Published in final edited form as:

Int J Pharm. 2017 October 05; 531(1): 118–133. doi:10.1016/j.ijpharm.2017.08.061.

Multipurpose Tenofovir Disoproxil Fumarate Electrospun Fibers for the Prevention of HIV-1 and HSV-2 Infections *In Vitro*

Kevin M. Tyo^{2,7}, Hung R. Vuong⁴, Danial A. Malik², Lee B. Sims¹, Houda Alatassi⁵, Jinghua Duan^{1,7}, Walter H. Watson^{2,6}, and Jill M. Steinbach-Rankins^{1,2,3,7,*}

¹Department of Bioengineering, Speed School of Engineering, University of Louisville, Louisville, KY, United States of America

²Department of Pharmacology and Toxicology, School of Medicine, University of Louisville, KY, United States of America

³Department of Microbiology and Immunology, School of Medicine, University of Louisville, KY, United States of America

⁴Department of Biochemistry, School of Medicine, University of Louisville, KY, United States of America

⁵Department of Pathology, University of Louisville, Louisville, KY, United States of America

⁶Department of Medicine, Division of Gastroenterology, Hepatology and Nutrition, School of Medicine, University of Louisville, KY, United States of America

⁷Center for Predictive Medicine, Louisville, KY, United States of America

Abstract

Sexually transmitted infections affect hundreds of millions of people worldwide. Both human immunodeficiency virus (HIV-1 and -2) and herpes simplex virus-2 (HSV-2) remain incurable, urging the development of new prevention strategies. While current prophylactic technologies are dependent on strict user adherence to achieve efficacy, there is a dearth of delivery vehicles that provide discreet and convenient administration, combined with prolonged-delivery of active agents. To address these needs, we created electrospun fibers (EFs) comprised of FDA-approved polymers, poly(lactic-co-glycolic acid) (PLGA) and poly(DL-lactide-co-ε-caprolactone) (PLCL), to provide sustained-release and *in vitro* protection against HIV-1 and HSV-2. PLGA and PLCL EFs, incorporating the antiretroviral, tenofovir disoproxil fumarate (TDF), exhibited sustained-release for up to 4 weeks, and provided complete *in vitro* protection against HSV-2 and HIV-1 for 24 hr and 1 wk, respectively, based on the doses tested. *In vitro* cell culture and EpiVaginal tissue tests confirmed the safety of fibers in vaginal and cervical cells, highlighting the potential of PLGA and PLCL EFs as multipurpose next-generation drug delivery vehicles.

*Corresponding Author: jill.steinbach@louisville.edu (JMSR).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Graphical Abstract

Keywords

electrospun fiber; microbicide; tenofovir (TFV); tenofovir disoproxil fumarate (TDF); drug delivery fiber; sexually transmitted infections (STIs); HIV; HSV-2; multipurpose prevention; MPT; sustained release fiber; antiretroviral (ARV)

1. Introduction

Sexually transmitted infections (STIs) are a global health challenge, with over one million new cases of STIs reported daily. Over 530 and 36 million people globally are infected by herpes simplex virus type-2 (HSV-2) and human immunodeficiency virus (HIV), respectively [1]. Compounding these statistics, HSV-2 infection has been shown to significantly enhance HIV infection by as much as 2 to 7-fold [2–4]. 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.

Despite the existence of multiple strategies to prevent and/or treat STIs, rates of infection among particular demographics remain high [5, 6]. Moreover, despite the numerous antivirals available to treat HIV and HSV-2 individually, to date there are no agents that completely prevent or cure these infections individually or together. In terms of prevention, pre-exposure prophylaxis (PrEP) has enabled high-risk individuals to prevent HIV-1 infection by taking oral medication daily. However, to date, only two compounds, Tenofovir Disoproxil Fumarate (TDF) and Emtricitabine (combined with TDF in Truvada), are approved for PrEP by the FDA [7].

Oral PrEP has demonstrated success in preventing HIV in clinical trials and is becoming increasingly acknowledged as a successful prevention platform [8, 9]. By frequent administration of oral antiretroviral (ARV) compounds, such as TFV and Emtricitabine, prevention rates from 44 to 75% have been achieved in clinical trials [10–12]. However, as exemplified in the VOICE (MTN-003) trial, frequent administration of oral tablets (or vaginal gels) and strict user adherence are critical to provide any meaningful protection [13].

Additional challenges of oral PrEP, based on the administration of ARVs, include renal and bone toxicity; associated decreases in condom use; the development of antiviral resistance; and reduction of drug concentration via first-pass metabolism [13]. Thus new topical delivery strategies are urgently needed to provide safe, effective, and long-term protection against multiple STIs.

