$\mathbf{6}_{25}$ respectively. The solid lines represent the fit to the model. All experiments were conducted at 25 °C in 25 mM phosphate buffer at pH 7.5.

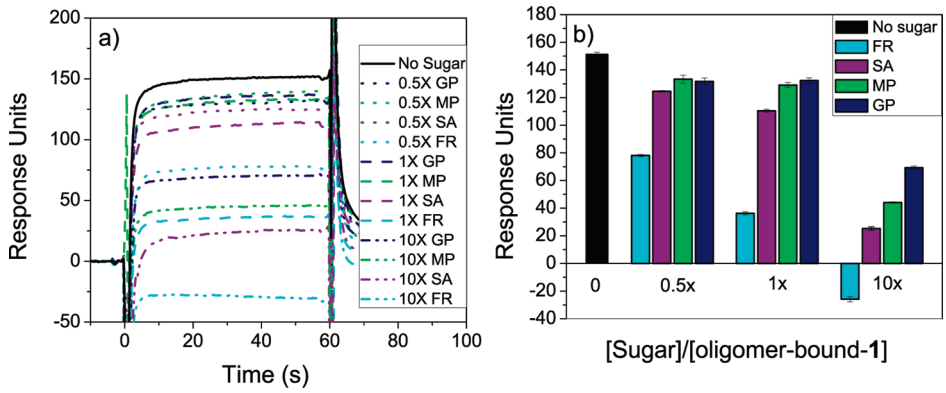

Figure 3. (a) Raw SPR analysis of the interaction between oligo-$\mathbf{6}_{25}$ (7.5 mg mL^{-1}) and immobilized HIV_{BAL} gp120 (1300 RU) on a C1, flat carboxymethyl sensor chip, in the presence of increasing ratios (0.5×, 1×, and 10×) of fructose (FR), methyl α-D-mannopyranoside (MP), *N*-acetylneuraminic acid (sialic acid, SA), or methyl β-D-galactopyranoside (GP) compared to the concentration of oligomer-bound benzoboroxole (**1**) at pH 7.5. A representative of four injections is displayed. (b) The average response for the affinity of oligo-$\mathbf{6}_{25}$ for gp120 at 30 s after injection is given as a function of the ratio of sugar concentration to oligomer-bound **1**. $N = 4$, mean ± SD.

29% at 0.5, 1, and 10 times the concentration of oligomer-bound **1**, respectively. Methyl β-D-galactopyranoside decreased the binding affinity to 87% at both 0.5 and 1 times the concentration of oligomer-bound **1**. At 10 times the concentration of oligomer-bound **1** the affinity was further reduced to 46%. Sialic acid decreased binding affinity to 82%, 73% and 17% at 0.5, 1, and 10 times the concentration of oligomer-bound **1**, respectively. The ability of sialic acid to reduce the binding affinity suggests that the sialic acid terminal residue moieties of the N-linked complex glycans of gp120 also play a role in the overall affinity of the benzoboroxole-functionalized oligomer.

The results of decreased binding affinity of the benzoboroxole-functionalized oligomer in the presence of seminal plasma sugar concentrations, as well as from competition with fructose, methyl α-D-mannopyranoside, methyl β-D-galactopyranoside and sialic acid further demonstrate that the affinity of the benzoboroxole-functionalized oligomers is due to boronic−diol interactions forming with the terminal N-linked glycan residues on gp120. The SPR results in the presence of sugars also indicate that concentrations of the benzoboroxole ligand will likely need to be increased in the in vivo environment to maintain affinity for the target ligands in the presence of physiological sugars and competing glycoproteins present in the vaginal lumen and seminal plasma.

