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Research paper

pH-responsive delivery of Griffithsin from electrospun fibers

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ABSTRACT

Human immunodeficiency virus (HIV-1) affects over 36 million people globally. Current prevention strategies utilize antiretrovirals that have demonstrated protection, but result in antiviral resistance, adverse toxicity, and require frequent administration. A novel biologic, griffithsin (GRFT), has demonstrated outstanding safety and efficacy against laboratory and primary HIV isolates and against intravaginal murine herpes simplex virus 2 (HSV-2) challenge, making it a promising microbicide candidate. However, transient activity and instability remain concerns surrounding biologic delivery, particularly in the harsh environment of the female reproductive tract (FRT). Recently, electrospun fibers (EFs) have demonstrated promise for intravaginal delivery, with the potential to conserve active agent until release is needed. The goal of this study was to fabricate and characterize pH-responsive fibers comprised of poly(lactic-*co*-glycolic acid) (PLGA) or methoxypolyethylene glycol-*b*-PLGA (mPEG-PLGA) with varying ratios of poly(*n*-butyl acrylate-*co*-acrylic acid) (PBA-*co*-PAA), to selectively release GRFT under pH-conditions that mimic semen introduction. Fibers comprised of mPEG-PLGA:PBA-*co*-PAA (90:10 w/w) demonstrated high GRFT loading that was maintained within simulated vaginal fluid (SVF), and pH-dependent release upon exposure to buffered and SVF:simulated semen solutions. Moreover, GRFT fibers demonstrated potent *in vitro* efficacy against HIV-1 and safety in vaginal epithelial cells, suggesting their future potential for efficacious biologic delivery to the FRT.

1. Introduction

Sexually transmitted infections (STIs) affect hundreds of millions of people globally, with more than one million new infections each day [1]. Infection with human immunodeficiency virus (HIV) has become increasingly common in women, with disproportionate rates seen in minority populations [2]. In addition, the majority of HIV-infected women also experience co-infection with herpes simplex virus type 2 (HSV-2), which impacts over 500 million people [3]. HSV-2 infection has been shown to significantly enhance HIV acquisition by as much as 2 to 7-fold [4,5]. Correspondingly, the challenges in HSV-2 prevention and treatment, combined with this high global incidence and propensity for co-infections, contribute to the need for multipurpose platforms that prevent both HSV-2 and HIV infections. With no cure currently in place, there is a crucial need to develop a safe and effective prevention method against a variety of STIs including HIV and HSV-2.

To date, oral and topical pre-exposure prophylaxis (PrEP) have relied primarily on antiretroviral (ARV) drugs to inhibit infection – while lacking the integration of biological agents. Furthermore, traditional oral and topical PrEP approaches have often focused on single indication products that have demonstrated efficacy against one type of viral infection, necessitating a combination of two or more agents in one therapy. The development of a platform that can prevent one or multiple viral/bacterial infections, while also increasing user compliance via a prolonged- or inducible-delivery platform, is urgently needed [6–11]. However, continued challenges of PrEP include the need for lifelong daily user adherence; potential renal and bone toxicity; associated decreases in condom use; and the long-term development of ARV resistance [12,13]. Moreover, to date, few biological agents have been explored to mitigate infection.

To address these multipurpose needs, an antiviral lectin, Griffithsin (GRFT), is currently being developed as a potent entry inhibitor against

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a variety of infection types. Originally identified and purified from the red alga *Griffithsia* sp. [14], GRFT is considered a promising microbicide candidate to prevent the sexual transmission of HIV. Of both synthetic drugs and biologically-based inhibitors, GRFT has potent anti-HIV activity in the picomolar range against both laboratory and primary isolates of HIV, and inactivates HIV-1 almost immediately upon contact [15]. Additionally, a 0.1% GRFT gel has demonstrated protection in mice against intravaginal HSV-2 challenge, demonstrating its potential against both HSV-2 and HIV-1 infections [16]. Moreover, GRFT has an excellent safety profile [14,17–20], negligibly induces pro-inflammatory cytokines [20,21], and has demonstrated synergy with ARV agents [22], suggesting the benefits of future co-administration strategies. Finally, GRFT is being developed as a low-cost alternative with the ability to manufacture in large-capacity and possesses environmental stability, prompting its current testing in clinical trials [15,16].

Despite the promise of GRFT and other microbicide candidates, significant hurdles face the clinical delivery of many active agents. A common theme, broadly and irrespective of application, is that existing topical dosage forms often necessitate daily administration; have low intravaginal residence time; and contribute to messiness via leakage. These issues adversely impact both user adherence and efficacy [6–11] and often provide only transient prophylactic or therapeutic benefit [23,24]. In particular, vaginally-administered gels are challenged with leakage from the vaginal cavity and remaining present to deliver active agents [25–27]. While, intravaginal rings offer longer delivery durations and durability [28], the high temperature processing conditions often associated with fabrication may prove challenging for the incorporation of biologics. Additionally, the administration of biological active agents may necessitate new considerations for delivery, to provide sustained- or inducible-release and protection of more rapidly degradable biologics. Given these challenges, next-generation topical formulations should be convenient and easy to administer, inexpensive to produce, and provide stability for and efficacy of incorporated agents for an appropriate time frame surrounding coitus.

Recently, polymeric electrospun fibers (EFs) have been investigated as a new delivery platform for reproductive applications, demonstrating both on-demand and sustained protection against HSV-2 and HIV-1 infections [29–34]. However, one of the challenges of delivery vehicles, including EFs, is that to provide adequate protection they must release therapeutically relevant concentrations of active agents for the duration of use. This often requires frequent administration and highly localized doses to maintain adequate release for prolonged durations. While user adherence may be increased by developing a product that necessitates less frequent application, designing a dosage form that is efficacious regardless of administration time is challenging. Many sustained-release formulations undergo a “burst” release phase, where a significant fraction of active agent is released within the early hours of delivery – regardless of whether this time frame is suitable for protection [35].

An alternative approach is to design a product that requires less frequent dosing, by inducing the release of active agent only when needed, in response to microenvironmental cues. This strategy has the potential to conserve active agent from unnecessary release, provide protection independently of administration time, and deliver active agents directly to the target site of virus entry. One such cue in the reproductive tract, increased pH, is associated with semen infiltration peri- and post-coitus. While the “normal” vaginal pH ranges from 4.0 to 5.0, exposure to semen (pH ~ 7.5) increases the local pH to more neutral levels. We expect a pH-responsive delivery vehicle that responds to increases in intravaginal pH, will only release active agent when triggered by semen, while maintaining the bioactivity and payload of encapsulated biologics under non-coital conditions.

While pH-responsive delivery has been used in a variety of drug delivery applications [36–42], thus far pH-responsive dosage forms are in the early stages of development for delivery to the female reproductive tract (FRT) [27,39,43–46]. Prior to the use of electrospun fibers for intravaginal applications, temperature and pH sensitive hydrogels were developed to impart the dual advantages of semen-triggered release and vaginal distribution and retention prior to intercourse [27]. Hydrogels with pH-responsive properties have been shown to release effective concentrations of antivirals. However, hydrogels tend to provide more transient protection due to their propensity for leakage from the FRT. Similarly, polymeric NP platforms comprised of poly(lactic-*co*-glycolic acid) (PLGA) and S-100 Eudragit® blends were evaluated to provide pH-responsive release of the antiretroviral reverse transcriptase inhibitors, tenofovir and TDF [43]. Increased S-100 ratios resulted in decreased encapsulation efficiency, while conversely providing improved pH-dependent release. Similar studies assessed the mucosal delivery of pH-sensitive Eudragit S-100 NPs loaded with hydrophilic or hydrophobic molecules [39], demonstrating retention of molecules within NPs under acidic intravaginal pH and releasedupon exposure to more neutral pH conditions. This study additionally demonstrated the uptake and biocompatibility of NPs in vaginal cells [39]. Most recently, spray dried mucoadhesive and pH-responsive TFV microspheres prepared from polymethacrylate salts were fabricated, resulting in ~90% release within the first hour, while demonstrating biocompatibility and mucoadhesivity to vaginal cells and porcine vaginal tissue [44].