Given the disadvantages of oral PrEP, several topical PrEP strategies have been developed that provide localized protection to overcome these limitations. Traditionally, topical PrEP has been administered in the form of gels, but films, tablets, and intravaginal rings (IVRs) have also demonstrated promise in safety and efficacy trials. Antiviral gels have the potential to confer protection when frequently applied. The potential of topical PrEP was demonstrated in the CAPRISA-004 clinical study, where a topical gel containing TFV was used to effectively reduce HIV transmission by 39% [14]. However, similar to the oral tablet arm of the VOICE (MTN-003) trial, topical gels containing TFV required strict user adherence both prior to and after sexual intercourse to maintain effectiveness [15, 16]. This strict dosing resulted in suboptimal user adherence, leading to decreased protection against infection. In addition, some users experience discomfort or leakiness, which may have further reduced adherence to the prescribed dosing regimen. Intravaginal films have also demonstrated protection against STIs; however, the rapid release of the encapsulated agents within hours of administration (burst release) remains a major hurdle for long-term administration [16–18]. In addition, challenges in self-administration of vaginal films, combined with reported irritation with long-term use has affected user adherence [16, 19]. Similar to vaginal films, tablets exhibit rather transient activity and a lack of long-term release; however, they offer a cost-effective platform for antiviral delivery [20].

Of these existing delivery technologies, intravaginal rings (IVRs) provide the current “gold standard” to prolong the release of active agents for 3 to 4 months. While IVRs have been utilized for hormonal contraceptive delivery for over a decade, the translation of IVRs to HIV PrEP has been recently demonstrated in the ASPIRE (MTN-020) and IPM clinical trials [21, 22]. Additionally, IVRs have demonstrated the ability to provide sustained-release of multiple ARVs such as Tenofovir (TFV) and Acyclovir, providing *in vitro* protection against HSV-2 and HIV, as well as protecting against HSV-2 *in vivo* [23]. However, concerns remain regarding the lack of complete protection provided in clinical trials, and their ability to incorporate less stable agents, such as proteins and oligonucleotides, due to the high processing temperatures utilized during the manufacturing process [24]. Lastly, similar to the above technologies, user adherence of IVRs, particularly in young age groups (18–25 yr), remains another major concern [21]. These results suggest that the development of alternative dosage forms may improve user adherence and achieve increased efficacy, by providing options that are more amenable to female preferences.

As a relatively new microbicide delivery technology, electrospun fibers (EFs) may provide a promising alternative for prolonged and localized agent delivery, with the potential to protect against multiple STIs. Some of the advantages of EFs include the ability to highly incorporate a diversity of active agents including drugs and biologics [25, 26], to tailor sustained-release by selecting different polymeric materials, and to maintain agent stability during the course of delivery [27, 28]. Biodegradable polymers, such as poly(lactic-co-

glycolic acid (PLGA) and poly(caprolactone) (PCL), are approved by the U.S. Food and Drug Administration (FDA) for therapeutic use, indicating their proven biocompatibility and potential for translation [29]. Together, these attributes have recently established polymeric EFs as an attractive platform for localized delivery against STIs.

Over the past decade, researchers have begun to incorporate antiviral agents into polymeric EFs to prevent HIV infection. One of the first studies to utilize electrospun fibers to combat HIV developed pH-responsive fibers that encapsulated cellulose acetate phthalate (CAP) [30]. While CAP EFs exhibited long-term stability in low pH environments characteristic of the female reproductive tract, the EFs quickly degraded with the introduction of semen, to release active CAP and neutralize HIV particles. Later research by the same group utilized surface-modified polystyrene and polypropylene fibers to bind to and inhibit HIV with higher efficacy than unmodified fibers alone [31].

In addition to pH-sensitive and surface-modified fibers, researchers have utilized EFs to provide tunable release of one or more incorporated active agents for HIV-1 prevention [32, 33]. Polymer blends of polyethylene oxide (PEO) and poly(L-lactic acid) (PLLA) were synthesized to encapsulate and tailor the release of the antivirals Maraviroc (entry inhibitor) and AZT for up to several weeks [33]. In another study, PLGA and PCL fibers were loaded with various concentrations of the antiretroviral TFV [32]. These fibers demonstrated sustained-release of TFV for 30 days as well as efficacy against HIV infection *in vitro*.

Similarly, but less extensively for HSV-2, sustained-release delivery vehicles have been recently developed. In one study, PCL molded matrices were fabricated using a heat-based injection molding technique to incorporate increasing concentrations of Acyclovir (10, 15, and 20% w/v) into IVR-like dosage forms [34]. The encapsulated ACV exhibited release up to 30 days and retained comparable 50% inhibitory concentration (IC₅₀) to free drug. In another study, ACV was incorporated into EFs. Release eluate collected up to 28 days post-release provided sustained protection against HSV-2 infection *in vitro* [35]. Other work that demonstrated protection against both HSV-2 and HIV-1 *in vitro* and *in vivo*, examined the incorporation of TDF, a newer and more potent prodrug of TFV, into IVRs. These IVRs demonstrated promise in multipurpose prevention and sustained-release due to the combined efficacy of TDF against both HIV and HSV-2; its increased oral and topical lipophilicity and cell permeability; and improved HIV-1 IC₅₀ (by 160-fold) relative to TFV [36]. In subsequent studies, these TDF IVRs prevented HIV infection in macaques for up to 4 months, with monthly IVR changes [36, 37].

