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Poly(styrene-*alt*-maleic anhydride) derivatives as potent anti-HIV microbicide candidates

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ABSTRACT

Topical microbicides offer women the opportunity to protect themselves from sexual HIV transmission under their own control. A series of poly[styrene-*alt*-(maleic anhydride)] derivatives were prepared by amidation or hydrolysis of the anhydride moiety. The derivatives were shown to be of low cell toxicity and effectively inhibited HIV-1 infections in an in vitro cellular model. Poly[styrene-*alt*-(maleic acid, sodium salt)] was the most potent inhibitor, being 100-fold more potent than dextran sulfate suggesting its potential application as a new class of polyanionic microbicides.

Acquired Immunodeficiency Syndrome (AIDS) caused by the human immunodeficiency virus type 1 (HIV-1) has become a worldwide concern. Currently, more than 4 million new HIV-1 infections occur each year, mainly through heterosexual contact.[1] Due to intrinsic physiological differences, women are twice as likely to be subjected to HIV-1 infection through unprotected heterosexual intercourse.[2] Social or behavioral factors also render women more vulnerable than men to sexual HIV-1 infection as women are less likely to determine condom use and are thus more likely to be subjected to non-consensual sex. Consequently, in sub-Saharan Africa women living with AIDS (13 million) outnumbered infected men (9 million) in 2005 while there were as many infected men as infected women in 1985.[3] Given the increasing population with AIDS and the dependence of women on male cooperation to prevent sexual HIV-1 transmission, methods that enable women to protect themselves under their own control would be valuable in combating the global HIV-1 epidemic.

Topical microbicides offer the promising options for women against sexual HIV-1 transmissions. Being natural or synthetic substances formulated as a gel, or a time-released suppository, etc., microbicides could be inserted directly into the vagina to prevent HIV-1 from entering host cells.[2] In the initial stage of HIV-1 infection, the viral envelope glycoprotein gp120 binds to host cell surface receptor CD4, leading to a conformational change in gp120. The conformation change enables gp120 to recruit its co-receptors CCR5 or CXCR4. The recruitment results in further conformational change of gp120 and the exposure of glycoprotein gp41. Gp41, non-covalently bound to gp120, is originally buried within the viral envelope. Once exposed, gp41 assists in the fusion of HIV and the host cell membranes, leading to effective viral infection.[3–5] Due to the essential roles of gp120 in HIV infections, inhibitors that can block the interaction of gp120 with its receptor or co-receptors have potential antiviral applications. Based on the inhibitory mechanisms, candidate microbicides have been categorized into detergents, pH modifiers, reverse transcriptase inhibitors, chemokine receptor blockers, antibodies, and polyanionic compounds.[6,2] Exemplary anionic compounds that have been considered as potential microbicides include sulfated polysaccharides,[7,8] dextran or dextrin sulfate,[9–12] poly(naphthalene sulfonate),[13,14] poly(styrene-4-sulfonate),[15] sulfated dendrimers[16] and carboxylate bearing compounds.[17,18] The inhibitory activity of anionic compounds have been attributed to the formation of salt linkages between the positively charged V3 loop of gp120 and the negatively charged groups on the polyanionic compounds,[19–21] which prevents viral entry into host cells. Here we report the potent