Relative to gel and NP delivery platforms, electrospun fibers have recently emerged as an alternative intravaginal delivery platform that offer a durable stationary reservoir of encapsulated agents. However, many of these studies have focused on the delivery of antibiotics or ARVs, relative to new biologics [39,43]. One of the first studies to investigate pH-responsive fibers for vaginal applications, demonstrated that cellulose acetate phthalate (CAP) fibers highly incorporated the reverse transcriptase inhibitors etravirine and TDF, and the hydrophilic dye rhodamine [46]. The CAP polymer, itself a potent antiviral, is minimally soluble in acidic conditions, and the addition of SSF rapidly dissolved the CAP fibers, releasing the encapsulated drugs. While this quick degradation was attributed to the natural (vs. synthetic) polymer chemistry, the fiber degradation raised concerns over long-term structural integrity as well as corresponding protection, prompting the development of fibers with improved mechanical properties. To address this need, coaxial fibers, comprised of a polyurethane core and CAP shell layer, were fabricated to provide pH-inducible release of rhodamine, while demonstrating enhanced mechanical properties [47]. Finally, fibers comprised of Eudragit L-100 encapsulating horseradish peroxidase and alkaline phosphatase were fabricated using emulsion electrospinning [48]. These fibers modulated protein release in response to pH, while preserving protein activity. In another study, pH-responsive fibers comprised of poly(methacylic acid-*co*-methyl methacrylate), encapsulating the ARVs, dapivirine and etravirine, were fabricated [49]. These fibers demonstrated sustained-release of therapeutics within acidic conditions, while the fibers rapidly dissolved in alkaline pH, to provide encapsulant release.

While a variety of pH-responsive platforms have demonstrated promise against STIs, many of these platforms, inclusive of electrospun fibers, have focused on the delivery of antibiotics or ARVs, relative to new biologics [39,43]. Recently, we and others have developed EFs as an efficacious platform to provide sustained-delivery of antiviral drugs to the FRT [29–34]. Building upon this work, the goal of this project was to develop and test pH-responsive EFs that incorporate the antiviral lectin, GRFT. Griffithsin fibers were designed to address the needs of an on-demand delivery system, while providing a delivery vehicle that may reduce the frequency of daily administration. It is well known that poly (acrylic acid) (PAA) has been used to fabricate a variety of pH-responsive dosage forms [50,51]. Moreover, due to its carboxylic acid groups that are deprotonated within acidic environments (here, vaginal), active agents are retained under slightly acidic conditions. Conversely, in neutral and alkaline environments, the carboxylic acid groups become ionized, inducing electrostatic repulsion, which results in fiber swelling and agent release into the surrounding medium [51]. Additionally, PAA as well as the polymer poly(*n*-butyl acrylate) (PBA) have been used to

produce mucoadhesive polymers, demonstrated in buccal delivery and other applications [52–57]. Given these properties, we selected the co-polymer, PBA-*co*-PAA, to blend with known sustained-release polymers, PLGA and methoxypolyethylene glycol (mPEG)-PLGA, to provide pH-dependent GRFT release. We hypothesized that the encapsulated GRFT released from these pH-responsive fibers would retain antiviral properties relative to free GRFT and that utilizing PBA-*co*-PAA fibers to deliver biological entry inhibitors, such as GRFT, may prove useful to conserve the payload and activity of active agent when needed.

2. Materials and methods

2.1. Materials

Carboxyl-terminated poly(D, L-lactic-*co*-glycolic acid) (PLGA, 50:50, 0.55–0.75 dL/g, 31–57 kDa MW) was purchased from LACTEL® Absorbable Polymers (Cupertino, CA, USA). Methoxy poly(ethylene glycol)-*b*-poly(lactide-*co*-glycolide) (mPEG-PLGA, 5,000:55,000 kDa) was obtained from PolySciTech® Akina Inc. (West Lafayetter, IN, USA). Poly(*n*-butyl acrylate-*co*-acrylic acid) (PBA-*co*-PAA, 50:50, catalog number 19911-10), an alkali-soluble 20% latex in alcohol was purchased from Polysciences Inc. (Warrington, PA, USA). Chemical solvents including 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), dichloromethane (DCM), and hydrochloric acid (HCl) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Sodium dodecyl sulfate (SDS) and MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] were purchased from Sigma Aldrich (St Louis, MO). Griffithsin (MW 12.7 kDa) was produced by Kentucky BioProcessing LLC (Owensboro, KY, USA) and was kindly provided by Dr. Kenneth Palmer (University of Louisville). Fetal bovine serum (FBS) and 100× penicillin-streptomycin solutions were purchased from VWR. Simulated vaginal fluid (SVF) and simulated semen fluid (SSF) were prepared as described in [58,59].

2.2. Cell lines and virus

Vaginal keratinocyte (VK2/E6E7), endocervical (End1/E6E7), and ectocervical (Ect1/E6E7) cell lines were used to assess fiber cytotoxicity (courtesy of Dr. Kenneth Palmer, originally from ATCC, Rockville MD). VK2/E6E7 (VK2), End1/E6E7 (End1), and Ect1/E6E7 (Ect1) are well-characterized immortalized cell lines derived from normal human vaginal, endocervical, and ectocervical epithelia, respectively. These cell lines were chosen as they are representative of the cell types found within the female reproductive tract. VK2, End1, and Ect1 cells were maintained in keratinocyte serum-free medium (KSFM) supplemented with recombinant human epidermal growth factor (0.1 ng/mL), bovine pituitary extract (50 µg/mL), calcium chloride (0.4 mM) (Thermo Fisher, Waltham, MA), with 1% penicillin and streptomycin (100 µg/mL each). During cell trypsinization, plating, and cell counting, cells were neutralized with Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 media (DMEM/F-12, 1:1, VWR) with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (100 µg/mL each).

TZM-bl cells, obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (ARRRP), were used to assess *in vitro* HIV-1 infectivity. TZM-bl cells, previously designated JC53-bl (clone 13), are derived from a HeLa cell clone engineered to express CD4, CCR5 and CXCR4. These cells have a Tat-driven luciferase or *E. coli* β-galactosidase reporter system, under the control of an HIV-1 long terminal repeat, permitting sensitive and accurate measurements of infection [60,61]. TZM-bl cells were cultured in DMEM containing 10% FBS, 25 mM HEPES, and 50 µg/mL gentamicin. These cells are highly permissive to infection by most strains of HIV, SIV and SHIV, including primary or molecularly cloned viral isolates and molecularly cloned Env-pseudotyped viruses.

The Env-pseudotype HIV-1 was produced in 293T/17 cells, using an envelope (env)-expressing plasmid (CCR5-tropic clade A strain, Q769.h5) and an env-deficient HIV-1 backbone vector (pNL4.3ΔEnv-Luc), both obtained from the NIH AIDS Reagent Program (11884 and 3418). HEK-293T (human embryonic kidney) cells were purchased from ATCC. Cells were maintained in MEM supplemented with 10% FBS, and 1% penicillin and streptomycin (100 µg/mL each).

2.3. Synthesis of electrospun fibers

Blank PLGA and mPEG-PLGA polymers (15% w/w) were dissolved in 0.6 mL HFIP overnight, while shaking at room temperature. Subsequent polymer blends, comprised of varying PLGA:PBA-*co*-PAA polymer ratios (100:0, 90:10, 85:15, 80:20, and 75:25 w/w) were prepared as follows (Fig. 1). Briefly, PLGA polymers (15–30% w/w)