Building upon this previous research, the goal of our work was to develop PLGA and poly(DL-lactide-co- ϵ -caprolactone) (PLCL) EFs containing TDF to demonstrate safe and efficacious inhibition of both HIV-1 and HSV-2 infections *in vitro*. TDF was selected as a model ARV to demonstrate proof-of-concept of our delivery vehicles, as at the time of this study, it was a next-generation, more lipophilic form of TFV, that had demonstrated strong protection after sustained-release from IVRs. Here we fabricated both PLGA and PLCL EFs to evaluate and compare two different biodegradable polymers known to impart the sustained-release of active agents. We synthesized 3 different formulations for each polymer, PLGA and PLCL, and characterized the loading and sustained-release of TDF from EFs. We

subsequently assessed the efficacy of fiber release eluates against both HSV-2 and HIV-1 infections *in vitro*, while demonstrating EF biocompatibility in vaginal keratinocytes, ectocervical and endocervical cells, and EpiVaginal tissue.

2. Materials and Methods

2.1 Materials

Poly(lactic-co-glycolic acid) (PLGA 50:50, 0.55–0.75 dL/g, 31–57k MW) and poly(DL-lactide-co-ε-caprolactone) (PLCL 80:20, 0.75 dL/g, 37k MW) were both purchased from Lactel Absorbable Polymers (Cupertino, CA). Solvents 1, 1, 1, 3, 3, 3—hexafluoro-2-propanol (HFIP) and trifluoroethanol (TFE) were obtained from Fisher Scientific (Pittsburgh, PA). TDF was purchased as Viread® (Tenofovir disoproxil fumarate, Gilead Sciences Inc., Foster City, CA) tablets from the University of Louisville Pharmacy. Other chemicals, including dimethyl sulfoxide (DMSO), acetonitrile, trifluoroacetic acid and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma Aldrich (St Louis, MO). Fetal bovine serum (FBS), antibiotics (penicillin/streptomycin and gentamicin), minimum essential medium (MEM, Corning), keratinocyte serum-free medium (KSFM, Gibco), and Dulbecco's modified Eagle medium (DMEM, Invitrogen) were all purchased from VWR and Thermo-Fisher. Simulated vaginal fluid (SVF) was prepared in house using a previously established protocol [38]. Finally, pure TFV and TDF were kindly provided by the NIH AIDS Reagent Program.

2.2 Cell lines, virus, and tissue culture

TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program (ARRRP). These cells are a genetically engineered HeLa cell clone that express CD4, CXCR4, and CCR5 and contain Tat-responsive reporter genes for firefly luciferase (Luc) and Escherichia coli β-galactosidase under regulatory control of an HIV-1 long terminal repeat [39, 40]. TZM-bl cells were maintained in DMEM containing 10% heat-inactivated FBS, 25 nM HEPES, and 50 µg/mL gentamicin, in a vented T-75 culture flask. Env-pseudotype HIV was kindly provided by Dr. Nobuyuki Matoba from the University of Louisville, and originally obtained from the NIH ARRRP. To conduct HSV-2 plaque assays, African green monkey kidney cells (Vero E6, originally obtained from ATCC), HEK-293T (human embryonic kidney cells originally purchased from ATCC) and HSV-2 (4674) were kindly provided by Dr. Kenneth Palmer from the University of Louisville. Cells were maintained in MEM supplemented with 10% FBS, and 1% penicillin and streptomycin (100 µg/mL each).

To assess cytotoxicity, endocervical, End1/E6E7 (End1); ectocervical, Ect1/E6E7 (Ect1); and vaginal keratinocyte, VK2/E6E7 (VK2) cell lines were used (courtesy of Dr. Kenneth Palmer, originally from ATCC). These cell lines were selected because they are representative of the cell types in the female reproductive tract that would be exposed to the topical EFs. End1, Ect1, and VK2 cells were maintained in KSFM supplemented with bovine pituitary extract (50 µg/mL), epidermal growth factor (0.1 ng/mL), and 1% penicillin and streptomycin. The media was further supplemented with calcium chloride (CaCl₂) to a final concentration of 0.4 mM. During cell trypsinization for plating and cell count, cells

were neutralized using DMEM/F12 (Gibco) with 10% FBS, and 1% penicillin and streptomycin. Organotypic EpiVaginal cultures of normal human vaginal-ectocervical epithelial cells were purchased from and cultivated as suggested by MatTek.