Viral Neutralization Assays. To determine whether multivalent benzoboroxole ligands incorporated into a polymeric backbone exhibit antiviral activity we synthesized higher molecular weight polymers. Increasing the molecular weight augments the potential for sterically hindering the interaction between gp120 and CD4 and/or the CCR5 coreceptor by creating polymers with a larger radius of gyration compared to the approximately ten- to twenty-monomer-containing oligomers used in the SPR binding assay. Larger molecular weight polymers also decrease cytotoxicity by preventing cellular uptake.[36]

The in vitro entry inhibitor activity of the functionalized polymers was determined for two R5-tropic, well-characterized, reference strains of HIV-1 isolated from acute, sexually transmitted infections and grown in peripheral blood mononuclear cells (PBMCs), HIV-1 DU156 (Clade C) and TORNO (TRO, Clade B), as well as the pseudotyped X4-tropic Clade B strain, WEAU. In North America and Western Europe, the HIV-1 subtype Clade B predominates, while Clade C dominates the southern and eastern regions of Africa. The highest rates of HIV-1 infection occur in southern Africa.[1] Both of the coreceptor tropisms were studied to determine if the tropic strain impacts activity. The assay was performed in single-cycle infection experiments using the TZM-bl luciferase reporter gene assay system.[37] These cells are CXCR4-positive HeLa cell clones that have been engineered to express high levels of CD4 and CCR5 and contain the integrated reporter gene for luciferase under control of the HIV long-term repeat sequence reporter, which is induced in *trans* by the viral protein Tat soon after single round infection.[37] Forty-eight hours post infection the luciferase activity in the cells was measured. The media used in the neutralization screen contained 25 mM glucose as well as the standard 10% fetal bovine serum.

In this HIV-1 entry assay the dose response curves for all three viral strains revealed consistent suppressed activity in the presence of all three benzoboroxole-functionalized polymers (Figure 4). The nonfunctionalized poly(HPMAm), $\mathbf{6}_0$, was tested as a nonactive control. $\mathbf{6}_0$ showed activity only at 20 mg mL^{-1} (see Figure 4), the highest concentration tested against all three strains, suggesting that high polymer concentration impedes cellular entry in this assay. $\mathbf{6}_{25}$ (∼150 benzoboroxoles per chain) showed only slightly higher

(36) Duncan, R. The Dawning Era of Polymer Therapeutics. *Nat. Rev. Drug Discovery* **2003**, *2* (5), 347–360.

(37) Wei, X.; Decker, J. M.; Liu, H.; Zhang, Z.; Arani, R. B.; Kilby, J. M.; Saag, M. S.; Wu, X.; Shaw, G. M.; Kappes, J. C. Emergence of Resistant Human Immunodeficiency Virus Type 1 in Patients Receiving Fusion Inhibitor (T-20) Monotherapy. *Antimicrob. Agents Chemother.* **2002**, *46* (6), 1896–1905.