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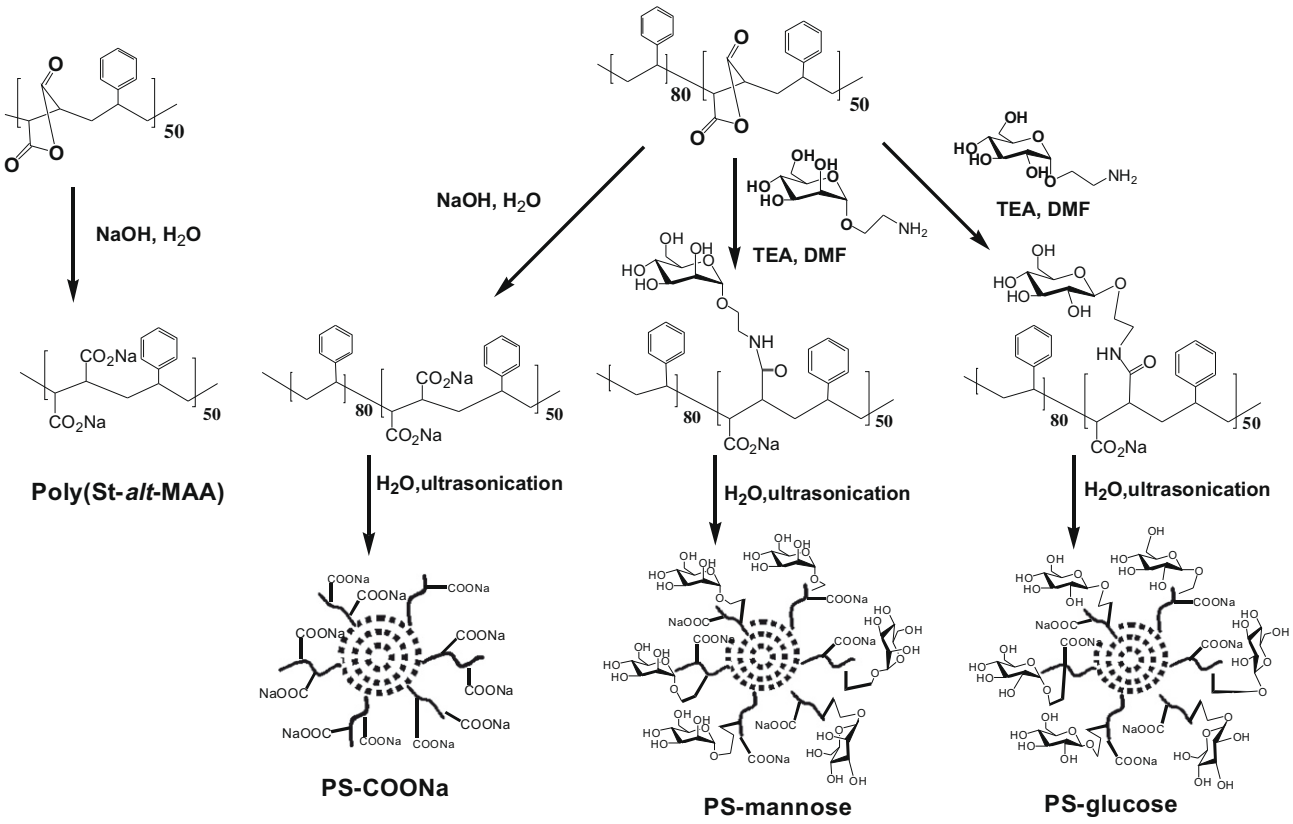

Scheme 1. Synthetic routes and morphologies of poly[styrene-*alt*-(maleic anhydride)] derivatives. Amidation of poly[styrene-*alt*-(maleic anhydride)]: Poly{styrene-co-[(maleic anhydride)-alt-styrene]} (20 mg), selected monosaccharide (30 mg) [e.g., β-1-*O*-(2′-aminoethyl)-D-glucopyranoside] and triethylamine (0.1 mL) were added to a flask containing anhydrous DMF (1 mL). The solution was stirred at room temperature overnight, followed by addition of water (9 mL) and was then ultrasonicated for 40 min at room temperature. The solution was dialyzed against deionized water using a dialysis tube (MWCO 3000) and then filtered through a 0.22 μm filter to produce the corresponding nanoparticles. Hydrolysis of the polymers: poly(styrene-*alt*-maleic anhydride) or poly{styrene-co-[(maleic anhydride)-*alt*-styrene]} (20 mg) was suspended in an aqueous solution of sodium hydroxide (1 M, 10 mL). The mixture was stirred at room temperature for 3 h, neutralized with acetic acid, and then dialyzed against deionized water using a dialysis tube (MWCO 3000).

inhibitory effects and cell toxicity of poly[styrene-*alt*-(maleic anhydride)] derivatives on cellular models and the possible use of .this class of anionic polymers as topical microbicides.