2.3 Synthesis of electrospun fibers

PLGA and PLCL EFs were prepared with different solvents and TDF concentrations spanning (1–20% wt drug/wt polymer (w/w)). Powder from crushed Viread tablets (700 mg tablets containing 300 mg TDF) were used as the source of TDF. The presence of inactive excipients in these samples was accounted for when determining the theoretical loading of TDF into polymer fibers. Blank fibers containing no TDF were prepared as negative controls. For blank EFs, 15–20% PLGA w/w and 12–20% PLCL w/w were dissolved in either 3 mL TFE or HFIP solvent overnight while shaking at 37°C. The following day, 2 mL of PLGA or PLCL solution was aspirated into, and electrospun from, a 3 mL plastic syringe as previously described [26, 35]. All formulations were electrospun with a flow rate of 2.0 mL/hr and an applied voltage of 20 kV. EFs were collected on a rotating 4 mm outer-diameter stainless steel mandrel, located 20 cm from the blunt needle tip. Sample flow rate was monitored by an infusion pump (Fisher Scientific, Pittsburgh, PA) and the voltage was applied using a high voltage power supply (Spellman CZE 1000R). For fibers incorporating TDF, either 1, 10, or 20% w/w TDF was dissolved in 1.2 mL solvent overnight. The next day the TDF solution was added to 1.8 mL polymer solution prior to electrospinning. After electrospinning, fibers were removed from the mandrel and dried overnight in a desiccator cabinet.

2.4 Electrospun fiber size and morphology

The impact of various parameters including: solvent choice, polymer composition, and TDF concentration on fiber size and morphology were evaluated using scanning electron microscopy (SEM). Desiccated EFs were placed on carbon tape, sputter coated with gold, and imaged using SEM (Supra 35 SEM Zeiss). SEM images were acquired at magnifications ranging from 1,000–5,000× to enable clear visualization of the fiber microstructure. 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 Fiber characterization: loading, encapsulation efficiency, and controlled release

Incorporated TDF was quantified via HPLC-UV/Vis using a modified established method [32]. Briefly, 10 mg of PLGA and PLCL fibers were dissolved in 1 mL of DMSO prior to analysis. Dilutions of these samples were injected into a Waters 515 HPLC pump using a Waters 717 Plus auto sampler with a Waters 2487 absorbance detector. The mobile phase was comprised of an isocratic mixture of 72% Milli-Q water with 0.045% trifluoroacetic acid and 28% acetonitrile with 0.036% trifluoroacetic acid. The column used for this procedure was a Waters Sun Fire C18 Column, (100Å, 5 µm, 4.6 mm × 250 mm). The instrument method comprised a 1 mL/min flow rate, 15 minute run time, UV/vis detection at 259 nm, and 20 µL sample injection volume. TFV was found to elute from the column 2.2 minutes after injection, while TDF eluted 12 min post-injection. The initial quantification of fibers was performed using combined TDF and TVF standards prepared in DMSO. Standard

curves of both TDF and TFV (0.7 – 100 µg/mL) were used to quantitate incorporated TDF and to assess TDF degradation. Samples from Viread tablets were assessed to verify TDF concentration and were used as standards in subsequent experiments. Controls included blank fibers and fibers spiked with a known concentration of TDF. TDF values determined from HPLC measurements were compared with the quantity of TDF added prior to electrospinning to obtain percent encapsulation efficiency (EE), where $EE = [(Mass\ of\ TDF\ Incorporated)/(Mass\ of\ TDF\ Initially\ Added)] \times 100$. Unless otherwise noted, all samples were analyzed in triplicate.

Controlled release experiments were performed to assess the release of TDF from EFs. Triplicate 10 mg fiber pieces were cut and suspended in 1 mL of simulated vaginal fluid (SVF) to represent intravaginal conditions *in vitro*. Samples were incubated at 37°C and constantly shaken. The complete volume of SVF was removed and replaced with fresh SVF at time points: 1, 2, 4, 6, 24, 48, 72 hr, and 1, 2, 3, and 4 wk. The amount of TDF in the supernatant was measured using HPLC. Quantification was performed using a Viread standard diluted in SVF, with eluate from blank fibers in SVF used as background correction. Statistical significance of both loading and release profiles between fiber formulations was determined by one-way ANOVA with the Bonferroni post hoc t-test ($p < 0.05$).

2.6 In vitro efficacy of PLGA and PLCL fibers against HIV-1 infection

HIV pseudovirus assays were used to assess the efficacy of TDF released from EFs against HIV infection *in vitro*. TZM-bl cells were infected with Env-pseudotype HIV, kindly provided by both Dr. Nobuyuki Matoba (University of Louisville) and the NIH ARRRP. To produce and propagate HIV Env-pseudovirus, HEK293T/17 cells were transfected with two plasmids, one containing an Env-defective HIV genome and a plasmid solely expressing Env. Transfection was facilitated with the use of FuGENE (Promega). HEK293T cells were allowed to incubate for 48 hr, after which viral particles were collected and titered using the 50% Tissue Culture Infectious Doses assay (TCID₅₀). Viral particles were stored at –80°C until use [41].