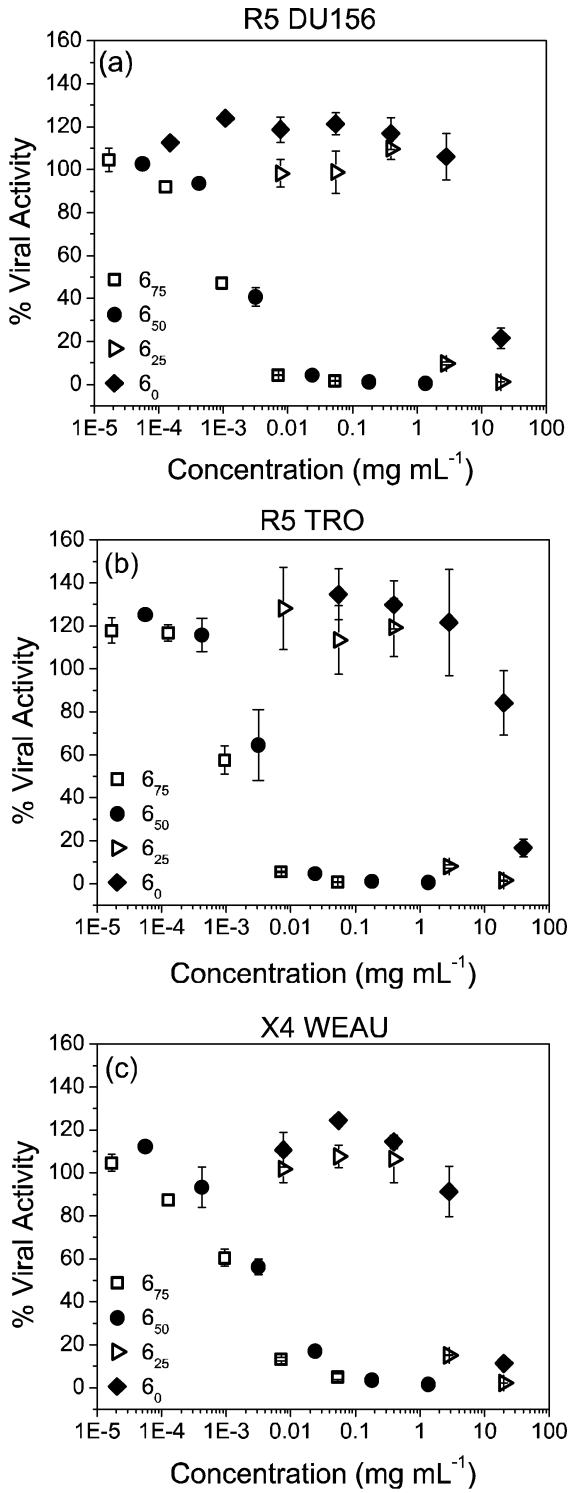

Figure 4. Results from the single-cycle HIV-1 infectivity inhibition in TZM-bl cells of polymer solutions $\mathbf{6}_{25}$, $\mathbf{6}_{50}$, and $\mathbf{6}_{75}$ against (a) R5 DU156 (Clade B), (b) R5 TRO (Clade C), isolated from acute sexually transmitted infections, and (c) the pseudotyped X4 WEAU (Clade B). The nonfunctionalized backbone, $\mathbf{6}_0$, showed antiviral activity at the highest concentration tested, 20 mg mL^{-1}, but at no other concentrations tested. $N = 3$, mean ± SD.

activity, with a 50% effective concentration for inhibiting viral activity (EC_{50}) of approximately 1 mg mL^{-1} (13,000 to 15,000 nM) against all three strains (Figure 5). However increasing the degree of functionalization to 75 mol % ($\mathbf{6}_{75}$, ~450 benzoboroxoles per chain) decreased the EC_{50} by 3 orders of magnitude against all three strains to ~10 nM (~1 μg mL^{-1}) (see SFigure 6 in the Supporting Information), with no statistically significant differences in the EC_{50} between the three strains and two coreceptor tropisms.

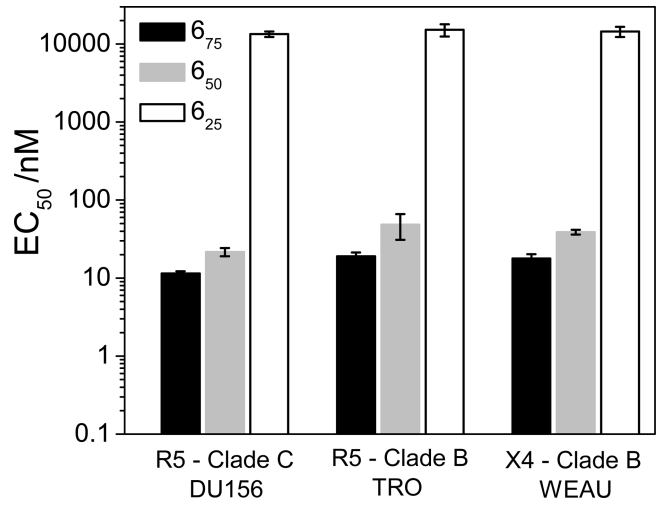

Figure 5. EC_{50} determined from the single-cycle HIV-1 infectivity inhibition in TZM-bl cells for $\mathbf{6}_{25}$, $\mathbf{6}_{50}$, and $\mathbf{6}_{75}$ against R5 DU156 (Clade B), R5 TRO (Clade C), isolated from acute sexually transmitted infections, and the pseudotyped X4 WEAU (Clade B) viral strains versus polymer concentration (nM). By ANOVA there was no statistical difference in the EC_{50} of each polymer compared across the three strains ($p < 0.05$). $N = 3$, mean ± SD.