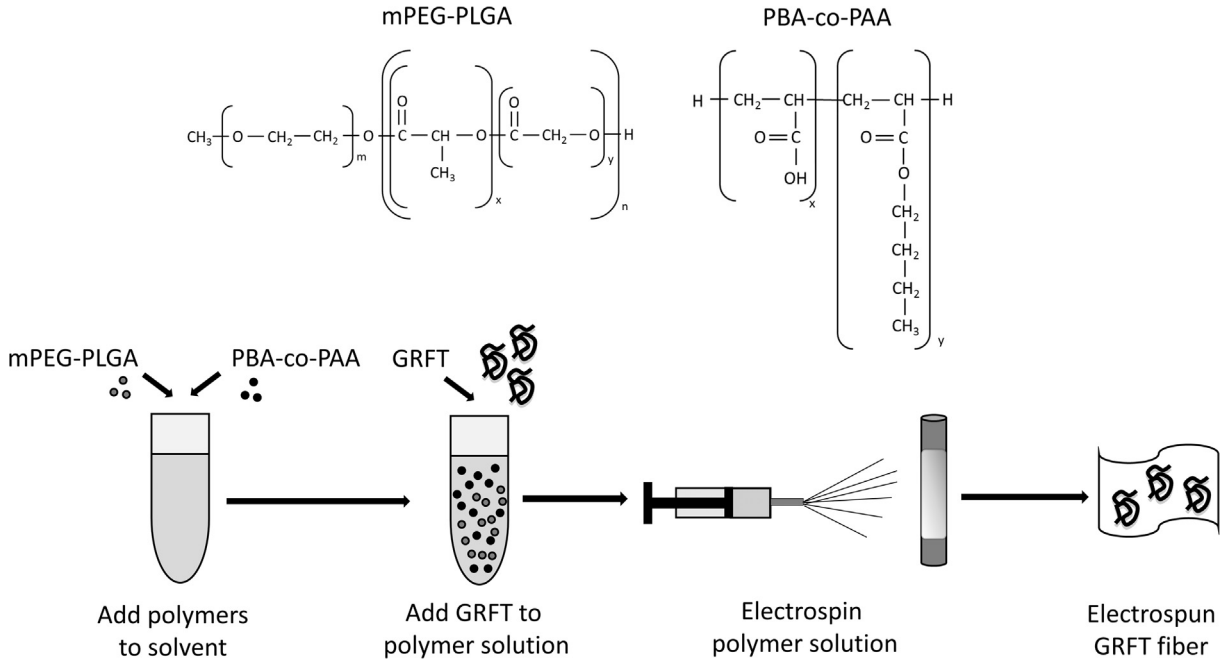

Fig. 1. Schematic of mPEG-PLGA and PBA-*co*-PAA co-polymers and the fiber fabrication process.

were first dissolved in HFIP. Corresponding mass to mass ratios of PBA-*co*-PAA polymer were added to the PLGA polymer mixture and allowed to solubilize overnight on a shaker at room temperature. Prior to electrospinning, 140 μL of Tris-EDTA buffer (TE, pH = 7.4) was added to the polymer solution and briefly vortexed.

For fibers incorporating GRFT, the GRFT stock solution was first concentrated with a Spin-X® UF concentrator (10 kDa MWCO, Corning Incorporated-Life Sciences, Oneonta, New York, USA) and resuspended to a volume of 1 mL in TE buffer. For a 90:10 polymer blend, 144 mg of either PLGA or mPEG-PLGA was weighed and added to 0.6 mL of HFIP. Immediately after, 80 μL (16 mg) of PBA-*co*-PAA ethanol solution (20% w/v) was added to this mixture, resulting in a polymer blend with a final concentration of 13.8% w/w (polymer/solvent). After the polymers solubilized overnight, 140 μL (4.8 mg) of GRFT solution (34.2 mg/mL) was added dropwise to the polymer mixture giving a final electrospinning volume of 820 μL, with theoretical GRFT loading of 30 μg GRFT/mg polymer. The solution was briefly vortexed and immediately electrospun.

Five hundred microliters of PLGA:PBA-*co*-PAA and GRFT suspension was aspirated into and electrospun from a 1 mL plastic syringe as previous described[33,34]. Flow rates spanning (0.3–1.0 mL/h) were optimized over a range of voltages (20–25 kV). The resulting fiber mat was collected on a rotating 4 mm outer diameter stainless steel grounded mandrel located 10–20 cm from the needle tip. All electrospinning processes were performed at room temperature (RT, ~25 °C). Sample flow rate was monitored using an infusion pump (Fisher Scientific, Pittsburgh, PA) while the voltage was applied using a high voltage power supply (Spellman CZE 1000R). Final electrospinning conditions applied a voltage of 25 kV, with a sample flow rate of 0.8 mL/h, with a distance of 20 cm, for the mPEG-PLGA:PBA-*co*-PAA 90:10 blend. After electrospinning, fibers were removed from the mandrel and dried overnight in a desiccator.

2.4. Fiber morphology

The morphologies of GRFT PLGA:PBA-*co*-PAA and mPEG-PLGA:PBA-*co*-PAA blended fibers were evaluated using scanning electron microscopy (SEM) (JSM-820 microscope, JEOL, Tokyo, Japan). Briefly, after drying, fibers were cut into 5 mm pieces and placed on double-sided adhesive carbon tabs (Ted Pella, Inc., Redding, CA, USA), which were then adhered to aluminum stubs. Samples were coated with a thin gold alloy film using a Bio-Rad E5100 sputter coat system. The coating process was operated at 20 mA for 90 s and images were captured at an accelerating voltage of 8 kV. The average fiber diameter was determined by analyzing SEM images in NIH ImageJ, and drawing line elements across a minimum of 50 fibers per image. Statistical significance between fiber diameters was determined using the Bonferroni post hoc *t*-test ($p < 0.05$).

2.5. Griffithsin loading and release

The amount of GRFT incorporated into each fiber was determined by dissolving of 3–5 mg fiber pieces in 500 μL DCM, and adding 500 μL TE buffer to extract GRFT. This extraction process was repeated twice by vortexing for 1 min and centrifuging for 5 min at 13,000 rpm to fully extract GRFT. The TE buffer was collected and analyzed using ELISA to determine GRFT loading. The encapsulation efficiency (EE) was defined as the ratio of actual GRFT loading to the theoretical loading (30 μg/mg).

To quantify GRFT release from pH-responsive fibers under different *in vitro* conditions, SVF (pH = 4.4), phosphate buffered saline (PBS, pH = 7.4), and SVF:SSF (1:3, v/v, pH = 7.4) were used as eluates. The SVF:SSF mixture was used to measure inducible GRFT release after exposure to semen-vaginal fluid mixture [59]. Samples of GRFT PLGA:PBA-*co*-PAA and mPEG-PLGA:PBA-*co*-PAA fibers were hole-punched with a Ribbel biopsy punch (7 mm diameter), resulting in an approximate fiber mass of 10 mg. These fibers were immersed in 1 mL SVF or PBS, in a shaker at 37 °C and 150 rpm. At pre-determined time points, the release buffer was completely replaced with 1 mL of either fresh SVF or PBS. In subsequent pH-dependent release studies, fibers were incubated in SVF for 24, 48, or 72 h. To create a pH "switch", the SVF was replaced with either PBS or (1:3) SVF:SSF to assess differences in GRFT release after a pH change. Fiber eluates were collected and replaced at 1, 4, 24, 48, 72, 96, and 120 h after the initial PBS or SVF:SSF "switch."

The concentration of GRFT released was determined using an established ELISA method. Briefly, Nunc Maxisorp ELISA plates were coated with 0.9 mg/mL influenza virus hemagglutinin (diluted in 0.1 M PBS) overnight at 4 °C for use as a coating buffer, which GRFT selectively binds to. The plate was washed three times with PBS containing 0.05% Tween-20 (PBST) using an Immunowash plate washer (Bio-Rad, Hercules, CA, USA). The wells were blocked by adding 3% (w/v) bovine serum albumin in PBST at room temperature (RT) for 2–3 h to block non-specific binding. A GRFT standard, loading extract and release eluates were added to the wells for 1 h at room temperature. A 1:10,000 dilution of both a primary antibody goat anti-GRFT (provided by Dr. Nobuyuki Matoba, University of Louisville) and secondary antibody goat anti-rabbit IgG-HRP (Sigma-Aldrich, St. Louis, MO, USA) were added to the wells for another 1 h to detect bound GRFT. Colorimetric values were derived using SureBlue Reserve TMB Peroxidase substrate (KPL, Gaithersburg, MD, USA), and the reactions were stopped by 1 N H_2SO_4. Absorbance was measured at 450 nm on a Synergy HT reader (BioTek, Winooski, VT, USA). Results are shown as the cumulative amount of GRFT released per mass fiber (μg/mg) and the cumulative release percentage, as a function of release time. The encapsulation efficiency (EE) was defined as the ratio of actual GRFT loading to the theoretical loading (30 μg/mg). All data are shown as the mean ± standard deviation. All experiments were conducted in triplicate, with a minimum of three independent experiments, unless otherwise noted.

