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BRIEF ARTICLE

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Activity and Safety of Synthetic Lectins Based on Benzoboroxole-Functionalized Polymers for Inhibition of HIV Entry

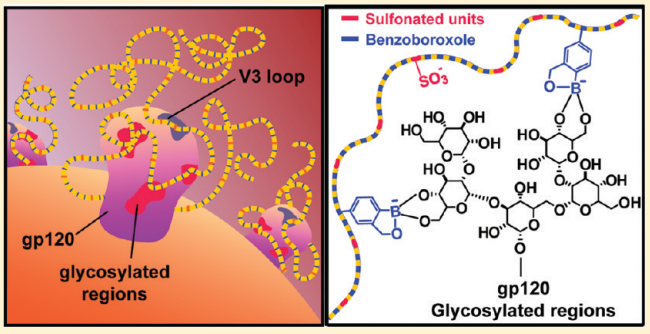
Alamelu Mahalingam,[†] Anthony R. Geonnotti,[‡] Jan Balzarini,[§] and Patrick F. Kiser*[,†]

[†]Departments of Pharmaceutics and Pharmaceutical Chemistry and Bioengineering, University of Utah, Salt Lake City, Utah 84112-5820, United States

[‡]Duke University Medical Center, Durham, North Carolina 27708-0281, United States

[§]Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium

ABSTRACT: Lectins derived from plant and microbial sources constitute a vital class of entry inhibitors that target the oligomannose residues on the HIV envelope gp120. Despite their potency and specificity, success of lectin-based entry inhibitors may be impeded by high manufacturing costs, formulation and potential mitogenicity. Therefore, there exists a gap in the HIV microbicides pipeline that underscores the need for mass producible, synthetic, broad-spectrum, and biocomptabile inhibitors of HIV entry. Here, we present the development of a polymeric synthetic lectin, based on benzoboroxole (BzB), which exhibits weak affinity ($\sim$25 M^{-1}) for nonreducing sugars, similar to those found on the HIV envelope. High molecular weight BzB-functionalized polymers demonstrated antiviral activity that increased with an increase in ligand density and molecular weight of the polymer construct, revealing that polyvalency improves activity. Polymers showed significant increase in activity from 25 to 75 mol % BzB functionalization with EC_{50} of 15 μM and 15 nM, respectively. A further increase in mole functionalization to 90% resulted in an increase of the EC_{50} (59 $\pm$ 5 nM). An increase in molecular weight of the polymer at 50 mol % BzB functionalization showed a gradual but significant increase in antiviral activity, with the highest activity seen with the 382 kDa polymer (EC_{50} of 1.1 $\pm$ 0.5 nM in CEM cells and 11 $\pm$ 3 nM in TZM-bl cells). Supplementing the polymer backbone with 10 mol % sulfonic acid not only increased the aqueous solubility of the polymers by at least 50-fold but also demonstrated a synergistic increase in anti-HIV activity (4.0 $\pm$ 1.5 nM in TZM-bl cells), possibly due to electrostatic interactions between the negatively charged polymer backbone and the positively charged V3-loop in the gp120. The benzoboroxole-sulfonic acid copolymers showed no decrease in activity in the presence of a seminal concentration of fructose ($p > 0.05$). Additionally, the copolymers exhibit minimal, if any, effect on the cellular viability, barrier properties, or cytokine levels in human reconstructed ectocervical tissue after 3 days of repeated exposure and did not show pronounced activity against a variety of other RNA and DNA viruses.



KEYWORDS: synthetic lectins, benzoboroxole, polyvalency, entry inhibitor, HIV

INTRODUCTION

From a therapeutic perspective, each step in the HIV life cycle provides an opportunity for pharmaceutical intervention. Among the initial steps in HIV infection is the binding event that occurs between the gp120 receptor on the viral envelope and surface proteins on the target cell. The HIV envelope is among the most heavily glycosylated proteins known to mankind.[1,2] The gp120 of HIV_{IIIB} has 24 potential N-linked glycosylation sites, of which 13 sites contain complex-type oligosaccharides, and the remaining 11 sites contain hybrid and/or high mannose-type structures.[3,4] Reports on the antiviral activity of lectin-based entry inhibitors suggest that targeting the oligomannose regions of gp120 can potentially produce broad spectrum entry inhibitors capable of inactivating HIV independent of tropism and strains. Prolonged exposure of these agents to virus-infected cells causes deglycosylations on the viral envelope, creating virions that are highly susceptible to neutralization by immunogenic responses. Such mutated virions also lack resistance to other small molecule entry inhibitors.[5−8] Therefore, lectin-based gp120 targeted entry inhibitors represent an interesting class of antiretroviral agents that potenatially can inactivate HIV in the vaginal lumen even before they reach susceptible CD4+ cells, preventing male-to-female heterosexual HIV transmission.[7,9]

All the entry inhibitors—with the exception of Maraviroc[10] and Fuzeon—[11] that have been tested in clinical trials have failed, most likely due to nonspecific interactions with the cell surface and/or suboptimal efficacy.[12−14] Consequently, there are a limited number of clinical candidates that target HIV entry in comparison to agents that target the enzymatic machinery involved in the viral replication.[15] Carbohydrate binding proteins

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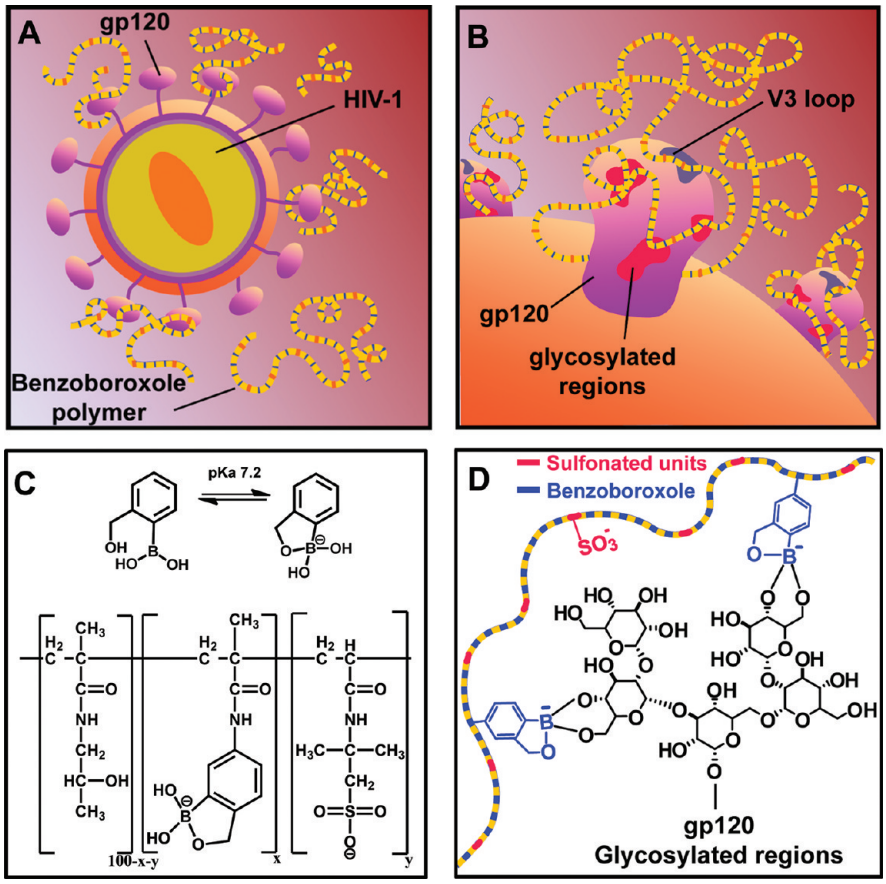