Determining the concentration of total benzoboroxole in the polymer solution (polymer-bound **1**) provides a means to compare the binding of the oligomers and polymer antiviral activity. A 1.0 mg mL^{-1} solution of $\mathbf{6}_{25}$ contains 0.588 mM of polymer-bound **1** compared to a 1.0 mg mL^{-1} solution of oligo-$\mathbf{6}_{25}$ that has 0.447 mM of oligomer-bound **1**. Solutions of the polymers and oligomers with the same feed ratio of **4** contained similar concentrations of bound **1**. However, the oligo-$\mathbf{6}_{25}$ was on average composed of only a few benzoboroxole moieties per chain whereas the polymers had as many as ~150 benzoboroxole moieties per chain. This may account for the weak affinity of oligo-$\mathbf{6}_{25}$ for the immobilized gp120 (K_D = 8.5 mM, concentration units in terms of bound **1**) as determined by SPR. However, the 25 mol % benzoboroxole functionalized polymer ($\mathbf{6}_{25}$) had an EC_{50} against HIV-1 that corresponds to a polymer-bound **1** concentration of ~200 nM (see SFigure 6 in the Supporting Information). It is interesting to note that the concentration of polymer-bound **1** is 125,000 fold lower than the 25 mM glucose concentration present in the DMEM media, indicating that glucose is not out competing the benzoboroxole groups' affinity for the terminal diol residues on gp120 N-linked glycans in the antiviral assay measurements.

Cytotoxicity Assay. Investigations of the individual polymer cytotoxic profiles were explored using Vk2/E6E7

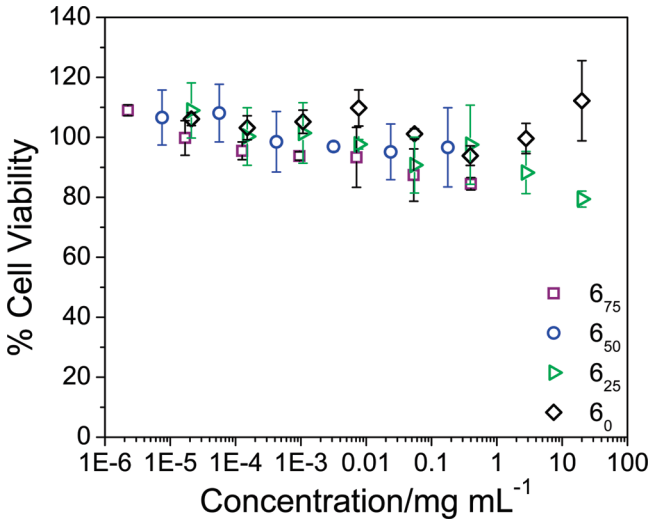

Figure 6. Results from cytotoxicity assay of polymer solutions $\mathbf{6}_0$, $\mathbf{6}_{25}$, $\mathbf{6}_{50}$, and $\mathbf{6}_{75}$ in Vk2/E6E7 human vaginal cells after 24 h exposure. $N = 3$, mean ± SD.