2.6. Nuclear magnetic resonance spectroscopy

For the quantification of the polymer composition, mPEG-PLGA, PLGA-*co*-PAA, empty-fiber, and GRFT-loaded fiber were dissolved with fully deuterated dimethyl sulfoxide for Nuclear magnetic resonance (NMR) spectroscopy. NMR spectra of all the samples were obtained at a temperature of 298 K using a 600 MHz proton frequency spectrometer equipped with a triple resonance prodigy probe (Bruker, Billerica, MA, USA). All the spectra were acquired with 2048 complex points and 4 number of scans and processed using TOPSPIN. Based on the chemical structures of mPEG-PLGA and PBA-*co*-PAA, peaks in the spectra of mPEG-PLGA and PBA-*co*-PAA were assigned to individual proton resonances. These assignments were consequently used to identify resonances in the fiber spectra. Although severe overlapped resonances from mPEG moiety in mPEG-PLGA and aliphatic moiety in PBA-*co*-PAA in the fiber spectra were observed, protons attached to carbons of lactic and glycolic moieties in mPEG-PLGA and proton in hydroxyl group in PBA-*co*-PAA could be unambiguously assigned to the resonances at ~5.0, ~5.5, and ~8.0 ppm, respectively (Supplemental Fig. 1). After the assignments, the relative composition of moieties in mPEG-PLGA were determined using integration of resonances in each spectrum and the determined number of chains for lactic and glycolic moieties in mPEG-PLGA were ~34 and ~54, respectively and the chain number of acrylic acid in PBA-*co*-PAA was ~34. The relative ratio of mPEG-PLGA and PBA-*co*-PAA in the fiber were determined as 1:0.1 using the integration of the peaks at ~ 5.0, ~5.5, and ~8.0 ppm in the spectra of fibers (Supplemental Fig. 1). Additional to the resonances from the fiber, GRFT resonances were observed only in the GRFT-loaded fiber (Supplemental Fig. 1) that confirms that GRFT is incorporated into the fiber using our protocol.

2.7. HIV pseudovirus-based neutralization assay

The antiviral activity of GRFT loading extract and fiber release eluates was measured relative to free GRFT, as a function of reduction in luciferase reporter gene expression after a single round of infection with Env- pseudotyped virus (CCR5-using clade A strain Q769.h5) in TZM-bl cells. The optimal virus dilution was established to yield ≥100,000 relative luminescence units (RLU) after infection. Assay stocks of molecularly cloned Env-pseudotyped viruses were prepared by transfection of 293T cells and were titrated in TZM-bl cells as previously described [62]. Briefly, all samples were diluted using serial dilutions (ranging from no dilution to 1:10,000 with PBS to a final volume of 50 µL within a 96 well plate. One hundred microliters of TZM-bl cell solution (10^4 cells in DMEM medium with 10 µg/mL DEAE-dextran) was subsequently added to each well, followed by the addition 50 µL of HIV pseudovirus virus dilution. Samples were then incubated at 37 °C for 48 h. After the 48 h incubation, 100 µL culture medium was carefully removed from each well. Luminescence was measured using the Bright-Glo™ luciferase assay system (Promega Corporation, Madison, WI, USA) by adding 100 µL Bright-Glo™ reagent solution to each well for 5 min. Plates were read via luminescence by the Synergy HT reader (BioTek, Winooski, VT, USA). All RLU values were corrected by subtracting the RLU of untreated/uninfected cells from the sample RLUs (treated infected cells). The percent virus inhibition was determined by normalizing the corrected RLUs of infected/treated cells to corrected untreated/infected cells: % Infection = [(sample RLU − untreated uninfected cells) ÷ (untreated infected cells –untreated uninfected cells)] × 100%. Antiviral activity is reported as the sample concentration at which RLUs compared with virus control wells RLUs.

2.8. In vitro cytotoxicity

The *in vitro* cytotoxicity of mPEG-PLGA:PBA-*co*-PAA (90:10), relative to free GRFT was evaluated in VK2, Ect1, and End1 cells using a colorimetric MTT assay. Briefly, each cell line was seeded into 12-well plate at a density of 600,000 cells per well. Eluates from 1 and 10 mg GRFT mPEG-PLGA:PBA-*co*-PAA fibers, suspended in 1 mL of media were incubated with the cells for 24, 48, and 72 h. After each time point, 100 µL MTT solution (5 mg/mL) was added to each well followed by incubation for 4 h at 37 °C. Lysis buffer (550 µL, 10% SDS in 0.01 M HCl) was then added to each well for and incubated for 16 h. Absorbance readings were performed at 570 nm. Ten percent DMSO was used as the positive control for cytotoxicity, with blank fibers eluate-treated and untreated cells as negative controls. All data are shown as the mean ± standard deviation.

3. Results

3.1. Fiber morphology

The morphology and microstructure of PLGA and mPEG-PLGA fibers were assessed with scanning electron microscopy (Fig. 2). In addition, the morphology of PLGA and PBA-*co*-PAA polymer fibers with blend ratios of: 100:0, 90:10, 85:15, 80:20 and 75:25 and mPEG-PLGA:PBA-*co*-PAA (90:10) fibers were evaluated (Fig. 3). As shown in Figs. 2 and 3, all formulations provided well-defined fiber morphologies. The fiber diameters of the different polymer blends tested for pH-responsive release were assessed using ImageJ software (NIH) (Table 1**)**. The fiber diameters of the PLGA:PBA-*co*-PAA and mPEG-PLGA:PBA-*co*-PAA (90:10) blends ranged from 204 to 407 nm. As the initial PBA-*co*-PAA ratio increased, the average fiber diameter decreased; however, only the 75:25 PLGA blend exhibited a statistically significant difference relative to the other PLGA blends. There was no evident relationship between polymer type, PBA-*co*-PAA ratio, and fiber diameter.

3.2. Griffithsin fiber loading and release characterization

To determine the impact of polymer blend ratio on GRFT loading, the extracts from 100:0, 90:10, 85:15, 80:20 and 75:25 PLGA:PBA-*co*-PAA and 90:10 mPEG-PLGA:PBA-*co*-PAA polymer blends were evaluated using an ELISA. Table 2 compares the actual amount of GRFT incorporated per milligram of fiber, to the theoretical loading of 30 µg/mg. The encapsulation efficiency of active GRFT in PLGA-only (100:0) EFs, measured via ELISA was over 90%, indicating the high loading potential of these fibers. The 90:10, 85:15, 80:20 and 75:25 PLGA:PBA-*co*-PAA blends exhibited GRFT encapsulation efficiencies of 62, 53, 51, and 80%, respectively, while the 90:10 mPEG-PLGA:PBA-*co*-PAA fibers demonstrated 54% loading efficiency. Similarly high GRFT loading was attained for both the PLGA-only and the 75:25 polymer blend, while GRFT loading of the other formulations was statistically lower. Although statistical differences in encapsulation efficiencies were observed between fiber formulations, there was no correlation between GRFT loading and increased PBA-*co*-PAA ratio.

To initially assess the ability of fibers to release GRFT under different pH conditions, the total cumulative release of GRFT from PLGA and mPEG-PLGA fibers was measured during 72 h incubation in SVF (pH 4.4) or PBS (pH 7.4). Although PLGA EFs demonstrated high GRFT encapsulation, negligible GRFT release was observed from PLGA-only fibers in either SVF or PBS (Fig. 4). However, GRFT release from mPEG-PLGA fibers in SVF and PBS exhibited burst release within the first 6 h (~33 and 45% of total GRFT loading, respectively), followed by minimal sustained-release during the remaining incubation period (Fig. 4). Based on these results, alternative polymer formulations were investigated to improve GRFT release.

In comparison to these initial release studies with PLGA and mPEG-PLGA fibers, it was observed that blending PLGA with a pH-responsive polymer, PBA-*co*-PAA, both enabled the formation of well-delineated fibers (Fig. 3), and provided enhanced GRFT release in PBS, relative to PLGA- or mPEG-PLGA-only fibers (Fig. 5). To mimic the more basic pH conditions of semen entry for pH-responsive applications, EFs were incubated in SVF for 24 h, and subsequently “switched” to PBS (Fig. 5). The same fiber formulations were also incubated in PBS alone as a control. While unblended PLGA-only fibers (100:0) released minimal

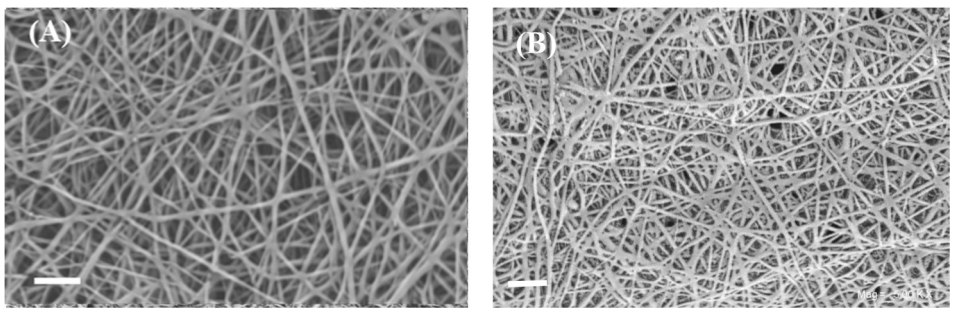

Fig. 2. SEM images of (A) PLGA and (B) mPEG-PLGA electrospun polymer fibers that incorporate GRFT. The scale bar represents 2 µm.