Figure 1. (A) Graphical depiction of the multivalent benzoboroxole-functionalized polymer interacting with the gp120 complex of HIV-1. (B) Schematic illustration of the binding between the polymers (shown in panel C) and the gp120 through interactions between benzoboroxole groups and the gp120 and/or through interactions between the anionic polysulfonate polymer and the cationic peptide fragments in the V3 loop of the gp120. (C) Chemical structure of benzoboroxole and the linear water-soluble polymers containing benzoboroxole and 2-acrylamido-2-methyl-1-propanesulfonic acid synthesized using a HPMA polymer backbone. (D) Hypothetical scheme of the binding chemistry between the multivalent polymers and the glycosylated regions on the gp120 heterotrimer.

(CBP), such as the non-human naturally occurring lectin protein (MBL),[16] cyanovirin-N (CV-N)[17] and griffithsin[18] that bind to the high-mannose oligosaccharides found on gp120, demonstrate the ability to inhibit HIV infection *in vitro* and *ex vivo*, irrespective of the cell type, or the coreceptor tropism of the viral strain.[19] However, the unacceptably high cost of large scale production and purification of these protein-based lectins, added to their low-stability and plausible immunogenic response,[20] may limit their use as a microbicide.[21]

Polyvalency—a mechanism capitalized by CBPs—pertains to simultaneous interactions between multiple-linked ligands and receptors with repeating epitopes, such as those found on the HIV envelope.[22] Due to translational entropic advantage[23] and steric stabilization, polyvalent interactions may enhance binding affinity of weak ligands.[22,24] Inspired by nature's adaptation to harness polyvalent interactions as a mechanism to enhance weak binding affinity between ligand−receptor pairs,[22] we aimed to develop a synthetic polyvalent inhibitor of the HIV entry by targeting the high-density glycosylation sites on the viral envelope (Figure 1A,B).

Phenylboronic acids (PBA) form reversible covalent complexes with *cis*-diols, a common chemical entity found on glycoproteins, e.g. mannose, galactose, *N*-acetylneuraminic acid, and fucose residues.[25] The presence of end-standing multiple mannose residues on each of the hybrid and/or complex-type N-linked glycans of gp120 suggests multiple binding sites for PBA-containing ligands. While PBA-containing polymers have demonstrated the ability to bind to glycosylated proteins, interactions of boronic acids with glycans have yet not been exploited as a mechanism to attenuate HIV entry. This is because the PBA and *cis*-diol form hydrolytically stable complexes only when the boronic acid exists in its tetrahedral form, mostly prevalent at a pH value greater than the pK_a of PBA.[26] PBA without any substitutions on the aromatic ring have a pK_a of 8.8 and, therefore, are found to bind strongly to diols only at alkaline pH's.[27] To improve binding at physiological pH, researchers have investigated various methods to facilitate the formation of stable tetrahedral boronate species at neutral pH by lowering the pK_a,[27] through dative bonds formed with boron,[28,29] and multivalency.[30] The addition of electron-withdrawing groups such as hydroxymethyl, fluoro and nitro to the phenyl ring of the PBA has been found to improve binding to diols at physiological pH compared to unsubstituted PBA.[26,27,30,31] Benzoboroxole (*o*-hydroxymethyl phenylboronic acid; BzB; Figure 1C) demonstrates strong binding to reducing sugars like fructose and is also capable of binding to glycopyranosides, like galactopyranose, a nonreducing sugar structurally similar to those found in the terminal glycan residues on gp120 at neutral pH.[32] Owing to the ease of synthesis, stability

and improved affinity to terminal sugar residues at neutral pH, this work focused on investigating BzB as a gp120-targeted HIV entry inhibitor.

In the past, our laboratory has shown that BzB ligands are capable of binding to the terminal sugar residues on the N-linked glycans of the gp120 and that multivalent polymers of BzB copolymerized with the water-soluble nontoxic 2-hydroxypropyl methacrylamide (HPMAm) monomer show cross-clade antiviral activity at nanomolar to low micromolar concentrations.[33] Based on our investigations to evaluate the effect of mole functionalization of BzB on antiviral activity of the synthetic lectins,[33] we chose the 50 mol % BzB polymers for further assessment. However, our previous system involved problems regarding solubility and activity, in addition to the need for biocompatibility evaluations. We hypothesized that incorporation of negatively charged moieties would improve affinity to gp120 through electrostatic interactions with the positively charged V3 loop, while improving solubility and steric availability of the reactive boronic moieties. This work therefore pertains to modulating the multiplicity, ligand density and charge of ligand presenting polymer backbone to augment the entry inhibition activity of synthetic lectins. In the current work, we investigate the effect of (a) ligand density (BzB mole % incorporation), (b) molecular weight of the BzB_{50} polymers and (c) activity of the polyvalent synthetic lectins with a sulfonated polymer backbone to inhibit HIV entry. Additionally, we also assayed the influence of fructose and the role of binding kinetics on the antiviral activity of the BzB_{50} polymers.

MATERIALS AND METHODS

Alizarin Red S., phenylboronic acid, 2-hydroxymethyl phenylboronic acid and all the diols were purchased from Acros and were used as received. Water was double distilled with a Milli-Q filtration system. 2-Acrylamido-2-methyl-1-propanesulfonic acid (AMPS) and AIBN were recrystallized from chloroform (dissolved at 60 °C, recrystallized at −80 °C) prior to use in free radical polymerizations. All other chemicals and reagents were purchased from Aldrich (St. Louis, MO) or Acros (Morris Plains, NJ) and used without further purification unless otherwise noted.