Following reported procedures,[22] poly{styrene-*co*-[styrene-*alt*-(maleic anhydride)]} and poly[styrene-*alt*-(maleic anhydride)] were synthesized from styrene and maleic anhydride via free radical polymerization. A group of poly[styrene-*alt*-(maleic anhydride)] derivatives were prepared as described in Scheme 1. Poly[styrene-*alt*-(maleic acid, sodium salt)], abbreviated as poly(St-*alt*-MAA), was prepared via hydrolysis of poly[styrene-*alt*-(maleic anhydride)] in an aqueous solution of sodium hydroxide. Poly(St-*alt*-MAA) exists in aqueous solutions as linear polymers with negatively charged carboxylate groups. Similarly, poly{styrene-*co*-[styrene-*alt*-(maleic acid, sodium salt)]} (designated as PS-COONa) was obtained from the alkaline hydrolysis of poly{styrene-*co*-[styrene-*alt*-(maleic anhydride)]}. PS-COONa, an amphiphilic diblock polymer, self-assembled in water into hairy micellar nanoparticles with surface anchored polymeric poly[styrene-*alt*-(maleic acid, sodium salt)] chains[22] (Scheme 1). The mean diameter of PS-COONa was found to be 35.2 nm as determined by dynamic light scattering (Fig. 1). Poly{styrene-*co*-[styrene-*alt*-(D-mannopyranosyl-α-*O*-ethylamidomaleic acid)]} (PS-mannose) and poly{styrene-*co*-[styrene-*alt*-(D-glucopyranosyl-α-*O*-ethylamidomaleic acid)]} (PS-glucose) were prepared by amidation of the anhydride moiety with β-1-*O*-(2′-aminoethyl)-D-mannosylpyranoside and β-1-*O*-(2′-aminoethyl)-D-glucopyranoside separately (Scheme 1).[23] The diameters of PS-mannose and PS-glucose were determined to be 31.4 and 20.0 nm separately (Fig. 1). The structurally related derivatives were evaluated for the effects of morphology (liner polymer vs hairy nanoparticles) and selected functional groups (mannose vs glucose) on antiviral activity and cell toxicity.

To assess the inhibitory efficacy of poly[styrene-*alt*-(maleic anhydride)] derivatives, TZM-bl cells were employed as the model host cells for quantitative analysis of HIV-1 infectivity.[24,25] TZM-bl, a Hela cell line stably expressing a large amount of CD4, CCR5 and CXCR4, is highly sensitive to infection with diverse isolates of HIV-1.[24] TZM-bl cells also harbor an integrated β-Gal reporter gene that codes for β-galactosidase under the control of the HIV-1 promoter.[25] Activation of the β-Gal reporter gene upon HIV-1 entry leads to expression of β-galactosidase. Enzymatic hydrolysis of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) catalyzed by β-galactosidase results in a blue precipitate inside cells. Experimentally, various doses of each derivative were first co-cultured with HIV-1_{pNL4-3} and TZM-bl cells for 48 h, and then TZM-bl cells were stained with X-gal. The pNL4-3 virus is subtype B of HIV-1 and infects host cells via the cell surface CXCR4 receptor. The percentages of cells stained blue, an indicator of HIV-1 infectivity, were recorded as functions of the doses of the derivatives (Fig. 2). As shown in Figure 2, HIV-1_{pNL4-3} infections on TZM-bl cells were effectively inhibited by all the poly[styrene-*alt*-(maleic anhydride)] derivatives at various concentrations. Among the derivatives, poly(St-*alt*-MAA) was the most potent with an IC_{50} value of 0.01 μg/mL (Fig. 2). The IC_{50} value is the compound concentration required to inhibit virus infection by 50%. For direct comparison of the inhibition efficiencies of the titled derivatives