To determine the *in vitro* efficacy of PLGA and PLCL TDF EFs against Env-pseudotype HIV infection, TZM-bl cells were seeded in 96-well plates at 100,000 cells/well in 100 µL of DMEM. Fifty microliters of fiber eluate media (DMEM 10% FBS) collected from time points: 1 and 24 hr; week 1 (release from days 0–7); week 2 (release from days 8–14), week 3 (release from days 15–21), and week 4 (release from days 22–28) were diluted by a maximum of 5 orders of magnitude from collected eluate (1:100,000 maximum dilution). Eluate dilutions were added to cells in triplicate, and 50 µL of diluted virus stock (1:8) was subsequently added to each well. The administered virus dose resulted in relative luminescence units (RLU) of at least twenty times that of background observed in untreated/uninfected cells, yielding an average of 100,000 RLUs in our experiments. Experimental controls included untreated/uninfected cells, untreated/infected cells, and blank fiber eluate-treated/infected cells. For wells containing untreated/uninfected and untreated/infected cell controls, 100 µL DMEM was added to the wells; for infected cells with blank fiber eluate, 50 µL DMEM was added to 50 µL blank fiber eluate, resulting in a final volume of 200 µL

for all wells. After infection, plates were incubated 48 hr at 37°C, and 100 µL of media was subsequently removed from each well (post-incubation), and replaced with 100 µL of Bright Glo Reagent (Promega). Cells were incubated at room temperature for another 5 minutes and the luminescence of each well was read at an integration time of 1 sec and a gain of 135 (Synergy HT luminometer). The amount of virus inhibition was determined by normalizing the RLUs of treated/infected cells to untreated/infected cells. Additionally, all RLU values were corrected by subtracting the RLU of untreated/uninfected cells. IC50 values were determined using GraphPad 6.0 sigmoidal regression analysis. Unless otherwise noted, all experiments were run with three or more replicates per treatment group. Statistical significance between the IC50s was determined using one-way ANOVA with the Bonferroni post hoc t-test ($p < 0.05$).

2.7 In vitro efficacy of PLGA and PLCL fibers against HSV-2 infection

HSV-2 plaque assays were conducted to test the efficacy of TDF EFs against HSV-2 infection *in vitro*. Fibers were incubated in 10 mL complete plating media (1% FBS MEM) for 1 and 24 hr. Additional fiber eluates were collected at week 1 (release from days 0–7); week 2 (release from days 8–14); week 3 (release from days 15–21); and week 4 (release from days 22–28) to assess the ability of PLGA and PLCL TDF EFs to provide prolonged delivery and corresponding HSV-2 protection. The antiviral activity of PLGA and PLCL TDF EF eluates was determined using HSV-2 (4674) plaque assays in Vero E6 cells. Vero E6 cells were seeded with 600,000 cells/well and grown to near confluence for 24 hr in a 6-well flat bottom plate. After 24 hr, the media was removed and cells were simultaneously administered 2 mL of fiber eluate serial dilutions from the above collected time points and 3,000 PFU of HSV-2 per well. Free TDF was used as a positive control for HSV-2 inhibition, in parallel with untreated/uninfected cells; whereas untreated/infected cells were used as a positive control of cell infection and death. After 48 hr, cells were fixed with methanol for 10 min, stained with 0.1% crystal violet for 30 min, and washed with DI water. Plaques were counted, and plaque numbers from experimental groups were normalized relative to the number of plaques in untreated/infected cells (~280–300 plaques). Samples were analyzed in triplicate, and GraphPad was used to determine the IC50 values of the TDF EF formulations. Statistical analysis was performed by comparing the average percent inhibition of HSV-2 using one-way ANOVA with the Bonferroni post hoc t-test ($p < 0.05$).

2.8 In vitro cytotoxicity of PLGA and PLCL fibers

Vaginal epithelial (VK2/E6E7), ectocervical (Ect1/E6E7), and endocervical (End1/E6E7) cells were incubated with TDF EFs in KSFM to assess the *in vitro* biocompatibility of TDF fibers. Cells were plated at a density of 300,000 cells/well in 12-well plates and incubated in triplicate with 10 mg fiber pieces placed in transwell inserts (10 mg/mL final concentration). No treatment (media alone) and 10% DMSO were used as positive and negative controls of cell viability, respectively. After 24, 48, and 72 hr incubation, 10 mL of MTT reagent was added to the cells, cells were lysed, and absorbance was read at 570 nm the following day. PLGA and PLCL EF-treated cell absorbance values were normalized to untreated cell absorbance to obtain percent viability.

2.9 EpiVaginal cytotoxicity of PLGA fibers

Full thickness vaginal epithelial (VEC-100 FT) EpiVaginal™ tissues (MatTek) were administered low (5 mg/mL) and high (50 mg/mL) concentrations of PLGA TDF fibers to best represent administration in a future *in vivo* model. PLGA samples were chosen due to our initial experiments demonstrating its enhanced TDF release profile and efficacy relative to PLCL. Control samples included untreated, blank PLGA fiber-treated, and toxic (0.2% nonoxynol-9)-treated control groups. The tissues were incubated at 37°C, 5% CO₂ for 2 and 3 days.