1. Colorimetric Assay To Determine Binding Constants for Boronic Acid−Diol Complexes. An ARS-based binding assay was performed as described by Wang et al.[26,34] Briefly, a 10 μM solution of ARS dye was freshly prepared in 100 mM pH 7.4 phosphate buffer. A 2 mM stock solution of BzB was prepared in 10 μM solution of ARS. The pH of both solutions was readjusted to 7.4. Using a 96-well plate, varying amounts of BzB solution were added to ARS solution to obtain a range of concentrations of BzB maintaining the concentration of ARS constantly at 10 μM. The plate was allowed to shake for 60 s prior to measurements. Fluorescence was measured at an excitation wavelength of 485 nm and emission at 620 nM. The experiments were carried out in triplicate. Absorbance spectra were collected over 300−600 nm. The association constant of PBA or BzB for ARS was determined by plotting the inverse of change in fluorescence vs the inverse of the concentration of boronic acid. The quotient of the *y*-intercept and the slope of the line were used to compute K_a for the boronic acid−ARS complex. To validate the assay we first determined the effect of varying concentrations of PBA on the absorbance and fluorescence intensity of ARS. ARS showed a color change, from red to yellow, and an 80-fold increase in fluorescence intensity at pH 7.4. The association constant for the PBA−ARS complex was determined to be 1260 M^{-1}, which is consistent with the values reported in the literature.[26,34] Following this, solutions with varying concentrations of the diol (D-fructose, glucose, sialic acid, methyl-β-D-galactopyranoside and methyl-α-D-mannopyranoside) were prepared in 100 mM pH 7.4 phosphate buffers containing 10 μM ARS and 2 mM PBA or BzB. Solutions containing excess of the diol were titrated against the boronic acid−ARS complex. As the diol replaces the ARS to form a boronic acid−diol complex, the concentration of free ARS in the solution increased, thereby showing a marked decrease in fluorescence. This shift in fluorescence was used to compute the association constant for the boronic acid−diol complex.[34]

Table 1. Association Constants of Phenylboronic Acid and Benzoboroxole Determined Using ARS Assay at pH 7.4 in 100 mM Phosphate Buffer

	association constant (M^{-1})	
diol	phenylboronic acid	benzoboroxole
ARS	1260	1307
D-fructose	160	664
N-acetylneuraminic acid	21	160
glucose	6	21
methyl-β-D-galactopyranoside	*a*	24
methyl-α-D-mannopyranoside	*a*	21

[a] Not measurable. Likely below 5 M^{-1} based on quantitative ARS assay

2. Polymer Synthesis and Characterization. BzB monomers and polymers were synthesized using methods similar to those described previously.[33] Briefly, polymers were prepared by free radical polymerization at 25 mol %, 50 mol %, 75 mol % and 90 mol % BzB feed ratio, in the presence of 5 mol % 2,2′-azobisisobutyronitrile (AIBN). Water-soluble and nontoxic 2-hydroxypropylacrylamide was used as a backbone monomer for copolymerization (Figure 1). The reaction mixture was purged with nitrogen and degassed for 1 h on ice. Following this, the reaction was moved to a 65 °C oil bath for 24 h. Polymers were precipitated in anhydrous ether using the fractional precipitation technique. Polymers were centrifuged and purified using dialysis across a 10,000 MWCO membrane using the VivaFlow dialysis system. Polymers with varying molecular weights were synthesized as described above, with 50 mol % feed ratio of BzB and varying feed ratios of AIBN (1−5 mol %). Polymers with sulfonic acid were also synthesized using the conditions described above. The degree of substitution was determined by ^{1}H NMR (Mercury 400 MHz spectrometer, Varian) and found to correlate with the feed ratios (Table 1). Molecular weight was determined by GPC (GPC 1100, Agilent Technologies, Santa Clara, CA) equipped with an organic column (PLgel mixed bed, 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).

3. Neutralization Assay To Evaluate the Activity of the Synthetic Lectins against Clinical HIV-1 Isoloates. Anti-HIV activity of the synthetic lectin polymers was evaluated using a single-cycle HIV-1 infectivity inhibition assay in TZM-bl cells.[33] A well-characterized R5 tropic DU156 (clade C) virus isolated from a sexually transmitted virus infection and cultured in PBMC was used in the neutralization assay. Additionally, R5 TRO

(clade C) and the pseudotyped X4 WEAU (clade B) viral strains were assayed to investigate cross-clade and cross-tropism activity. Polymer solutions were prepared at 10 mg/mL in DMEM (4.5 g/L D-glucose, 110 mg/mL sodium pyruvate and L-glutamine, Invitrogen, Carlsbad, CA), with the pH adjusted to 7.5 as necessary, and pasteurized for 5 min at 70 °C. After pasteurization, the stock solution was diluted 1:1 with DMEM containing 2× nutrients (DMEM supplemented with 20% fetal bovine serum (Hyclone, Logan, UT), 50 mM HEPES (Gibco/Invitrogen, Carlsbad, CA) and 100 μg/mL gentamicin (Sigma, St. Louis, MO) under sterile conditions. Preparation was designed to pasteurize the polymers without proteins being present in the medium. The final medium composition after 1:1 dilution was 10% FBS, 25 mM HEPES, and 50 μg/mL gentamicin. The assay was conducted in a 96-well plate such that the polymer solution at the highest concentration was added to the bottom row. From the bottom row of the plate 20 μL of the test sample was added to the prior row with 100 μL of growth medium to create a 6-fold dilution. This scheme of serial dilution was repeated five times to obtain a dose−response curve. Appropriate cell controls and virus controls were included in each plate. Following the serial dilutions, 50 μL of cell-free virus (200 $TCID_{50}$) was added to each well, with the exception of the wells that served as cell control. The plate was then incubated for 1 h in a 37 °C, 5% CO_2 incubator. After the preincubation, 100 μL of TZM-bl cell suspension prepared at a density of 1×10^5 cells/mL in growth medium containing DEAE dextran (37.5 μg/mL) was added to each well and incubated for an additional 48 h. Luminescence was measured after 2 min incubation with Britelite Reagent (PerkinElmer, Waltham, MA). Toxicity of the samples was simultaneously analyzed using an identical plate layout in the absence of virus. To determine loss in cell viability, luminescence from the sample-treated wells was compared to that of the cell controls. EC_{50} is reported as the concentration of the sample which reduced the relative luminescence units (RLUs) by 50% compared to the virus control. Dose−response curves were fit to the percent neutralization data using GraphPad Prism software (Graph Pad, LaJolla, CA).

In the assay evaluating the effect of preincubation time on the antiviral activity of the synthetic lectins, the assay was conducted using a procedure similar to the one described above, with the exception of the preincubation time. The polymer solutions were incubated for 0, 15, 30, and 60 min with the virus prior to addition of TZM-bl cells. For the assay evaluating the effect of fructose, 30 mg/mL fructose solution was prepared in DMEM medium. To the polymer solution, 10 μL of the fructose solution was added along with the virus to create a fructose concentration of 3 mg/mL. The remainder of the steps were performed as described above.

4. Broad-Spectrum Antiviral Assays and Activity against Laboratory HIV-1 Strains. The anti-HIV activity and cytotoxicity were also evaluated against the laboratory HIV-1 strain IIIB and HIV-2 strain ROD in human T-lymphocyte CEM cell cultures. Briefly, virus stocks were titrated in human T-lymphocyte CEM cells and expressed as the 50% cell culture infective dose ($CCID_{50}$, 1 $CCID_{50}$ being the virus dose to infect 50% of the cell cultures). CEM cells were suspended in culture medium at ~3×10^5 cells/mL and infected with HIV at ~100 $CCID_{50}$. Immediately after viral exposure, 100 μL of the cell suspension was placed in each well of a flat-bottomed microtiter tray containing various concentrations of the test compounds. After a 4 day incubation period at 37 °C, the giant cell formation was microscopically determined. Compounds were tested in parallel for their potential cytostatic effects in uninfected CEM cell cultures.