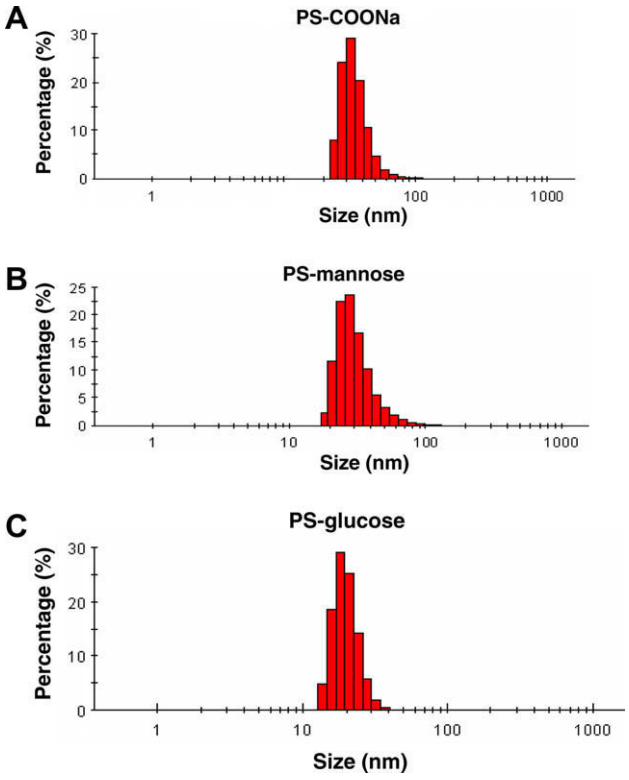

Figure 1. Determination of the diameter size of nanoparticles assembled from poly{styrene-*co*-[styrene-*alt*-(maleic anhydride)]} derivatives by dynamic light scattering.

with the well studied candidate microbicides, heparin[26] (1.5 kDa) and sulfated dextran[27,28] (500 kDa) were included in our inhibition assays. The IC_{50} values of heparin and sulfated dextran on HIV-1_{pNL4-3} infectivity were 0.5 μg/mL and 1.0 μg/mL, respectively (Fig. 2), which were in good agreement with reported values.[27,28] Of the titled derivatives, poly(St-*alt*-MAA) was 50–100 times more potent than heparin and sulfated dextran against HIV-1_{pNL4-3} infection. PRO2000 is composed of naphthalene sulfonate polymers and is currently in clinical trial phase III.[14] PRO2000 was reported to inhibit HIV-1 infections with an IC_{50} value of 15 μg/mL using similar assays.[14] Compared with PRO2000, poly(St-*alt*-MAA) is three orders of magnitude (1500-fold) more potent against HIV-1 infection. Based on the in vitro cellular assay, poly(St-*alt*-MAA) is far more superior than most-if not all-of the reported polyanionic microbicides in inhibiting HIV-1_{pNL4-3} infection.

Several subtypes of HIV-1 circulate among human beings worldwide, including subtype B in the United States and subtype C in Africa. Subtype C binds to host cell surface CCR5 in the initial stage of its life cycle while Subtype B targets cell surface CXCR4 receptor.[29] To probe the inhibition efficacy of poly(St-*alt*-MAA), the most potent inhibitor we have identified, against different HIV-1 strains, the infectivity of the pMJ4 virus on TZM-bl cells was determined using the aforementioned assay. The pMJ4 virus is subtype C of HIV-1. Poly(St-*alt*-MAA) inhibited HIV-1_{pMJ4} infection approximately 300-fold less effectively (IC_{50}: 3.0 μg/mL, Fig. 3) than HIV-1_{pNL4-3}. Although capable of inhibiting both subtype B and C of HIV-1, poly(St-*alt*-MAA) is more effective against subtype B, which targets cell surface CXCR4 receptor.