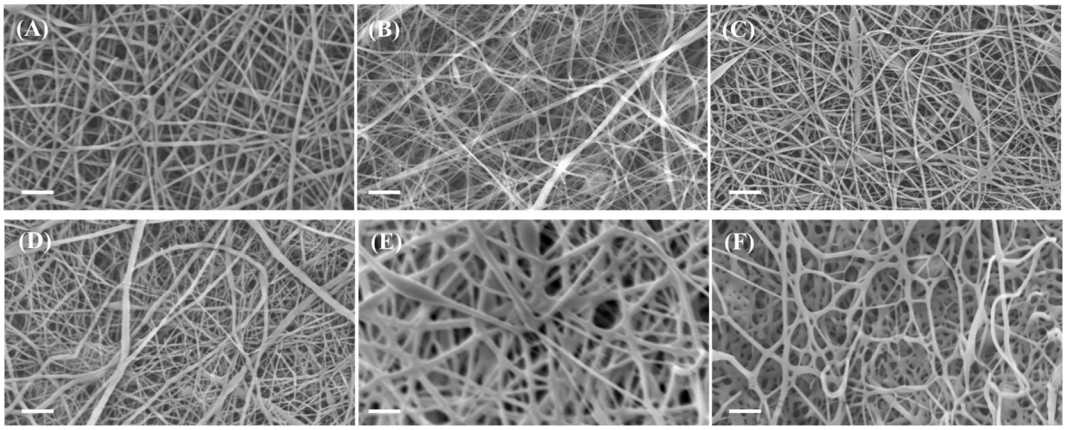

Fig. 3. SEM images of different pH-responsive electrospun fiber blends that incorporate GRFT. (A) PLGA:PBA-*co*-PAA (100:0, w/w); (B) PLGA:PBA-*co*-PAA (90:10); (C) PLGA:PBA-*co*-PAA (85:15); (D) PLGA:PBA-*co*-PAA (80:20); (E) PLGA: PBA-*co*-PAA (75:25); and (F) mPEG-PLGA:PBA-*co*-PAA (90:10). The scale bar represents 2 μm.

Table 1
pH-responsive fiber diameters measured from SEM images.

Fiber Formulation	Weight Ratio	Fiber Diameter (nm)
PLGA:PBA-*co*-PAA	100:0	263 ± 81
	90:10	236 ± 82
	85:15	239 ± 106
	80:20	204 ± 104
	75:25	407 ± 150
mPEG-PLGA:PBA-*co*-PAA	90:10	336 ± 100

Table 2
GRFT loading and encapsulation efficiency based on formulation.

Quantification of GRFT EF loading and encapsulation efficiency				
Fiber Formulation	Weight Ratio	Theoretical Loading (μg/mg)	Actual Loading (μg/mg)	Encapsulation Efficiency (%)
PLGA:PBA-*co*-PAA	100:0	30	29.6 ± 1.6	98.7 ± 5.0
	90:10		18.7 ± 0.9	62.3 ± 2.8
	85:15		16.0 ± 3.8	53.4 ± 12.9
	80:20		14.5 ± 1.8	51.3 ± 6.1
	75:25		24.0 ± 0.8	80.0 ± 2.5
mPEG-PLGA:PBA-*co*-PAA	90:10		16.2 ± 1.2	54.0 ± 13.7

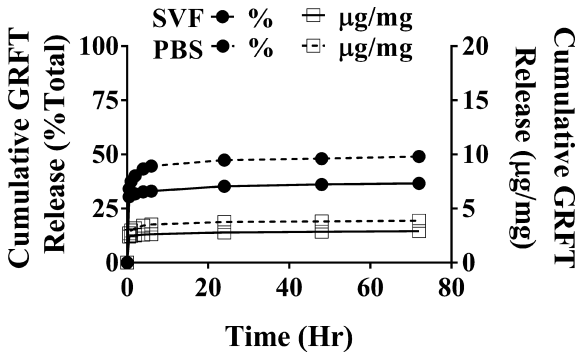

Fig. 4. *In vitro* release profiles of GRFT from mPEG-PLGA fibers. Negligible GRFT release was detected from PLGA-only fibers (data not shown, release coincides with x-axis). Cumulative release data is shown as a function of percent total loading (solid circles) and mass released per mass of fiber (open squares). Fibers were incubated in either SVF (solid lines) or PBS (dashed lines) for 72 h at 37 °C. Data are expressed as the mean ± SD of triplicate samples.

amounts of GRFT, polymer blends containing increased PBA-*co*-PAA (10–25% w/w), released more GRFT within the first 24 h in either PBS or SVF. Of these blends, only the 90:10 PLGA:PBA-*co*-PAA blend exhibited increased GRFT release in PBS only (relative to SVF), indicating its potential for pH-responsive applications. In contrast, blends with increased PBA-*co*-PAA ratios (15, 20, and 25%) released GRFT in both PBS as well as SVF, deeming them less discerning for a pH-responsive delivery platform.

Based on these release results (Figs. 3 and 4), it was hypothesized that incorporating the properties of the 90:10 PLGA:PBA-*co*-PAA blend with the increased hydrophilicity of mPEG-PLGA, may increase GRFT release in more neutral conditions due to increased hydrophilicity, while retaining GRFT loading at low intravaginal pH (e.g., SVF). To evaluate this, mPEG-PLGA:PBA-*co*-PAA (90:10) fibers were formulated and incubated in SVF for either 24, 48, and 72 h. After 24, 48, and 72 h, the fibers were "switched" to PBS (Fig. 6**A**) or simulated vaginal-semen fluid (SVF:SSF (1:3) w/w, pH 7.4)) (Fig. 6B**)**. The mPEG-PLGA:PBA-*co*-PAA blend provided negligible release of GRFT in SVF after 24, 48, and 72 h, releasing only 0.27, 0.41, and 0.47 μg/mg GRFT respectively. However, when switched to PBS, the blend exhibited a nearly thirty-fold increase in GRFT release, releasing 10–12 μg/mg (66–74%) over a subsequent 120 h (Fig. 6A). Moreover, within the first hour post-switch, the fibers that were incubated for 24, 48, and 72 h in SVF released 6.2, 3.5, and 4.2 μg GRFT/mg fiber, respectively. Similar release was observed when fibers were switched from SVF to a more representative intravaginal environment of SVF:SSF (1:3), with 1.3, 1.3, and 1.4 μg GRFT/mg fiber release 1 h post-switch from the 24, 48, and 72 h SVF incubations, respectively (Fig. 6B). Overall, GRFT release from fibers switched to SVF:SSF showed a lesser and more gradual release curve relative to fibers switched to PBS. While fibers switched to PBS released approximately 11 μg GRFT/mg fiber 24 h post-switch, fibers switched to SVF:SSF released ~5 μg GRFT/mg fiber within the same duration. Moreover, the PBS switched fibers exhibited minimal release after 24 h, while the SVF:SSF switched fibers continued to release, resulting in a total of ~7 μg GRFT released per mg fiber, 72 h post-switch.