In a cocultivation assay, 5×10^4 persistently HIV-1 infected HUT-78 cells (designated HUT-78/HIV-1) were mixed with 5×10^4 SupT1 cells, along with appropriate concentrations of the test compound. After 20 h, marked syncytium formation was noted in the control cell cultures, and the number of syncytia was determined under the microscope. The EC_{50} was defined as the compound concentration required to prevent syncytium formation by 50%.

The other antiviral assays were based on inhibition of virus-induced cytopathicity in HEL [herpes simplex virus type 1 (HSV-1), HSV-2 (G), vaccinia virus, and vesicular stomatitis virus], Vero (parainfluenza-3, reovirus-1, Coxsackie B4, and Punta Toro virus), HeLa (vesicular stomatitis virus, Coxsackie virus B4, and respiratory syncytial virus) and CrFK (feline corona virus (FIPV) and feline herpes virus) cell cultures. Confluent cell cultures in microtiter 96-well plates were inoculated with 100 $CCID_{50}$ of virus in the presence of varying concentrations (5,000, 1,000, 200 nM) of the test compounds. Viral cytopathicity was recorded as soon as it reached completion in the control virus-infected cell cultures that were not treated with the test compounds.

5. Biocompatibility Evaluation in MatTek VEC-100 Reconstructed Human Vaginal Tissue. Triplicate solutions of BzB_{50}-$AMPS_{10}$-$HPMA_{40}$ at 1 mg/mL were prepared in serum containing medium. Polymer solutions were pasteurized at 70 °C for 5 min. Media containing the polymers were applied to the apical surface of tissues cultured in 24-well plate inserts. Three repeated exposures to the test sample were performed in intervals of 24 h. After 72 h of exposure, tissue viability was determined using MTT assay and change in tissue morphology was evaluated through histological examination of the epithelium. On days 1−3, cytokine levels were assayed using ELISA. Levels for IL-1α, IL- 8, IL-6 and TNF-α were measured in tissue culture supernatant by utilizing human cytokine kits (R&D System). Tissue integrity was monitored through measurements of transepithelium electrical resistance (TEER). Triplicates of control solutions of pHPMA (1 mg/mL), nonoxynol-9 (0.02 mg/mL) and culture medium were also performed.

RESULTS AND DISCUSSION

1. Comparative Evaluation of the Binding Affinity of PBA and BzB. To measure the affinity of the boronic acid ligands for reducing and nonreducing sugar residues, we used the ARS-based three-component colorimetric binding assay.[34] Affinity for glucose, fructose, sialic acid, methyl-β-D-galactopyranoside and methyl-α-D-mannopyranoside was assayed. To ensure the viability of the assay, the association constant of PBA-ARS was compared to values reported in the literature by Wang and coworkers.[34] Table 1 summarizes the association constants of PBA and BzB for various diols. Comparison between PBA and BzB affinities for simple reducing sugars revealed 4-fold stronger affinity of BzB for fructose (~600 M^{-1}) when compared to PBA (160 M^{-1}); however, the affinity of BzB and PBA for glucose was comparable. Unsubstituted PBA demonstrated minimal affinity for nonreducing sugars such as methyl-β-D-galactopyranoside or methyl-α-D-mannopyranoside at neutral pH. In contrast, BzB revealed higher, but still weak, affinity (~25 M^{-1}) for nonreducing complex sugars. To develop a boronic

acid based synthetic entry inhibitor for HIV, we have exploited the ability of the BzB to bind to nonreducing sugars,[32] which are structurally similar to the terminal sugar moieties found on the high mannose and complex-type N-linked glycans of gp120. We have previously confirmed this observation also using surface plasmon resonance (SPR), in which BzB showed a K_d of ~180 mM for HIV_{BaL} gp120, whereas no measurable binding was observed with PBA.[33]

Unsubstituted PBA-containing polymers have demonstrated the ability to bind to glycosylated proteins only at alkaline pH; whereas BzB binds to glycosides at neutral pH.[29] This difference has been ascribed to the presence of oxygen from the *ortho* substituent that stabilizes the boronate ester toward hydrolysis[28] and decreases the energy barrier for tetrahedral formation.[35] While the BzB shows superior binding affinity for sugar residues, when compared to the unsubstituted PBA, the binding is fairly weak for practical applications. In this regard, polyvalency has commonly been exploited to improve binding affinity of weak ligands. This effect is well-known in natural protein−ligand interactions as well as for other synthetic protein mimics.[22,23] Kaur et al. demonstrated almost a double increase of affinity for glucose for a tweezerlike bis-boronic acid molecule compared to a monovalent boronic acid.[30] Incorporating ligands into polymer backbones provides a synthetically accessible mechanism for increasing the affinity of ligands for glycoproteins.

Table 2. Polymer Composition and Molecular Weight Distribution

		mole functionalization[a]			mol wt[b] (kDa)		
polymer	AIBN (mol %)	BzB	AMPS	HPMA	M_w	M_n	PDI
BzB_{25}-$HPMA_{75}$	5.0	29		71	103	88	1.17
BzB_{75}-$HPMA_{25}$	5.0	70		30	74	50	1.48
BzB_{90}-$HPMA_{10}$	5.0	91		8	98	76	1.30
BzB_{50}-$HPMA_{50}$	5.0	48		52	110	91	1.21
	2.5	54		54	226	159	1.42
	1.0	51		51	382	285	1.34
$AMPS_{10}$-$HPMA_{90}$	5.0				153	111	1.38
BzB_{50}-$AMPS_{10}$-$HPMA_{40}$	5.0	45	10	55	131	109	1.20

[a] Degree of substitution was determined by ^{1}H NMR and found to correlate with the feed ratios. [b] Molecular weight was determined by GPC attached to a light scattering and a refractive index detector.

2. Polymer Synthesis and Characterization. Table 2 summarizes the molecular weight of the polymers synthesized by free-radical polymerization, determined using size exclusion chromatography (Agilent Technologies, Santa Clara, CA) equipped with a PLgel mixed-B column (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). Mole functionalization of BzB in the polymer was determined using ^{1}H NMR.

3. Effect of BzB Mole Functionalization (Ligand Density) on the Entry Inhibition Activity of the Synthetic Lectins. While the binding affinity of BzB for nonreducing sugars determined by the ARS-based colorimetric assay and for gp120 from SPR revealed weak affinity (Table 1), high molecular weight polymers with polyvalent presentation of BzB showed ≥4 log scale increase in activity (EC_{50} in low micromolar to nanomolar range). As seen in Figure 2, ~2 log scale increase in antiviral activity was observed with an increase in BzB mole functionalization from 25 mol % to 50 mol % in the polymer (2-tailed Student's t test $p < 0.001$), and further increase in BzB functionalization from 50 mol % to 75 mol % showed only a marginal increase in activity (2-tailed Student's t test $p = 0.01$). On the contrary, an increase in ligand density from 75 mol % to 90 mol % showed a reduction in the ability of the polymers to neutralize HIV entry (2-tailed Student's t test $p = 0.01$), with an anti-HIV activity that was comparable to the 50 mol % BzB-functionalized polymers.