Nature utilizes multivalency to achieve stable protein-glycan binding for the initiation of subsequent biological activities despite the fact that glycan-binding proteins typically bind their monovalent ligands with low affinity.[30] Poly(St-*alt*-MAA) (MW 1 Kd) is too small to bridge trimeric gp120 (MW 120Kd) or multiple gp120 on the virus surface. Given the size of the HIV-1 viron (120 nm), we were interested to see if poly[styrene-*alt*-(maleic acid, sodium salt)] displayed on a nanoparticle surface could allow simultaneous multivalent binding of the anionic polymers with multiple gp120 molecules on HIV-1. Multivalent interactions between polyanionic nanoparticles and multiple gp120 molecules might be beneficial for enhanced inhibition activity. Poly{styrene-*co*-[(maleic acid, sodium salt)-*alt*-styrene]} is an amphiphilic diblock copolymer capable of forming hairy micellar nanoparticles with corona comprised of multimeric hydrophilic linear polymers and a hydrophobic core in aqueous solutions. The nanoparticles (NP-COONa) were formed with an average diameter of 35 nm (Fig. 1A). To our surprise, the potency of PS-COONa against HIV-1 infection (IC_{50}: 0.5 μg/mL, Fig. 2) was 50 times weaker than poly(St-*alt*-MAA). The decreased inhibition efficiency indicated that the anticipated multivalent nanoparticle–virus interactions did not occur in the assay conditions.

Microbicides conferred with dual or multiple biomedical functions, like contraceptive or mucoadhesive properties, are

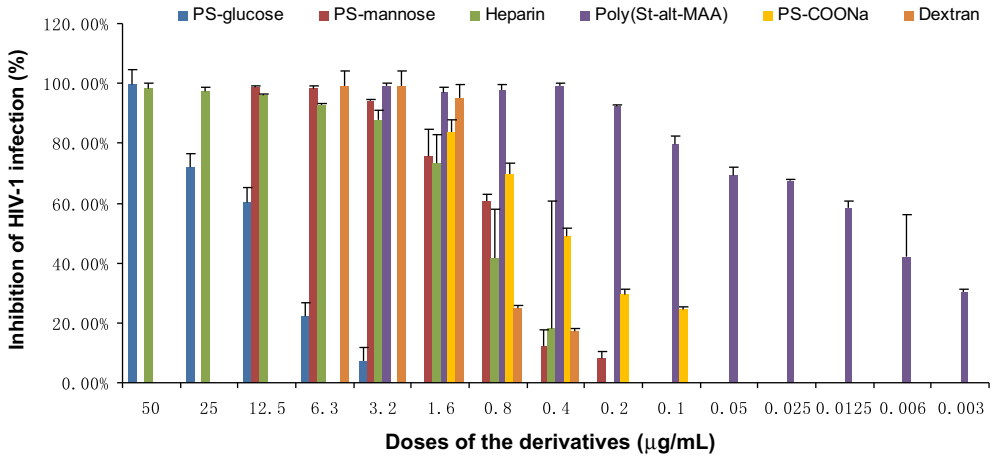

Figure 2. Inhibitory effects of poly[styrene-*alt*-(maleic anhydride)] derivatives on HIV-1_{pNL4-3} infection of TZM-bl cells. The assay was performed according to a reported procedure.[42] Briefly, HIV-1_{pNL4-3} was produced from 293FT cells and quantitated using a P24 enzyme-linked immunosorbent assay.[43] TZM-bl cells were seeded in 48-well plates (1×10^5/well) and cultured in DMEM at 37 °C overnight in a CO_2 incubator. The medium was then removed and then 75 μL of DMEM was added to each well containing HIV-1_{pNL4-3} (2.96×10^4 pg/mL), DEAE (20 μg/mL), and 25 μL of serially twofold diluted compounds. The plates were cultured at 37 °C for 2 h followed by addition of 500 μL DMEM. The plates were further incubated for 48 h and then the cells were fixed. After aspiration of the fixing solution, cells were washed with PBS buffer and then stained with X-Gal staining solution for 20 min. Infected cells were counted through an ELISPOT reader.