3.3. HIV-1 inhibition studies

Based on the release results, mPEG-PLGA:PBA-*co*-PAA (90:10) GRFT fibers were evaluated to provide pH-dependent protection against HIV-1 infection *in vitro*. First, the activity of GRFT, extracted from electrospun fibers, was assessed to determine whether GRFT is inactivated during electrospinning. Previous studies have shown that harsh solvents and electric field used during the electrospinning process may denature protein and decrease biologic activity [63,64]. Therefore, the inhibitory potential of extracted, relative to free GRFT, was tested against HIV-1

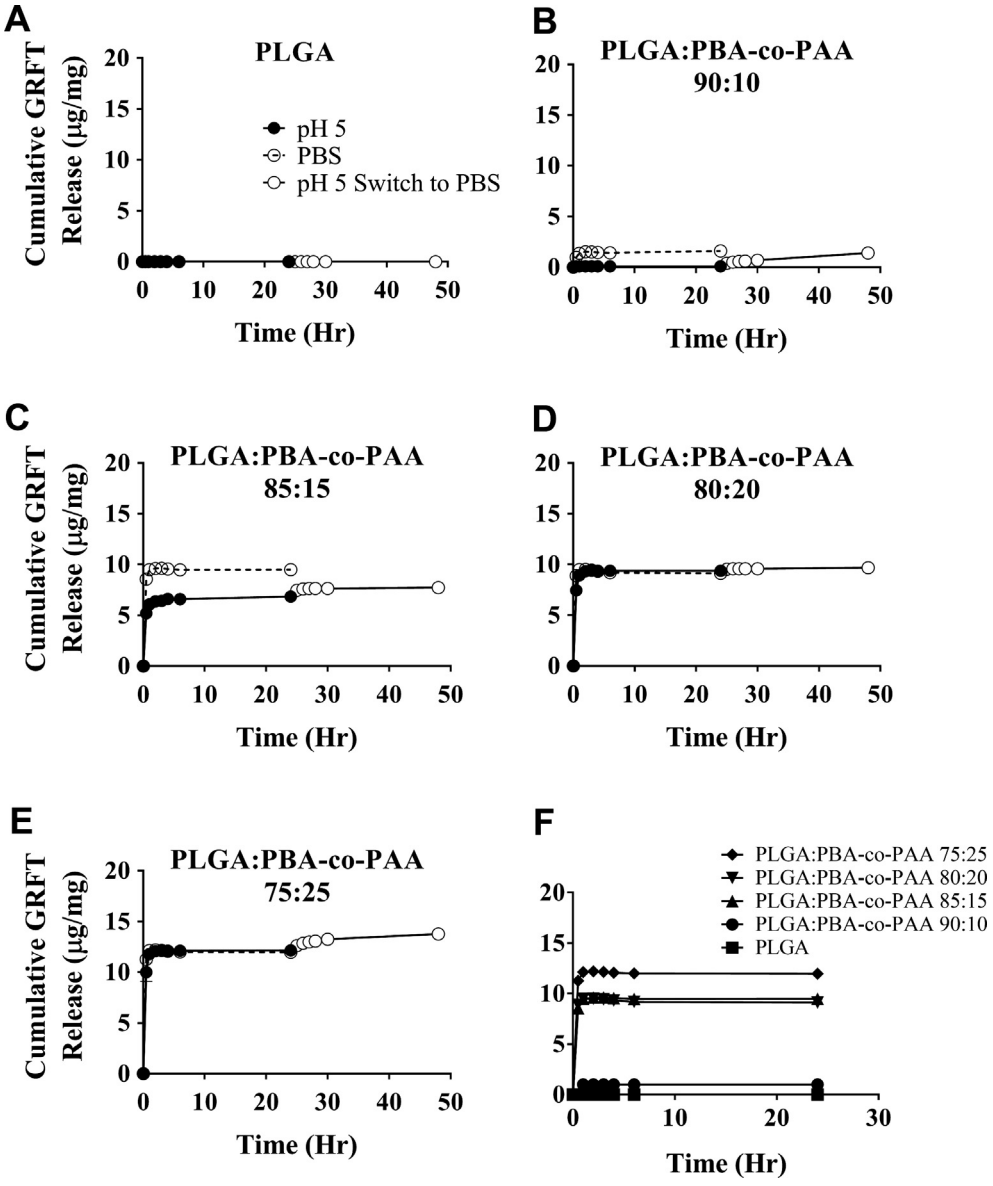

Fig. 5. *In vitro* pH-responsive release profiles of GRFT from different PLGA:PBA-*co*-PAA blended fiber formulations, each theoretically loaded with 30 µg GRFT/mg polymer. The cumulative release of GRFT from: (A) PLGA:PBA-*co*-PAA (100:0, w/w); (B) PLGA:PBA-*co*-PAA (90:10); (C) PLGA:PBA-*co*-PAA (85:15); (D) PLGA:PBA-*co*-PAA (80:20); and (E) PLGA: PBA-*co*-PAA (75:25) were monitored at 37 °C. Fibers were initially incubated in SVF (pH = 4.5) for 24 h and switched to PBS (pH = 7.4) for an additional 24 h. Release in PBS (pH 7.4) is shown (without a switch) for comparison. A summary of GRFT release from all blends in PBS is provided in (F). As the ratio of PLGA decreases, the cumulative amount of GRFT release increases. Release data are expressed as the mean ± SD of triplicate samples.

infection. Similar antiviral activity was observed between free GRFT and GRFT extracted from mPEG-PLGA:PBA-*co*-PAA (90:10) fibers (Fig. 7). Complete protection against HIV-1 infection was achieved from undiluted fiber extracts (IC_{50} 10.6 ng/mL) and free GRFT (15.5 ng/mL), with a dose-dependent decrease in protection observed for both fiber extract and free GRFT dilutions.

To assess the inhibitory activity of GRFT fibers after an induced pH change, mPEG-PLGA:PBA-*co*-PAA (90:10) fibers were incubated in SVF for either 24, 48, or 72 h, followed by a switch to PBS or SVF:SSF, for an additional hour. After 1 h, the eluates were collected and assessed in HIV-1 inhibition assays. As seen in Fig. 8A and B, despite different initial incubation periods in SVF (24, 48, or 72 h), GRFT maintained bioactivity and completely inhibited HIV-1 infection after exposure to PBS. The IC_{50}s for the 1 h PBS release eluates, after an initial 24, 48, and 72 h in SVF were 28.3, 29.6 and, 23.5 ng/mL respectively. Similarly, complete inhibition of HIV-1 infection was achieved from mPEG-PLGA:PBA-*co*-PAA fibers that were switched to a more physiologically relevant environment of SVF:SSF after the same durations (24, 48, 72 h) (Fig. 8C and D). The IC_{50} values from 1 h post-switch SVF:SSF release eluates, were 23.2, 23.7, and 21.3 ng/mL respectively. The IC_{50}s of both PBS and SVF:SSF 1 h post-switch release eluates were not statistically different, demonstrating that GRFT maintains bioactivity regardless of incubation time.

3.4. *In vitro* safety

To determine the biocompatibility of pH-sensitive GRFT EFs, vaginal epithelial cell lines: VK2, Ect1, and End1 E6/E7 were incubated with 1 and 10 mg/mL fibers *in vitro*. As shown in Fig. 9, all cell lines demonstrated greater than 95% cell viability after 24, 48, and 72 h fiber administration, relative to untreated and DMSO-treated cells. There were no statistically significant differences noted in cell viability, as a function of cell line or administration duration.

4. Discussion

While previous work has demonstrated the potential to provide pH-responsive release of traditional antivirals, here we present a method to induce the pH-responsive release of the antiviral lectin, GRFT. Griffithsin is a promising new biologic for use against HIV, HSV-2, human papillomavirus, and a variety of other viruses, due to its potent binding and antiviral activity [15,17,65–67]. Additionally, GRFT has

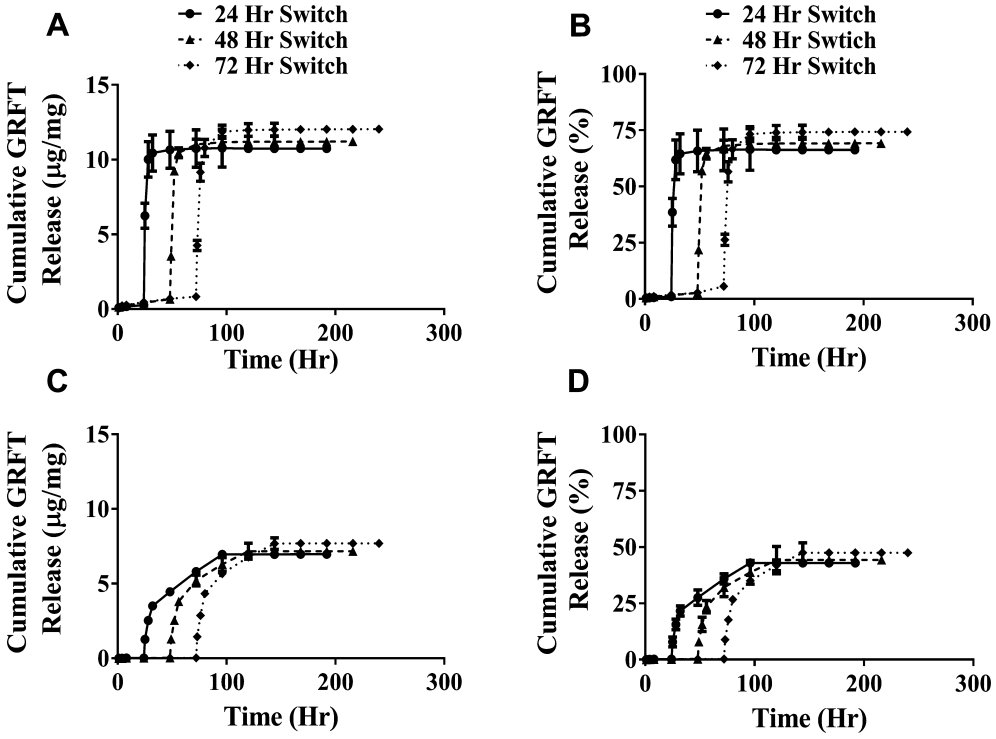

Fig. 6. *In vitro* pH-responsive release profiles of GRFT from mPEG-PLGA:PBA-*co*-PAA (90:10, w/w) fibers. The cumulative release of GRFT is shown as a function of mass released (A, C) and percent total loading (B, D). Fibers were incubated in SVF for 24, 48, or 72 h. The media was subsequently "switched" to (A, B) PBS or (C, D) SVF:SSF (1:3) mixture, and fibers were incubated for an additional 72 h. Release values are expressed as the mean ± SD of triplicate samples.