We believe that the increase in antiviral activity with increasing mole functionalization can be explained by at least two distinct mechanisms described by Mammen and co-workers:[22,24] polyvalency and steric stabilization. The combined effect of the increase in the number and density of ligands likely improves the probability of binding, thereby enhancing binding and antiviral activity through avidity and polyvalency. As a secondary mechanism, we suspect that, when the BzB presenting polymers bind to gp120, they escort along a large water-swollen polymer layer which possibly makes the viral envelope sterically inaccessible for attachment and entry into the host cell.[24,36] If we assume that it is these two mechanisms that collectively determine the activity of synthetic lectins, the gradual increase in activity from 50 to 75 mol % and the decrease in activity from 75 to 90 mol % can be explained

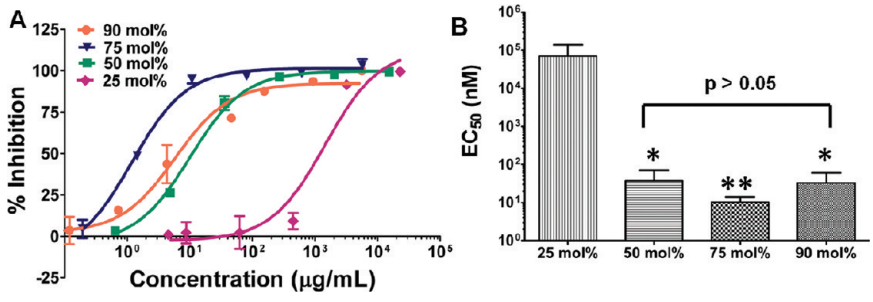

Figure 2. Effect of BzB mole functionalization on the entry inhibition activity of BzB_{50} polymers. Results from the single-cycle HIV-1 infectivity inhibition in TZM-bl cells against R5 DU156 (Clade B) isolated from acute STIs. (A) Dose−response curve of BzB functionalized HPMA polymers at varying mole incorporation. (B) EC_{50} was determined by fitting a sigmoidal dose−response curve with variable Hill's slope. Single factor ANOVA showed significant differences between the groups ($p < 0.05$). Two tailed Student's t test revealed significant increase in activity from 25 to 50 and 75 mol %. Further increase from 75 to 90 mol % showed reduction in activity; EC_{50} of BzB_{90}-$HPMA_{10}$ was comparable to EC_{50} of BzB_{50}-$HPMA_{50}$ ($N = 3$, mean ± SD; $^*p < 0.05$; $^{**}p < 0.01$).

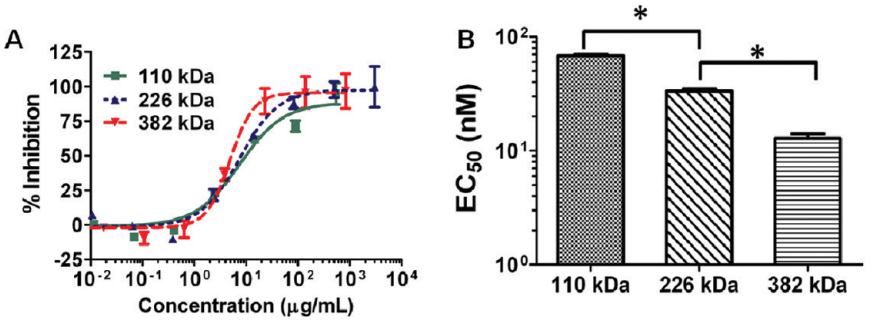

Figure 3. Effect of molecular weight on the entry inhibition activity of BzB_{50} polymers. Results from the single-cycle HIV-1 infectivity inhibition in TZM-bl cells against R5 DU156 (clade B) isolated from acute STIs. (A) Dose−response curve of BzB_{50}-$HPMA_{50}$ polymers at varying molecular weights and (B) EC_{50} determined by fitting a sigmoidal dose−response curve with variable Hill's slope. Two tailed Student's *t* test was conducted ($N = 3$, mean ± SD; $^*p < 0.05$). Significant increase in activity was observed with increase in MW of the polymer from 110 to 226 kDa and further to 382 kDa ($p = 0.03$ and 0.01, respectively). Highest activity was observed with 382 kDa MW polymer (11.3 ± 2.9 nM).

through loss in the steric stabilization effect of these polymers. As the ligand density in the polymers has been increased, the negatively charged borons could repel each other, thereby distorting the polymer conformation from an effective random coil structure to a less effective rigid elongated structure.[24] On the other hand, increasing the number of available ligands and, therefore, points of attachment between the polymer and the gp120 could result in a collapsed polymer structure, compromising the ability of the polymer to sterically stabilize the binding surface.[24] Although the relative contribution of the above-mentioned two mechanisms toward antiviral activity is yet to be elucidated, it is reasonable to assume that steric stabilization plays a critical role[24] in governing the ability of BzB-functionalized polymer to block HIV entry. Based on these results, further assessments were performed on 50 mol % functionalized BzB polymers.

Depending on the coreceptor tropism and clade, HIV displays significant variations in the viral envelope,[37,38] making it important to evaluate cross-clade and -tropism activity of new therapeutic agents.[39,40] For example, agents targeting the positively charged V3 loop of the gp120, such as the nonspecific sulfated polymers, show severely compromised activity against R5 tropic strains.[41] This is because the coreceptor tropism of the virus impacts the extent of the positive charge on the gp120 V3 loop, with the CXCR4 coreceptor-tropic HIV-1 strains (X4) exhibiting more positive charges than the CCR5-tropic HIV-1 strains (R5). We have previously reported the antiviral activity of the BzB-HPMA copolymers at 25, 50, and 75 mol % BzB mole incorporation, against HIV strains across clades and tropism.[33] The assays were performed using clade B R5 DU156 and clade C R5 TRO, isolated from acute sexually transmitted infections, and the pseudotyped clade B X4 WEAU. Independent of the ligand density, BzB polymers showed comparable antiviral activity across the clades and tropisms of HIV.[33] Although the HIV envelope exhibits domains of heterogeneity across viral strains, many N-linked glycans are believed to be fairly conserved, providing a potential mechanism for the observed broad spectrum activity of lectins.