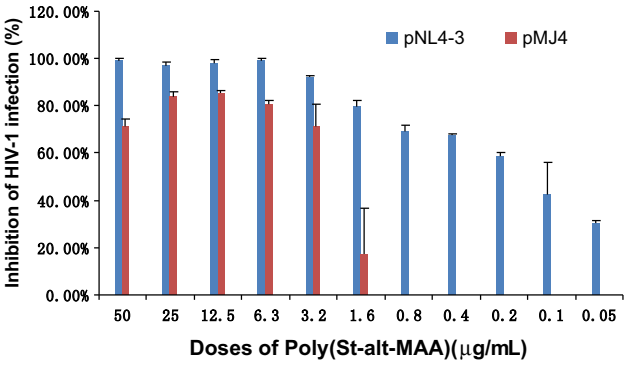

Figure 3. Inhibitory effects of Poly(St-*alt*-MAA) on the HIV-1_{pMJ4} infectivity of TZM-bl cells as compared to HIV-1_{pNL4-3}.

advantageous for practical applications. Genital HIV-1 infection and sperm fertilization share the same anatomical and functional context.[31] It is valuable to develop anti-HIV microbicides which could simultaneously target sperm cells to prevent unintended pregnancies. Located on the sperm cell surface, a mannose binding lectin is essential for sperm-oocyte fusion.[32,33,31] Blocking of this receptor by D-mannose monosaccharide has been shown to be able to prevent fertilization.[34,35] PS-mannose assembled into hairy micellar nanoparticles with multivalent mannose displayed on the surface with mean diameter of 31 nm (Scheme 1, Fig. 1). PS-mannose has been shown to bind to human sperm cells with high affinity and caused aggregation of the sperm cells, indicating its potential application as a contraceptive agent.[23] Hence inhibition of PS-mannose of HIV-1 infection on TZM-bl cells was performed. The IC_{50} value was 0.8 μg/mL and was comparable to the inhibition efficiency of PS-COONa (Fig. 2). Due to the potent inhibition of HIV-infections and its ability to bind sperm cells with high affinity, PS-mannose might find a dual application as a contraceptive microbicide. As the structural analog of PS-mannose, PS-glucose was assayed as the control of PS-mannose against HIV-1 infection. The IC_{50} value for the inhibition of HIV-1 infection by PS-glucose was 9 μg/mL, 10-fold weaker than PS-mannose. Mannose differs from glucose by inversion of the C2 chiral center. This apparently simple change leads to the drastically different inhibition efficiencies of PS-mannose and PS-glucose, which is probably due to their differential interactions with some cell surface receptors. It is viable to introduce functional molecules into poly[styrene-*alt*-(maleic anhydride)] to produce derivatives with new therapeutic functions while maintaining various extent of antiviral activity. Since thiol containing polymers have been shown to be mucoadhesive,[36,37] incorporating thiol-containing molecules into the poly[styrene-*alt*-(maleic anhydride)] might produce mucoadhesive microbicides that can attach to mucins in the genital tract for sustained anti-HIV-1 activity.

In addition to inhibition efficiency, safety is another important factor needed to be taken into account for practical microbicides. To evaluate the cell toxicities of poly[styrene-*alt*-(maleic anhydride)] derivatives, time course studies were performed to examine the effects of each derivatives on T cell growth and cell viability at various doses. In all the cases, cell growth slowed down moderately (20–30%), even after 6 days of co-culture with doses that can effectively suppress HIV-1 infections. No significant cell death was observed (Fig. 4), suggesting that the derivatives are of low cell toxicity. Particularly, in the presence of poly(St-*alt*-MAA) cells remains fairly healthy at the dose (1.6 μg/mL) that is four times higher than the threshold dose amount required to fully inhibit virus infections (0.4 μg/mL). Biodistribution and immunogenicity studies have shown that poly(St-*alt*-MAA) was nontoxic and nonimmunogenic in vivo.[38] Conjugation of poly(St-*alt*-MAA) with the antitumor protein neocarcinostatin has been marketed in Japan to treat primary hepatoma and secondary tumor of the liver.[38] The low toxicity of poly(St-*alt*-MAA) as shown in our assays