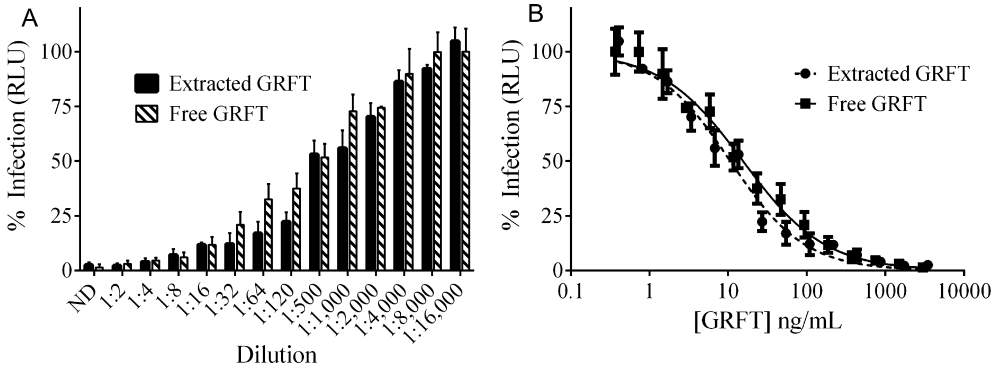

Fig. 7. HIV-1 inhibition assays were conducted to assess the functional activity of extracted GRFT after electrospinning into fibers, relative to free GRFT. GRFT extracted from mPEG-PLGA:PBA-*co*-PAA (90:10) fibers demonstrates complete efficacy against HIV-1 infection, and similar efficacy to free GRFT. The percent of HIV-1 infected cells, relative to untreated cells, is shown as the mean ± SD of triplicate samples, as a function of (A) eluate dilution or (B) GRFT concentration.

demonstrated stability and safety, prompting its development in clinical trials. As such, we hypothesized that the incorporation of GRFT within an electrospun fiber may provide an effective antiviral delivery vehicle to satisfy the on-demand needs of virus entry inhibition. In this work we demonstrate that these fibers provide pH-inducible release of GRFT while preserving GRFT activity and payload for up to 3 days, prior to exposure to PBS or SVF:SSF solutions.

In our initial studies, fibers comprised of either PLGA or mPEG-PLGA polymers were fabricated to provide sustained-release of GRFT. Based on the biocompatibility of PLGA and its previous utilization in sustained-release applications [68], it was reasoned that similar sustained-release of GRFT may be achieved. However, GRFT release from PLGA fibers was minimal, and substantially below prophylactic needs, beyond 6 h application. We hypothesized that the diminished release of GRFT from PLGA fibers may be attributed to the isoelectric point of GRFT (pI = 5.4), facilitating GRFT adhesion to, or hydrophobic interactions with, the negatively-charged PLGA polymer. In contrast, GRFT release increased from the slightly more hydrophilic mPEG-PLGA fibers. Despite slightly improved initial release, mPEG-PLGA fibers exhibited sub-optimal release at later time points, highlighting the need for an improved formulation that retains GRFT under relevant intravaginal conditions, yet releases GRFT in response to coital cues.

In combination with the challenges of obtaining sustained-release, one of the issues with any sustained-release system, is that cargo is released under temporal conditions that may not prophylactically (or therapeutically) necessitate release. For more expensive and labile biologics, conventional sustained-release platforms (not triggered by stimuli) may be inefficient to deliver active agent, particularly an entry inhibitor like GRFT that should be present in high concentration, to protect against virus entry and exposure. Moreover, from an economic perspective, the premature release of biological molecules that have shorter half-lives or are more expensive to produce may adversely impact overall cost and feasibility. Given these considerations, we sought to develop a vehicle that retains GRFT, and only releases GRFT upon exposure to the more neutral pH conditions of semen infiltration. We hypothesized that this would be a desirable option, given the role of GRFT as an entry inhibitor.

To test the pH-responsive properties, 100:0, 90:10, 85:15, 80:20, and 72:25 of PLGA:PBA-*co*-PAA GRFT fibers were evaluated. Increases in the length of hydrophobic alkyl groups on acrylic acid, often result in increases in pKa, affecting the pH of polymer switch [69]. In addition, pendant acidic groups are typically ionized at a pH dependent on pKa, in neutral and alkaline solutions, and their induced repulsion affects the physical properties of the polymer. Thus, the ionizable carboxylic acid groups in both PBA and PAA increase solubility at neutral/basic pH [50]. In our studies, it was observed that increased ratios of PBA-*co*-

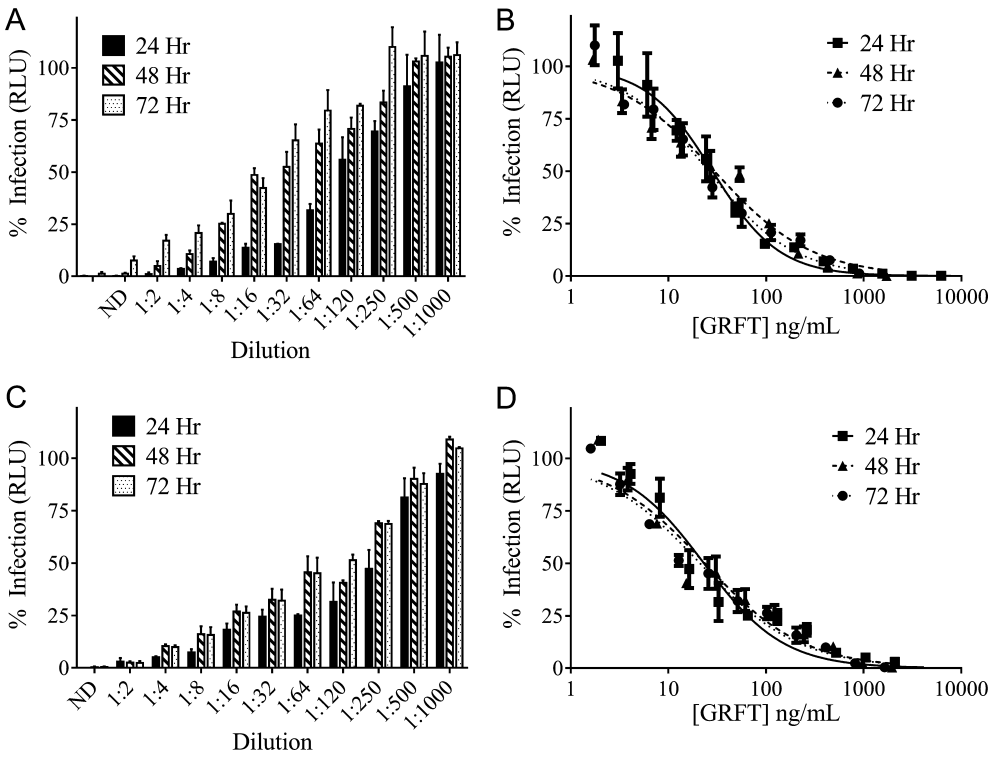

Fig. 8. HIV-1 inhibition assays were conducted to assess the antiviral activity of GRFT mPEG-PLGA:PBA-*co*-PAA (90:10) fiber release eluates against HIV-1 infection. GRFT mPEG-PLGA:PBA-*co*-PAA fibers were incubated in SVF for 24, 48, or 72 h. (A) After each time point, SVF was "switched" to PBS, and serial dilutions of fiber eluates were collected to assess antiviral efficacy after 1 h in PBS. (B) The percent of HIV-1 infected cells, relative to untreated cells, is shown as the mean ± SD of triplicate samples. (C and D) The study was repeated with fibers "switched" from SVF to SVF:SSF (1:3). The serial dilutions of GRFT release eluates were evaluated against HIV-1 and IC_{50} values were calculated.