4. Effect of Molecular Weight of the BzB_{50} Polymers on the Entry Inhibition Activity of the Synthetic Lectins. Having an understanding of how ligand density affects activity of the BzB functionalized polymers, we were next interested in exploring the influence of the molecular weight of the polymer at a fixed BzB functionalization on antiviral activity of the synthetic lectins. BzB functionalized polymers at 50 mol % were synthesized using varying concentrations of the initiator, yielding polymers with a range of molecular weights. As shown in Figure 3, EC_{50}'s of the polymers decreased (thus, higher activity) from 68 nM for 132 kDa BzB_{50} to 12 nM for 382 kDa BzB_{50} (2-tailed Student's *t* test $p = 0.01$). Owing to the increased number of ligands available for binding per polymer chain, increase in molecular weight of the polymers at a fixed percent BzB incorporation showed improved activity. However, the improvement in activity found with molecular weight variations was less dramatic than that seen with variation in percent BzB functionalization.

5. Antiviral Activity of BzB_{50} Polymers against Viruses Bearing Glycosylated Envelopes. Entry inhibition activity of lectins originates from their ability to bind to glycans spiked on the viral surface. Several of the enveloped viruses bear carbohydrate shields on the envelope,[42] which could be effectively targeted using lectins that nonspecifically bind to carbohydrates. Therefore, we and others in the field have tested the activity of plant, bacterial and/or synthetic lectins against lentiviruses[43] (HIV-1, HIV-2, SIV, MSV), RNA viruses[44] (VSV, Coxsackie virus B4, and respiratory syncytial virus; and parainfluenza type 3 virus, reovirus type 1, Sindbis virus, and Punta Toro virus, ebola, coronaviruses, influenza viruses), and DNA viruses[43] (herpesvirus such as cytomegalovirus) with glycosylated envelopes. Table 3 summarizes the inhibitory concentration of $BzB_{50(382kDa)}$ against the viruses we tested. We notably observed activity solely against HIV. Except for HIV, none of the other viruses were inhibited by the BzB polymers even at concentration 100-fold higher than the EC_{50} for HIV. These results are in contrast with UDA that inhibit viruses such as HIV-1, HIV-2, RSV, influenza, CMV, SIV and FIV. CV-N, an extensively reviewed and tested CBP derived from the cyanobacterium *Nostoc ellipsosporum*, demonstrated marked inhibition of viruses such as HIV-1,[39,45] SIV, ebola,[44] HCV,[46] influenza A virus[47] and HSV. The HIV-specific activity we observed with the synthetic lectins can be attributed to the fact that no other virus that was tested in this assay exhibited comparable extent of glycosylation sites and/or a similar nature of the glycans (i.e., high-mannose-type) as HIV. Since the interactions between a polyvalent construct such as the BzB-based synthetic lectin and the viral envelope depend on a multitude of factors such as the density of glycosides on the viral surface, the degree of oligomerization of the glycoprotein, spatial arrangement of saccharides and flexibility or both, it is conceivable that the synthetic lectins selectively inhibit viruses, in this particular case HIV and not just all types of viruses that bear a glycosylated envelope.

Table 3. Antiviral Activity of BzB_{50}-$HPMA_{50}$ (382 kDa) against Viruses with a Glycosylated Envelope

virus	EC_{50} (nM)[a]	virus	EC_{50} (nM)[a]
HIV-1	1.1	herpes simplex virus-1 (KOS)[b]	>1000
feline corona virus (FIPV)[c]	1000	herpes simplex virus-2 (G)[b]	>1000
feline herpes virus[c]	5000	herpes simplex virus-1TK (KOS)[b]	>1000
Punta Toro virus[d]	>40	vesicular stomatitis virus[b,e]	>1000
parainfluenza-3 virus[d]	>40	respiratory syncytial virus[e]	>1000
reovirus-1[d]	>40	Coxsackie virus B4[e]	>1000
vaccinia virus[b]	>1000		

[a] 50% Effective concentration or compound concentration producing 50% inhibition of virus-induced cytopathic effect. [b] HEL cells. [c] Crandell−Rees feline kidney cells (CRFK cells). [d] Vero cells. [e] HeLa cells.

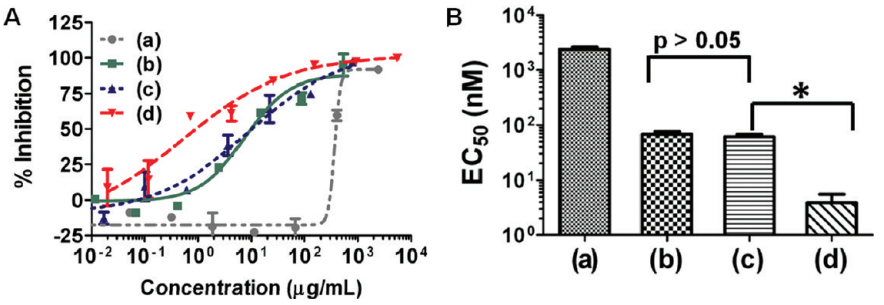

Figure 4. Effect of incorporation of sulfonic acid on the entry inhibition activity of BzB_{50} polymers. Results from the single-cycle HIV-1 infectivity inhibition in TZM-bl cells on (a) $AMPS_{10}$-$HPMA_{90}$, (b) BzB_{50}-$HPMA_{50}$, (c) physical mixture of $AMPS_{10}$-$HPMA_{90}$ and BzB_{50}-$HPMA_{50}$ and (d) copolymer BzB_{50}-$AMPS_{10}$-$HPMA_{40}$ against R5 DU156 (clade B) isolated from acute STIs. (A) Dose−response curve of the polymers and (B) EC_{50} determined by fitting a sigmoidal dose−response curve with variable Hill's slope. Two tailed Student's t test was conducted ($N = 3$, mean ± SD; $p < 0.05$). $AMPS_{10}$ showed an EC_{50} of 2361 nM ± 60 (~350 μg/mL). Physical mixture of BzB_{50}-$HPMA_{50}$ and $AMPS_{10}$-$HPMA_{90}$ showed no significant increase in antiviral activity over BzB_{50}-$HPMA_{50}$ ($p = 0.70$). However, copolymers of HMPBA and AMPS at 50 and 10 mol % functionalization, respectively, showed a dramatic increase in antiviral activity with EC_{50} of 4.0 nM ± 1.6 (~1 μg/mL), suggesting the presence of synergistic activity between HMPBA and AMPS ($^*p = 0.004$).

6. Incorporation of Sulfonic Acid in the Polymer Backbone Affords a Synergistic Increase in Viral Entry Inhibition Activity of BzB_{50} Polymers. Lead polymers from the above studies with an antiviral activity of ~10 nM ($BzB_{75(75kDa)}$ and $BzB_{50(382kDa)}$) showed poor aqueous solubility (<5 mg/mL). Considering that the activity of the BzB polymers could be compromised in the presence of proteins and other components in the vaginal environment, improving aqueous solubility of the polymers may be required in order to deliver the required drug concentrations. Kataoka et al. and others have demonstrated that the incorporation of amines into a PBA-containing polymer could lower the pK_a of the boronic acid to around 7, thereby enhancing solubility.[48,49] Incorporation of amines was also utilized by Winblade et al., who reductively aminated 4-formylphenylboronic acid to a poly-lysine-PEG backbone. The pK_a of this polymer was below 6.[36,50] To address the poor aqueous solubility of the polymers at high benzoboroxole functionalization (≥50 mol %) and/or high molecular weight polymers (≥125 kDa), we have synthesized polymers with 10 mol % 2-acrylamido-2-methylpropanesulfonic acid (AMPS), which is an anionic monomer. Incorporation of AMPS into the copolymer adds charge to the otherwise neutral HPMA backbone, increasing the aqueous solubility of the polymer at neutral pH's. Nearly 100-fold increase in aqueous solubility was observed with the incorporation of AMPS.