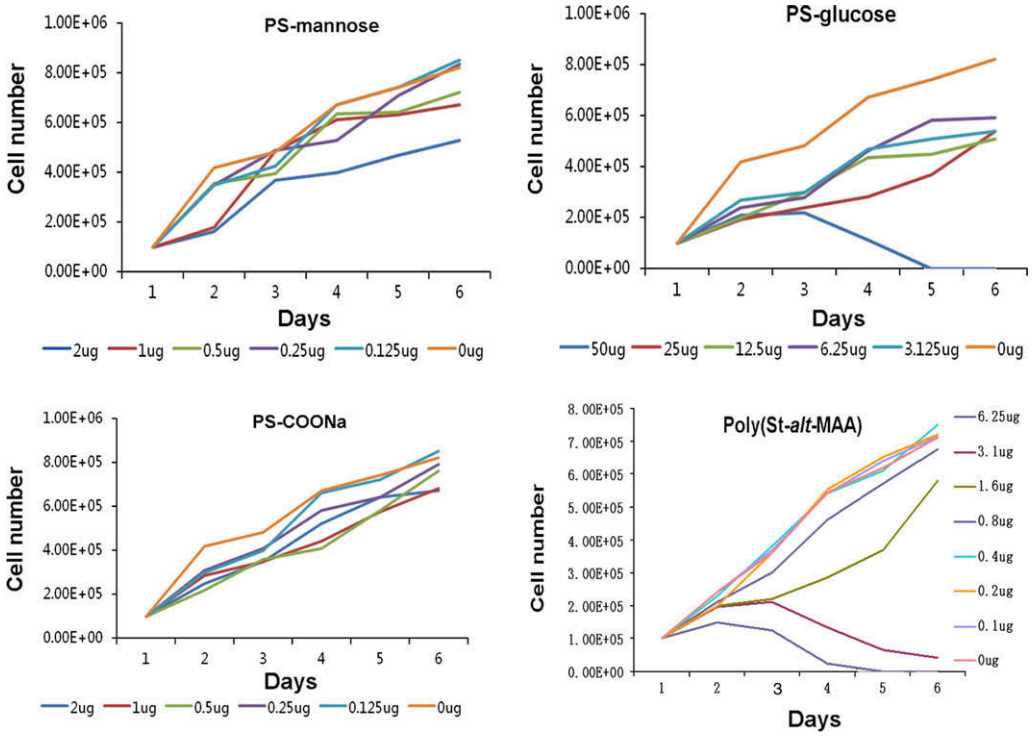

Figure 4. Cell toxicities of poly[styrene-*alt*-(maleic anhydride)] derivatives on T cells. MT4 cells were seeded in 48-well plates (1×10^5 cells/well) in DMEM medium (500 μL) supplemented with various amounts of the derivatives. The cells were cultured at 37 °C with 5% CO_2. Every 24 h, an aliquot of the cells was taken out and stained with trypan blue. Cell number and cell viability were determined using the trypan blue exclusion test.

strongly suggest that poly(St-*alt*-MAA) is safe as a potential topical microbicide.

Successful microbicides need to be potent, safe, as well as affordable. There has been dramatic success in AIDS treatments largely achieved through the use of antiretroviral therapy.[39–41] Although the success has tremendously improved survival and quality of life, current therapies remain largely inaccessible to low-income populations. Poly[styrene-*alt*-(maleic anhydride)] can be easily prepared in a large scale via a one-step reaction under mild conditions. The derivatives can be produced in high yields via alkaline hydrolysis of the anhydride moieties. The low-cost production of poly[styrene-*alt*-(maleic anhydride)] derivatives make it suitable for applications in developing countries.

In conclusion, these preliminary data demonstrate that poly[styrene-*alt*-(maleic anhydride)] derivatives are of low cell toxicity and are active against HIV-1 infections in an in vitro cellular model. To our knowledge, poly(St-*alt*-MAA) was the most potent inhibitor against HIV-1 infection with efficacy superior to most microbicide candidates.[14,27] Given the in vivo safety demonstrated by the success of its bioconjugate in anticancer therapy[38] and its low-cost production, poly(St-*alt*-MAA) derivatives are potential microbicides worthy of further biological evaluations. Novel biomedical properties could be conferred to the derivatives by coupling a selected functional group with the anhydride moiety via reliable amidation chemistry, which might be beneficial for practical applications using the derivatives to control the global HIV-1 epidemic.

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