human vaginal cells (Figure 6). Nonoxynol-9 (N-9) was selected as a toxic control as it has demonstrated cytotoxicity in in vitro studies[38] and in vivo clinical studies[39] and was tested at concentrations ranging from 0.025−0.00125 mg mL^{-1} resulting in 40% cell viability at the lowest concentration tested. The unfunctionalized pHPMAm, $\mathbf{6}_0$, polymer was tested as the negative control due to a history of noncytotoxicity in in vitro assays.[27] A dose profile identical to that performed in the antiviral study was conducted. $\mathbf{6}_0$ exhibited no cytotoxicity up to 20 mg mL^{-1}. $\mathbf{6}_{25}$ exhibited cytotoxicity at 20 mg mL^{-1} with cell viability near 80%. Concentrations higher than 0.18 mg mL^{-1} for $\mathbf{6}_{50}$ and 0.4 mg mL^{-1} for $\mathbf{6}_{75}$ could not be tested due to gelation of the polymer after addition to the cells that impeded readouts in the MTS assay. Greater than 90% cell viability occurred in the presence of $\mathbf{6}_{50}$ up to 0.18 mg mL^{-1} in the vaginal cell line, two orders of magnitude higher than its EC_{50} against all three viral strains. Monolayer cell based toxicity assays are more sensitive than tissue based assays that use normal stratified epithelia found in ex vivo and in vivo tissue studies.[40] The lack of cytotoxicity in this assay suggests that these polymers may have minimal toxicity in vivo.

Discussion

In this study we have shown that the small molecule benzoboroxole, **1**, exhibited weak affinity to gp120 by SPR. In accord with other investigations using boronic acid moieties for sensing diols[14] the affinity increased at alkaline pH likely due to further stabilization of a tetrahedral boronate−diol complex above the molecules pK_a of 7.2 suggesting that the mechanism of polymer binding is benzoboroxole-sugar mediated.[16]

The SPR of **1** at pH 7.5 and pH 9.5 indicated that the number and affinity to the binding sites changes as a function of pH. This result may likely be explained by the pH sensitive affinity displayed by arylboronic acids. In general arylboronic acids exhibit increasing affinity above pH 7 for most sugar moieties except sialic acid,[41] a terminating moiety on some of gp120s complex glycans. Sialic acid binds to the nonfunctionalized phenylboronic acid at acidic pH, with decreasing affinity above pH 7.[41] While the affinity of **1** has not been tested for sialic acid or for hexopyranosides above pH 7.8 yet, it may display a similar affinity profile as phenylboronic acid. Thus at pH 7.5, **1** may have a weak affinity for the terminal nonreducing mannose, galactose and sialic acid providing the SPR signal that indicates a large number of binding sites with weak affinity. At pH 9.5, **1** may then exhibit a higher binding affinity for only the terminal nonreducing mannose and galactose while the affinity for sialic acid dramatically drops. A dramatically weak affinity for sialic acid would decrease the number of binding sites while an increase in affinity for mannopyranoside and galactopyranoside due to the more alkaline pH would yield an SPR signal indicating a higher affinity. This would provide one explanation for the apparent low response signal, indicating fewer binding sites, with higher affinity at pH 9.5 compared to the greater response signal with weaker affinity displayed at pH 7.5 by the SPR signal of **1** binding to the gp120 functionalized surface.

Our results complement those of Bérubé et al. by demonstrating affinity of benzoboroxoles for glycoprotein diols at physiological pH. The synthetic incorporation of the benzoboroxole moiety by free radical polymerization of a benzoboroxole containing vinylic monomer with the water-soluble and nontoxic HPMAm monomer created multivalent oligomers. The 25 mol % functionalized oligomer, oligo-$\mathbf{6}_{25}$, resulted in an approximate 10-fold improved of affinity for gp120 over the small molecule **1**. Incorporation beyond 25 mol % to 50 mol % rapidly decreased solubility of the resulting oligomer and prevented us from measuring affinity as a function of benzoboroxole incorporation in the oligomers.

Exposure to seminal sugar concentrations of fructose (16 mM) and glucose (6 mM) efficiently reduced binding of oligo-$\mathbf{6}_{25}$ to surface-bound gp120. Additionally, the reduced binding affinity that occurred in the presence of increasing