Since both AMPS and BzB bind to gp120, we tested both polymers individually, as a physical mixture and a copolymer of AMPS and BzB. BzB was functionalized at 50 mol % and AMPS at 10 mol % in a HPMA polymer. As shown in the dose−response curve shown in Figure 4, the physical mixture of the two polymers, BzB_{50} and $AMPS_{10}$, did not demonstrate any significant increase in activity (2-tailed Student's t test $p = 0.70$). This data suggests that the presence of $AMPS_{10}$ only in the polymer solution has no influence on the binding of BzB to gp120. In contrast, when the AMPS and BzB were copolymerized, the resulting polymer showed a more than 2 log scale decrease in EC_{50} (2-tailed Student's t test $p = 0.004$). We believe that the presence of (a) simultaneous ionic and covalent interactions; (b) steric stabilization; and (c) entropic advantage with multiple binding events may contribute to the observed synergistic improvement in antiviral activity of the copolymers over the physical mixture. The charged sulfonated polymer backbone likely facilitates initial ionic interactions with the V3 loop on gp120, thereby sterically facilitating covalent interactions between the BzB on the polymer and the glycans on gp120. This implies that, irrespective of the binding affinity, the binding event would bring along a large polymer drape to the viral surface, thus inhibiting interactions with the host cell. The BzB-AMPS copolymers show activity at concentrations (~1 to 4 nM) comparable to CBPs, such as CV-N (~0.6 nM). The BzB HPMA copolymer has also been evaluated for its potential to prevent syncytium formation in cocultures of persistently $HIV\text{-}1_{IIIB}$ infected HUT-78 and uninfected Sup T1 cells, and found to display an EC_{50} of 50 ± 2.6 nM.

Sulfated anionic polymers have previously been evaluated as a microbicide that targets the V3 loop on gp120.[51] We are aware of

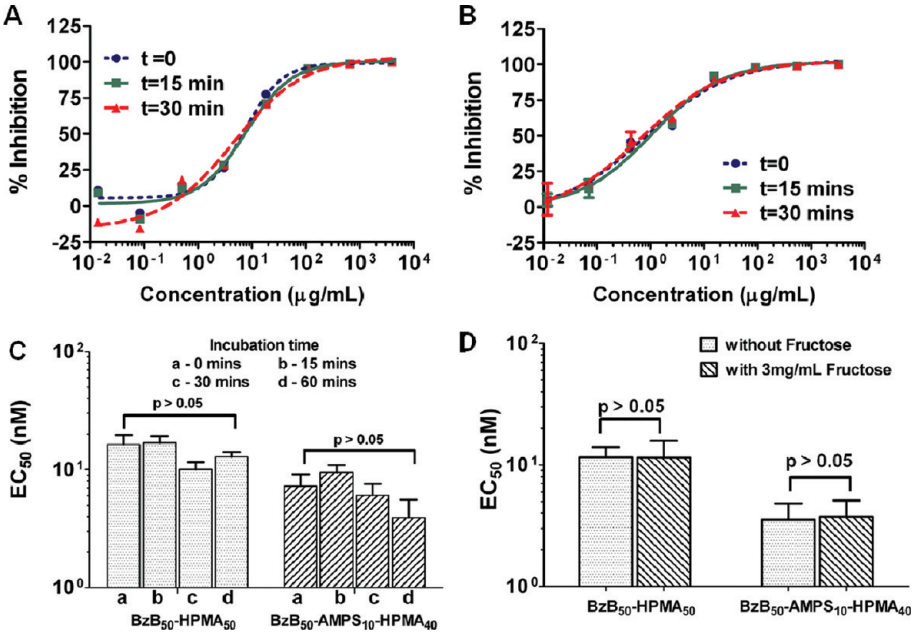

Figure 5. Effect of incubation time and fructose on the entry inhibition activity of BzB_{50} polymers. Dose−response curves of (A) BzB_{50}-$HPMA_{50}$ (382 kDa) and (B) BzB_{50}-$AMPS_{10}$-$HPMA_{40}$ against R5 DU156 as a function of preincubation time. (C) EC_{50} of both polymers as function of preincubation time. Using single factor ANOVA, independent of the incubation time, no significant difference in EC_{50} was observed ($p = 0.72$ and 0.55). (D) EC_{50} of BzB_{50}-$HPMA_{50}$ and BzB_{50}-$AMPS_{10}$-$HPMA_{40}$ in the presence of seminal concentration of fructose. Both polymers showed no significant loss in antiviral activity in the presence of fructose. Using 2-tailed, Student's t test $p = 0.44$ and 0.53 for BzB_{50}-$HPMA_{50}$ and BzB_{50}-$AMPS_{10}$-$HPMA_{40}$, respectively.

the issues associated with the sulfonated polymers for microbicide applications which include toxicity due to nonspecific binding, tropism-specific activity[41] and loss of antiviral activity in the presence of semen.[51] In an attempt to address the above issues, we synthesized polymers with small amounts of AMPS copolymerized with the biocompatible HPMAm, most likely reducing potential toxicity. Furthermore, the primary mechanism of action of the synthetic lectins is through the ability of BzB to covalently bind to gp120, which demonstrates clade and tropism independent activity.[33] The ionic interactions between AMPS and the V3 loop are an auxiliary mechanism of action which augments the overall activity of the BzB-based synthetic lectins. In summary, incorporation of AMPS in BzB_{50} yields a polymer with enhanced aqueous solubility and generates a heteromeric polyvalent presentation of ligands with superior binding to gp120.

7. Effect of Binding Kinetics on the Activity of the Synthetic Lectins. While the BzB polymers retained activity in the presence of seminal concentrations of fructose, we were next interested in asking whether the activity of these polymers is governed by thermodynamics alone or whether binding kinetics also influences the entry inhibition activity of the BzB. To evaluate the effect of binding kinetics, the BzB-APMS copolymer samples were preincubated with the virions for 0, 15, 30, and 60 min. As shown in Figure 5, irrespective of the incubation time, the copolymer samples showed 50% inhibition of HIV at ~4.5 ± 2.0 nM (2-tailed Student's t test $p > 0.05$). Unlike the α-(1−3)- and α-(1−6)-D-mannose oligomer-specific plant lectins, whose antiviral activity is up to 20-fold more pronounced upon a 1 h preincubation time with the virus,[43] the BzB-based synthetic lectins show superior and rapid inhibition of HIV entry, irrespective of preincubation with the virus particles.