To monitor the tissue viability following exposure to TDF PLGA EFs, the basal side tissue culture media was collected on days 2 and 3. Cytotoxicity was measured using a lactate dehydrogenase (LDH) cytotoxicity assay kit (Pierce). The viability of the TDF EF treated tissues was determined by normalizing the absorbance of the treated tissues to the absorbance of the untreated tissue. Percent cell viability was expressed as: % Viability = [OD (treated tissue)/OD (untreated tissue)] × 100. Transepithelial electrical resistance (TEER) was measured using an EVOM2 Epithelial Voltohmmeter equipped with an Endohm electrode chamber (World Precision Instruments, Sarasota, FL) on days 0, 1, 2, and 3 of the treatment.

To assess inflammatory markers resulting from fiber exposure, tissue media was analyzed for cytokine production. Based on previous work [42–45], cytokines: IL-1α, IL-β, IL-6, IL-8, and TNF-α were assessed in all collected media via Luminex assay. Additionally, GM-CSF, IFN-γ, and MCP-1 expression were assessed based on previous microbicide studies using Luminex [46–48]. Cytokine expression in samples was compared with untreated EpiVaginal tissue via fold increase. The fold-increase was calculated by dividing the sample expression level by the untreated tissue values.

To visually examine the structural integrity of the tissue after 3 days of consecutive treatment, the tissue samples were washed with PBS and fixed with 4% paraformaldehyde. The tissue specimens were embedded in a paraffin block, stained with hematoxylin and eosin (H&E), and cross-sections were observed under 20× magnification using an Aperio Imagescope (Leica Biosystems Inc., Buffalo Grove, IL). Tissue samples were subjected to histological analysis by a pathologist blinded to treatment group assignment.

3. Results

3.1 Electrospun fiber size and morphology

Fiber morphology was evaluated using SEM, and NIH ImageJ software was used to assess fiber diameters. Blank PLGA and PLCL fibers fabricated using either HFIP or TFE solvent are shown in Fig. 1. For fibers electrospun in HFIP, 15% w/w PLGA or 12% w/w PLCL provided well-defined fiber morphologies. However with TFE, both polymers required an increase in concentration to 20% (w/w) to produce well-delineated microstructures. The average diameters were 2.0 ± 0.8 and 1.7 ± 0.4 μm for 15% PLGA and 12% PLCL fibers made with HFIP, and 1.9 ± 0.9 and 1.9 ± 0.8 μm for 20% PLGA and 20% PLCL fibers made with TFE (Table 1). Once well-delineated fibers were established, the effect of TDF

incorporation on PLGA and PLCL EF morphologies electrospun with HFIP was evaluated (Fig. 2).

The morphologies and diameters of TDF EF formulations are shown in Figs. 2 and 3, respectively. Prior to TDF incorporation, the average diameters of all blank fiber formulations were similar, ranging from 1.7 to 2.0 μm , with no statistically significant differences observed between formulations (Fig. 3A). In comparison, PLGA fibers incorporating TDF showed no particular trend in fiber diameters; whereas PLCL fiber diameters decreased with increased TDF concentration. The average fiber diameters for HFIP 15% PLGA 1%, 10%, and 20% TDF were 1.1 ± 0.3 , 0.8 ± 0.3 , and 1.1 ± 0.4 μm . For HFIP 12% PLCL 1%, 10%, 20% TDF fibers, the resulting fiber diameters were 1.1 ± 0.5 , 0.9 ± 0.3 , 0.7 ± 0.2 μm (Table 1). TDF-incorporated fibers electrospun with TFE solvent, displayed a similar decrease in diameters to 1.2 ± 0.4 μm and 0.6 ± 0.2 μm , for PLGA and PLCL respectively. All TDF fibers exhibited statistically significant decreases in fiber diameter relative to blank PLGA (1.7 and 2.0 μm for HFIP and TFE blank EF respectively) and PLCL (1.7 and 1.9 μm for HFIP and TFE blank EF respectively) EFs. However, no statistical significance was observed between the 1, 10, and 20% TDF fiber formulations as a function of TDF incorporation. Thus, TDF incorporation resulted in decreased fiber diameter relative to blank fibers; whereas variation in the amount of TDF incorporation had no significant effect on fiber diameter.

3.2 Fiber characterization: loading, encapsulation efficiency, and controlled release

To determine the loading of TDF in PLGA and PLCL fibers, different concentrations of TDF (1, 10, and 20% w/w) were incorporated. The TDF, TFV, and Viread standard curves, used to quantify TDF in fibers, maintained linearity and similar peak intensities in both DMSO as well as SVF (Supp. Fig. 1). For all samples examined, TDF incorporated into fibers remained stable against degradation or hydrolysis. Although TFV peaks were present in many loading samples, they were either below the limit of quantification or comprised less than 2% of TDF sample concentration. In addition to TFV peaks, a minor peak eluting at 3.5 min was present in all samples and standards containing TDF. This peak, dubbed “minor TDF” comprised an area that was 4% of the TDF peak area (Supplemental Fig. 1) and may be attributed to the monoester derivative of TDF (mPTFV). This proportionality was observed in all loading samples as well as controlled release samples collected during the first week of release. After 1 wk, the proportion of mPTFV increased, reaching a 1:1 ratio with TDF in some samples (data not shown). This increased ratio of mPTFV:TDF is attributed to the increased exposure of fibers to aqueous solution at later time points, coupled with an overall decrease in TDF release. The mPTFV concentration was quantified using the TDF standard.