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concentration of fructose, sialic acid, methyl α-D-mannopyranoside or methyl β-D-galactopyranoside demonstrates that the interaction of benzoboroxole with diols is responsible for the oligomer's affinity to the glycoprotein. The presence of sialic acid reduced binding more extensively than either methyl α-D-mannopyranoside or methyl β-D-galactopyranoside indicating that this terminal residue on the complex N-linked glycans of gp120 impacts affinity. Additionally, the reduced affinity of the benzoboroxole-functionalized oligomer in the presence of sialic acid likely indicates that sialic acid may have a higher affinity for benzoboroxole compared to other two pyranoside sugar moieties tested. While the small molecule benzoboroxole had similar affinity for methyl α-D-mannopyranoside and methyl β-D-galactopyranoside,[16] a more rapid reduction of affinity occurred in the presence of methyl α-D-mannopyranoside compared to methyl β-D-galactopyranoside. It may be that the difference in structure between the high mannose and complex N-lined glycans impacts affinity. The polymer-bound concentration of benzoboroxole in a microbicide formulation will thus need to take into account competing glycoprotein and monosaccharide concentrations present in the vaginal lumen and seminal plasma to maintain activity against HIV-1. Further testing of the antiviral activity in the presence of seminal plasma will need to be undertaken to further understand and address these concerns.

We suspect that the capacity of benzoboroxole to bind terminal hexopyranosides present on the N-linked glycosylated sites of gp120 as demonstrated by SPR provides the broad spectrum entry inhibition exhibited by the benzoboroxole-functionalized polymers against the two clades and two coreceptor tropic strains of HIV-1 tested. The dramatic increase in activity from $\mathbf{6}_{25}$ to $\mathbf{6}_{50}$ may occur in part due to the pattern and orientation of the benzoboroxole moiety on the polymer chain as well as antagonistic binding with glucose and glycoproteins from the fetal bovine serum present in the DMEM media.[16] However, $\mathbf{6}_{50}$ revealed activities against all three strains even in the presence of a large excess of glucose compared to the concentration of polymer-bound **1**, indicating that an increase in avidity may occur due to incorporation of 50 mol % benzoboroxole compared to the 25 mol % benzoboroxole-functionalized polymer. Thus the increased multivalent presentation of the benzoboroxole ligand in $\mathbf{6}_{50}$ appears to improve the affinity for gp120 compared to $\mathbf{6}_{25}$.

The low polymer concentration required to inhibit HIV-1 entry for $\mathbf{6}_{50}$ and $\mathbf{6}_{75}$ may be due to random incorporation of **1** into the polymer backbone during free radical polymerization that yields a diverse library of spatial arrangement between two or more benzoboroxole moieties—out of the more than 100 that are present in an average chain—that augment affinity for unique geometric conformation of the terminal residues of the N-linked glycans on gp120. The nature and length of linker separating two arylboronic acid moieties has demonstrated great influence in the boronic acid binding selectivity and affinity[42] and may account in some degree for the 3-fold increase in antiviral activity of $\mathbf{6}_{50}$ compared to $\mathbf{6}_{25}$. Increasing the degree of functionalization from 50 to 75 mol % likely displays only a gradual increase in activity owing to an improved probability of binding events due to the increased number of benzoboroxole ligands present on the polymer backbone.

Like CV-N the synthetic CBA behavior of the benzoboroxole-functionalized polymers demonstrated similar activity against the two tested clades and the R5 and X4 coreceptor tropisms of HIV-1 suggesting that these polymers could have topical broad spectrum activity across a breadth of sexually transmitted viral strains. This level of activity against both coreceptor tropisms was not exhibited by the anionic sulfate polymers initially tested in microbicides, which displayed a higher activity against the X4 tropic strains compared to the R5.[43] The N-linked glycosylated sites are conserved to a large extent between different viral isolates potentially providing a mechanism for the observed broad spectrum activity.[44]