8. Effect of Seminal Concentrations of Fructose on the Activity of the Synthetic Lectins. Several antiviral agents such as sulfated polymers,[51] intended to be delivered vaginally, show compromised or complete loss of antiviral activity in the presence of seminal fluid. Seminal fluid is a rich source of proteins, enzymes, salts, immune cells and sugars.[52] Of these, the presence of high concentrations of simple sugars such as fructose especially may raise concerns for lectin-based therapeutics. As seen by our results from the ARS assay, BzB show exceptionally high affinity for fructose. Therefore, we tested the ability of the BzB_{50}-$AMPS_{10}$-$HPMA_{40}$ copolymers and BzB_{50}-$HPMA_{50}$ (383 kDa) to neutralize HIV entry in the presence of average seminal fructose concentration (3 mg/mL) of fructose. As shown in Figure 5D, activity of the synthetic lectins was fully preserved in the presence of fructose, suggesting that fructose from seminal fluid would not compromise the antiviral activity of the synthetic lectins (2-tailed Student's t test $p > 0.05$). This feature of the synthetic lectins is especially attractive, as some of the microbicides tested in the past, including CV-N,[39] have shown compromised activity in the presence of seminal fluid.[51]

9. Biocompatibility Evaluation in MatTek VEC-100 Reconstructed Human Vaginal Tissue. Enabling this research for microbicide development requires careful determination of how interactions of BzB polymers with the epithelial cells of cervicovaginal tissue may impact their viability and cytokine production. Owing to the ability of the BzB to bind to glycoproteins and the ability of sulfonic acid to nonspecifically bind to positively charged surfaces; it is critical to assess the safety of the BzB-AMPS copolymers in vaginal tissue. The VEC-100 tissue purchased from MatTek Corporation serves as a suitable model for evaluating the tissue-level toxicity of topical microbicides.[53] As can be seen in Figure 6, BzB-AMPS copolymers showed no significant loss in tissue viability when compared to the nontoxic HPMA control even at concentrations ~1000-fold the in vitro EC_{50}, after 72 h of exposure (≥90% viability). The irritation potential of test polymers was evaluated by ELISA for inflammatory cytokines associated with mucosal toxicity (i.e., IL-8, IL-1, IL-6,

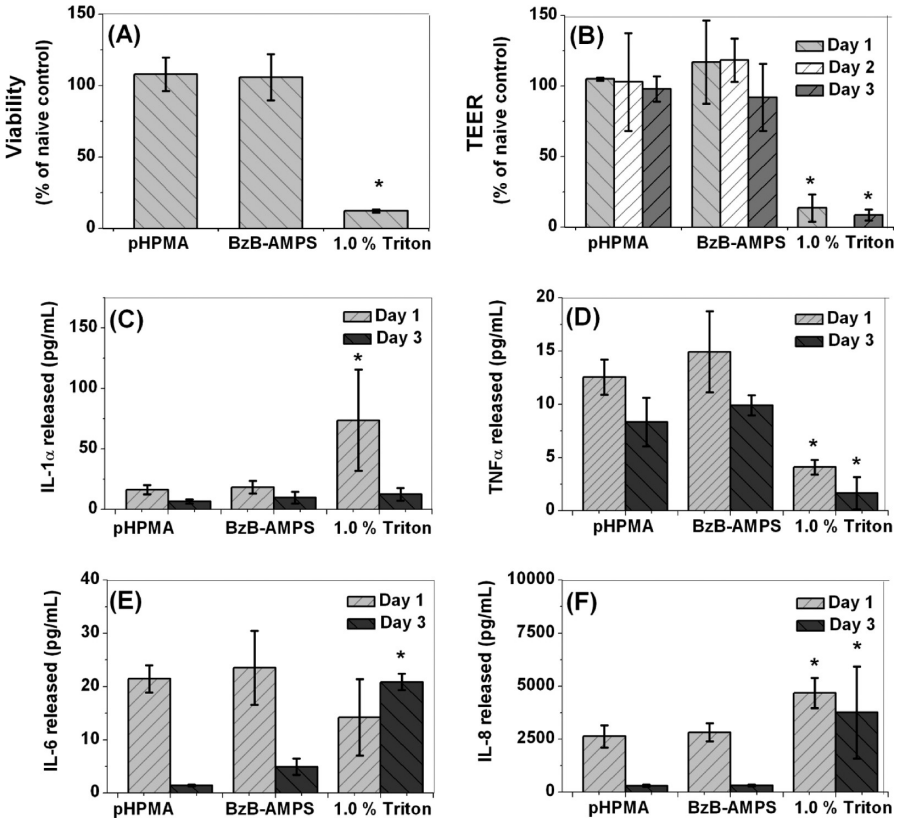

Figure 6. Safety evaluation of BzB_{50}-$AMPS_{10}$-$HPMA_{40}$ on VEC-100 reconstructed human ectocervical tissue after three repeated exposures. pHPMA and Triton served as nontoxic and toxic controls, respectively. (A) Percentage viability of the tissue determined using the MTT assay at the end of day 3, (B) TEER measurements graphed as percentage of the no-treatment control for days 1−3. (C−F) Proinflammatory cytokine IL-1α, TNFα, IL-6 and IL-8 release for days 1−3. Tissues treated with BzB_{50}-$AMPS_{10}$-$HPMA_{40}$ showed no symptoms of loss in tissue viability or elevation in cytokine levels when compared to pHPMA-treated nontoxic control (N = 3, mean ± SD, 2-tailed Student's t test; $^*p < 0.05$).

TNFα) using human cytokine kits (R&D System). Our investigations on biocompatibility revealed no significant alterations in tissue viability, cytokine levels, tissue morphology or the barrier properties of the epithelium. In summary, these results suggest that polymers functionalized with BzB moieties are likely to be nontoxic and are likely to have a high therapeutic index, allowing higher concentrations of the polymer to be dosed safely, thereby minimizing the impact of competing binding sites that may be present in the vaginal environment.

CONCLUSION

This work is relevant to the development of new agents capable of rendering the virus inactive during female-to-male heterosexual transmission of HIV-1. An ideal microbicide candidate must exhibit potency, cross-clade broad spectrum activity, selective inhibition of the pathogen, mass producibility and biocompatibility. While current research on CBPs suggests that they possess several of the above favorable properties to qualify as potential microbicide candidates, their success is largely impeded by the potential mitogenic properties, cost of production and isolation and purification in mass quantities. In this regard, our approach of developing synthetic lectins using the BzB moieties meet all of the above criteria of an ideal microbicide. The BzB moiety can readily be incorporated into a variety of biocompatible polymer backbones and polymeric constructs which can be synthetically modified to enhance activity and specificity. Additionally, the BzB-based synthetic lectins also present an affordable and scalable product that could be delivered to pandemic regions.

AUTHOR INFORMATION

Corresponding Author

*University of Utah, Department of Bioengineering 20 S. 2030 E., Rm. 108, Salt Lake City, UT 84112-9458. Tel: (801) 505-6881. Fax: (801) 585-5151. E-mail: patrick.kiser@utah.edu.

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