Table 2 summarizes the total loading (μg TDF/mg fiber) and encapsulation efficiency (EE) achieved for the various fiber formulations. Overall, we observed that fibers electrospun with HFIP resulted in high EEs spanning 60–89%. Furthermore, proportional increases in loading were observed based on the amount of TDF added to PLGA and PLCL formulations. However, comparing polymer formulations electrospun with different solvents, PLGA and PLCL fibers electrospun with HFIP demonstrated higher loading and encapsulation

efficiency, relative to PLGA and PLCL fibers electrospun with TFE. Ten percent TDF fibers electrospun with HFIP showed high encapsulation efficiencies spanning 76 to 89%, relative to fibers electrospun with TFE (~60%). Considering the higher polymer concentration required to fabricate well-delineated fibers with TFE (20% for PLGA and PLCL vs. 12 and 15% for PLGA and PLCL, respectively) and the lower EE, HFIP was selected to electrospin subsequent formulations.

After determining PLGA and PLCL fiber loading, we assessed the release of TDF from PLGA and PLCL EFs in SVF for up to 4 weeks (Fig. 4). Figure 4A demonstrates increased TDF release per mass of fiber, and corresponds with increased incorporation of TDF in 1, 10, and 20% PLGA and PLCL fibers. While both the 1% TDF PLGA and PLCL formulations exhibited release near the limit of TDF detection, the cumulative release of TDF from the 10% and 20% PLCL fibers resulted in 16 and 26 $\mu\text{g}/\text{mg}$ (31 and 29% total release), while the PLGA fibers averaged only 9.3 and 19.7 $\mu\text{g}/\text{mg}$ (20 and 22% total release) within 1 hr. Within the first 24 hr, the 10% and 20% formulations demonstrated a burst release, with the amount of TDF release increasing with increased TDF incorporation. Overall, the 10 and 20% PLGA fibers released up to 40% more TDF than PLCL fibers. Although appreciable increases in release were only observed for approximately 72 hr, PLGA fibers released more TDF than PLCL fibers for the 10 and 20% TDF formulations. The 10% PLGA and PLCL fibers released 66 and 39% of their cargo, respectively, while the 20% PLGA and PLCL fibers released 64 and 43% of their cargo after 4 wk.

3.3 In vitro efficacy of PLGA and PLCL fibers against HIV-1 infection

3.3.1 Short-term efficacy—To assess the antiviral activity of TDF PLGA and PLCL fibers, HIV inhibition assays were performed using fiber eluates collected at different release time points. Both short- and long-term release samples were collected to assess efficacy. For short-term assessment of antiviral activity, 10 mg fibers were incubated in 1 mL DMEM for 1 or 24 hr. A histogram of the HIV inhibition after administration of the 1 hr (Fig. 5A) or 24 hr fiber release eluates (Fig. 5B) is shown, and the corresponding IC₅₀s are shown in Table 3. All 10 or 20% TDF fibers (PLGA or PLCL) completely inhibited viral infection in TZM-bl cells down to a 1:100 eluate dilution. For 1 and 24 hr eluate dilutions exceeding 1:100, viral inhibition was more pronounced after administration of the 24 hr eluates, relative to 1 hr eluates. The increased efficacy observed with 24 hr eluates can be attributed to the higher amount of TDF released within 24 hr. However, for both time points, the IC₅₀s of both fibers were similar against *in vitro* infection (Table 3).

Overall, the antiviral activities of these eluate dilutions demonstrate that the amount of TDF in the fiber corresponds with increased viral inhibition. For the 1% TDF PLGA and PLCL fibers, 1 hr undiluted eluates decreased infection to 27% and 10%, relative to untreated/infected controls. However, subsequent dilutions of the 1% TDF 1 hr eluates yielded decreased protection against HIV-1 infection. In contrast, after 24 hr release, the 1% TDF PLGA and PLCL fibers completely inhibited virus infection, with subsequent decreases in virus inhibition corresponding with increased eluate dilution. Full infection resulted after administration of the 1% PLGA and PLCL fibers at a dilution of 1:10 and 1:1000,

respectively for the 1 hr eluates; and 1:1000 and 1:100 for the PLGA and PLCL 24 hr eluates.