The EC_{50} of CN-V against HIV-1 R5 Clade B (BR/92/003) and C (ZA/97) isolates in TZM-bl cells was 3.9 nM and 15.6 nM (0.04 to 0.17 μg mL^{-1}) respectively.[45] These values compare favorably to the EC_{50} of ~10 nM for $\mathbf{6}_{75}$ (~1 μg mL^{-1}) against the R5 Clade B and C HIV-1 strains we screened. The antiviral activity of these polymers in the TZM-bl luciferase reporter gene assay indicates that they act as entry-inhibitors although we have not conclusively determined whether they prevent binding to CD4 or the CRR5 coreceptor or both. Based on these results we have thus demonstrated the successful use of multivalent-benzoboroxoles to create a polymer based synthetic CBA entry inhibitor with broad spectrum activity and similar efficacy *in vitro* as CN-V. We also believe that linear polymer-based CBA produced by free radical polymerization can more likely be economically scaled up and formulated. Even considering the recent breakthrough in the production of griffithsin, a protein based CBA, which required a 5,000 square-foot green house to produce 60 g of recombinant protein,[46] industrial scale up for polymer synthesis has been readily accomplished on a multi-kilogram scale. These polymers may therefore act as a potential broad-spectrum entry inhibitor at economi-

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cal prices required for microbicide distribution in resource-poor pandemic regions.

The use of benzoboroxole binding to terminal nonreducing hexopyranoside residues has several potential benefits for use as a microbicide entry inhibitor. First, they may be capable of targeting other glycosylated sexually transmitted virions like herpes-simplex virus[47] against which several protein CBA are inactive,[48] and the human papillomavirus. Protein CBAs have also demonstrated the potential to block the binding of HIV-1 to disseminating cells like DC-SIGN-expressing dendritic cells[49] which transfer HIV-1 to T-cell lymphocytes as a dissemination pathway in the male-to-female heterosexual transmission of HIV-1.[50] Similarly the benzoroboxole based synthetic CBA may also be capable of preventing this dissemination pathway of HIV-1. Also, recent research into the drug resistance profiles of CBAs like CV-N demonstrate that these compounds select for mutant HIV-1 strains that predominantly contain deleted N-glycosylated sites on the envelope gp120.[51−53] These deletions expose previously hidden immunogenic epitopes and promote the possibility of mutant strains that are more susceptible to immunogenic attack. These mutant strains were also not resistant to other entry inhibitors.[53] These results may be pertinent for investigation of future constructs targeted toward systemic delivery for treatment of HIV-1 infection.

Our investigation into cytotoxicity of the benzoboroxole-functionalized polymers against a human vaginal cell line revealed that no gross cytotoxicity occurred for the 50 mol % functionalized polymer ($\mathbf{6}_{50}$) at 2 orders of magnitude above the EC_{50}. Testing at higher concentrations was prevented due to gel formation upon polymer interaction with the cell media. Cell-based cytotoxicity assays are sensitive indicators of topical toxicity in an intact vaginal epithelia, therefore suggesting that these polymers may be safe. However, CBAs have the potential to induce inflammatory activity and to stimulate various differential markers[53] warranting further investigation into the cellular response induced by these polymers. We envision these polymers delivered to the vaginal lumen, rather than systemically, and that they would likely not be absorbed by the tissue potentially preventing adverse side effects.

Conclusion

The strategy presented herein has potential advantages over current protein CBAs. Specifically we have demonstrated the potential of a polymer based synthetic lectin to act as an entry inhibitor against HIV-1. Incorporation of benzoboroxoles into a multivalent polymer architecture improved affinity to gp120 by a factor of 10. Incorporation of benzoboroxole moieties into polymers with approximately ten times the molecular weight of the oligomers tested in the SPR assay generated antiviral activity across two clades and two corecepter tropisms of HIV-1 with EC_{50}'s. The activity increased by 3 orders of magnitude from a 25 mol % benzoboroxole-functionalized polymer to a 75 mol % benzoboroxole-functionalized polymer with an EC_{50} of ∼10 nM. We envision that benzoboroxole-functionalized polymers with this or other polymer architectures will lead to a new class of polymer-based entry inhibitors for use as vaginal microbicides that will exhibit broad spectrum activity against HIV-1, with economical production and ease of formulation.

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Supporting Information Available: Complete profile of raw SPR responses for **1** and oligomers binding to gp120 HIV-1_{BaL} functionalized surfaces; EC_{50} in μg mL^{-1} and polymer-bound **1** (nM). This material is available free of charge via the Internet at http://pubs.acs.org.

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