PAA resulted in increased GRFT release after exposure to PBS and SVF:SSF solutions. We expect this trend is due in part to fiber swelling upon exposure to neutral PBS and SVF:SSF solutions. We additionally observed that even small increases in the ratio of PBA-*co*-PAA, relative to PLGA, resulted in increased release under both neutral and acidic conditions. In fact, increased GRFT release corresponded with increased ratio of PBA-*co*-PAA under acidic conditions, which was undesirable for a pH-responsive application. The pKa values of PBA (7.4) and PAA (4.28), indicate that the amount of PBA is driving this release, in both neutral (PBS, SVF:SSF) and acidic (SVF) conditions. Of these selected formulations, the 90:10 blend exhibited the most desirable pH-dependent release profile, and release in PBS and SVF:SSF was improved by utilizing the more hydrophilic mPEG-PLGA fibers.

In addition to the pH-responsive properties imparted by PBA-*co*-PAA, PLGA provides a biocompatible polymer that lends mechanical stability for use in implants [68]. Moreover, it is well known that PLGA fibers specifically impart high mechanical strength for a variety of applications, such as scaffolds [70]. Given these favorable properties, blending with other polymers can add complementary attributes [71], here pH-responsive behavior. The anionic polymers PBA and PAA have been applied to a variety of biomedical applications [72–78]. Together, it was anticipated that mPEG-PLGA and PBA-*co*-PAA may provide a stable mechanical scaffold for administration and longevity in the FRT, while imparting properties that enable prompt dissolution when needed. While not addressed in this immediate work, we seek to assess the mechanical properties in addition to the mucoadhesivity often

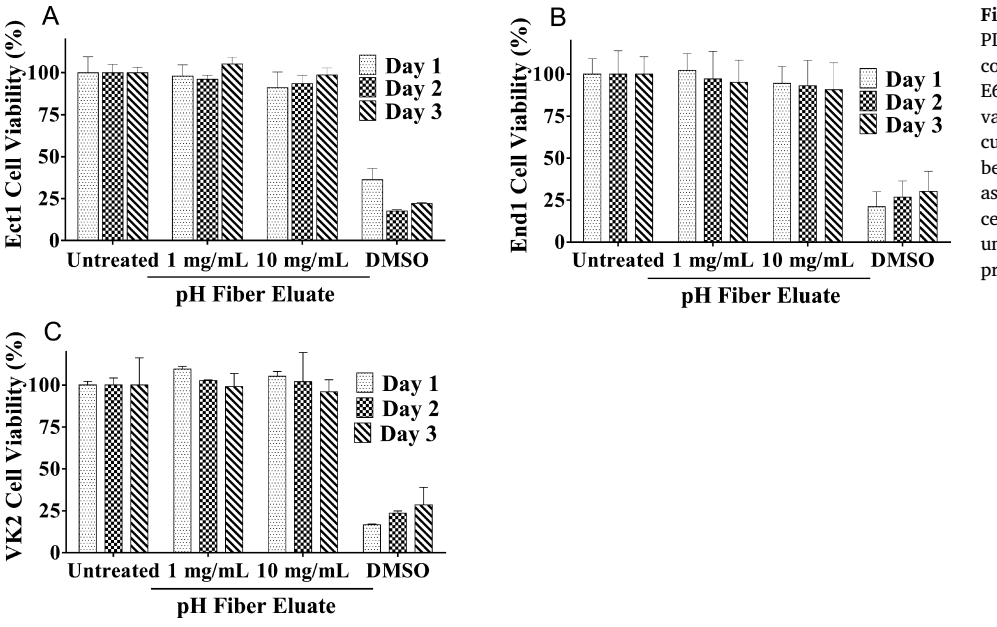

Fig. 9. *In vitro* safety evaluation of mPEG-PLGA:PBA-*co*-PAA (90:10) fibers on cervicovaginal cell viability. (A) Ectocervical (Ect1/E6E7), (B) endocervical (End1/E6E7), (C) and vaginal (VK2/E6E7) epithelial cells were incubated with GRFT mPEG-PLGA:PBA-*co*-PAA fibers (1 and 10 mg/mL) for 24, 48, and 72 h and assessed for cytotoxicity using the MTT assay. All cells had greater than 90% viability, relative to untreated positive controls. Cell viability is expressed as the mean ± SD of triplicate samples.

provided by blending with PBA or PAA [79]. For long-term applications, these features would be useful to provide immediate protection with retention and subsequent release of GRFT.

In this work we show that pH-responsive fibers retain GRFT for up to 72 h in SVF, suggesting that this platform may provide greater active agent stability and tailored release for less frequent administration. In addition, we evaluated GRFT release from fibers exposed to both PBS and SVF:SSF after initial incubation in SVF. Fibers switched to PBS exhibited complete release of GRFT within the first 24 h (Fig. 6), whereas fibers incubated in SVF:SSF exhibited a more gradual release, for up to 72 h after SVF:SSF exposure. This difference in release may be attributed to the increased viscosity and osmolality of simulated seminal fluid relative to PBS [59,80]. The daily release of GRFT from fibers switched to SVF:SSF was enough to provide protection throughout the 72 h post-switch incubation period, indicating that these fibers may provide activity for up to 6 days administration in the female reproductive environment. Moreover, previous studies have shown that human semen may be present within the FRT for up to 72 h post-coitus, during which time multiple exposures to HIV may occur. A platform that provides pH-dependent release coupled with sustained short-term release post-switch may address the administration challenges of short- versus long-term (or unknown) exposure. This platform demonstrates the ability to rapidly release GRFT under pH conditions of semen exposure, while also preserving GRFT after 3 days in SVF, demonstrating exciting utility relative to traditional delivery platforms.

In addition to conserving GRFT payload and providing inducible release, mPEG:PBA-*co*-PAA (90:10) EFs provided corresponding immediate and complete protection against HIV-1 *in vitro*. Fiber eluates maintained antiviral activity against HIV-1 after different incubation durations, demonstrating their potential utility for administration every few days. Building upon this work, we expect to conduct more prolonged release and efficacy studies in the future. Furthermore, the *in vitro* release profiles indicate that the fibers retain GRFT for short duration, unlike traditional sustained-release delivery platforms, which often exhibit an initial burst release whether or not it is needed. This design feature may provide enhanced protection relative to the administration of free GRFT alone or other traditional delivery platforms, in which prematurely released GRFT may be shed with mucus, or may be locally diluted when needed. Considering that GRFT acts as an entry inhibitor by interacting with viral envelope proteins, the ability of this pH-responsive platform to provide GRFT release immediately after exposure to SSF, may increase the success rate of viral inhibition by releasing a timely localized concentration of GRFT to inactivate virus.

In addition to relevant release times and efficacy, the design of intravaginal delivery vehicles requires that safety and biocompatibility are considered early in the development process. Particularly for GRFT, which has demonstrated outstanding safety [20,81], similar safety must be assessed with its integration in new delivery vehicles. In our studies with vaginal epithelial cells, incubation with fiber eluates resulted in greater than ~95% cell viability after 1, 2, and 3 day exposure, indicating the preliminary safety of these pH-responsive fibers. Future work will assess if these fibers induce inflammation or enhance cytokine production in *in vivo* experiments.

5. Conclusions

Drug delivery systems in which active agent release can be tailored to release in response to incoming stimuli are particularly promising for biologics that may lose activity quickly and be expensive to produce. In this study, pH-responsive fibers comprised of PLGA, mPEG-PLGA, and PBA-*co*-PAA polymer blends were fabricated to provide pH-responsive release of GRFT. Of the formulations tested, the mPEG-PLGA:PBA-*co*-PAA (90:10) blend provided the optimal release of GRFT, exhibiting increased release under more neutral conditions while maintaining minimal release in acidic SVF. In addition, fiber release eluates provided immediate activity against HIV-1 infection while simultaneously retaining GRFT activity. Furthermore, the release profiles demonstrated that fibers provided pH-induced release for at least 72 h, further indicating the utility of this delivery platform to preserve active agent for a minimum of 3–6 days post-administration.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ejpb.2018.04.013.

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