For the 10% TDF formulations, both PLGA and PLCL fibers exhibited complete protection against HIV down to 1:100 and 1:1000 eluate dilutions, respectively after 1 hr. After administration of the 24 hr eluates, complete protection was observed even after a 1:1000 dilution of each formulation. Subsequent dilutions of PLGA eluates for 1 hr and 24 hr time points exhibited an increase in infectivity (decrease in prevention) to 31% and 13% for 1:1000, and complete infectivity for 1:10,000 dilutions, respectively. For PLCL, complete protection was achieved with the 1:1000 eluate dilutions at both 1 and 24 hr time points. Additionally, these fibers showed efficacy even at eluate dilutions of 1:10,000; with 48% and 69% infectivity at 1 and 24 hr. The corresponding IC₅₀s for 10% TDF PLGA and PLCL EFs were 2.4 and 3.1 ng/mL TDF after 1 hr, and 7.2 and 7.4 ng/mL after 24 hr (Table 3).

As expected, the 20% TDF fibers exhibited the highest efficacy against HIV infection per mass of fiber tested. Similar to the 10% TDF PLGA and PLCL EFs, 20% TDF fibers completely inhibited infection after a 1:1000 dilution, and exhibited partial efficacy (58–100%) between 1:10,000 and 1:100,000 dilutions. The corresponding IC₅₀s for 20% TDF PLGA and PLCL EFs were 4.6 and 3.1 ng/mL after 1 hr release, and 5.1 and 1.9 ng/mL after 24 hr release (Table 3). Despite these small differences, the PLGA and PLCL TDF fibers were equally efficacious at their respective time points (Table 3). While statistical analysis was performed between each formulation and time point, no clear pattern emerged. There was no statistical significance between any of the formulations after 1 hr release, with the exception of free TDF, which had a significantly higher IC₅₀ than the TDF fiber formulations. However, after 24 hr there was marked decrease of the IC₅₀ of free TDF (12.1 and 5.3 ng/mL at 1 and 24 hr respectively) which resulted in no statistical difference between free TDF and fibers. However, most of the IC₅₀s at 24 hr possessed statistical significant differences at that time point. Furthermore, the IC₅₀s at 24 hr generally were larger than their 1 hr counterparts, although this trend was not seen in all formulations. Considering that all EF formulations possessed similar IC₅₀ to their free TDF counterparts, suggests that any formulation could be used to provide short-term protection for 1 or 24 hr.

3.3.2 Long-term efficacy—To assess the long-term efficacy of the fibers against HIV infection *in vitro*, eluates were collected from 10 mg of 20% TDF PLGA and PLCL fibers after 1, 2, 3, and 4 wk. Twenty percent TDF fibers were selected due to their greater encapsulation, release, and applicability to future dosing *in vivo*. The resulting HIV inhibition after administration of 1 and 2 wk fiber release eluates is shown in Fig. 5C and the corresponding IC₅₀s are quantified in Table 4. After 1 and 2 wk, eluates from 10 mg/mL PLGA and PLCL EFs completely inhibited HIV infection. However, only the 1 wk eluates completely inhibited HIV infection, after a 1:100 or 1:1000 dilution. Two week eluates demonstrated weaker activity, showing only marginal protection (14% and 27%) at 1:10 dilutions of PLGA and PLCL fiber eluates, respectively. The corresponding IC₅₀s were 1.9 and 11.9 ng/mL for 1 wk PLGA and PLCL eluates and 10.2 and 72.3 µg/mL for 2 wk PLGA and PLCL eluates. Free TDF controls showed a similar decrease in inhibition, relative to their IC₅₀s after 1 and 24 hr exposure to media (12 and 5.2 ng/mL), to 4.5 and 4.9 µg/mL at 1 and 2 wk, suggesting the hydrolysis of free TDF into its monoester derivative after

prolonged exposure to media (Fig. 5E, which has been documented in similar studies [36]). Undiluted eluates from weeks 3 and 4 showed minimal protection against HIV at the doses tested (Fig. 5D). Blank fiber eluates were tested as a negative control for inhibition (Fig. 5F). The decreased efficacy of TDF EFs against HIV may be attributed to increased levels of mPTFV within the solution as well as decreased TDF release from the fiber.

As the incubation time increased, the level of protection seen from both TDF EFs and free TDF decreased. Although 1 wk PLGA fiber eluates showed higher efficacy (1.9 ng/mL), relative to PLCL (11.9 ng/mL), the IC₅₀s were not statistically significant. However, when compared to free TDF, the IC₅₀s of both PLGA and PLCL fibers demonstrated statistically significant increases in protection ($p < 0.05$, Table 4). Furthermore, all IC₅₀s of the 1 wk eluates were statistically significant ($p < 0.05$) relative to the 1 hr, 24 hr, and 2 wk time points. Thus for applications spanning one week, TDF fibers demonstrated prolonged activity, relative to free TDF. Additionally, less PLGA fiber was needed, relative to PLCL, to release therapeutically relevant amounts of TDF.

3.4 *In vitro* efficacy of PLGA and PLCL fibers against HSV-2 infection

To evaluate the potential of these fibers to inhibit HSV-2 infection, the antiviral efficacy of the 20% TDF EFs was also assessed in HSV-2 plaque assays. Similar to the HIV infection assay, eluate from 5 mg/mL fibers at 1 and 24 hr was shown to completely inhibit viral plaque formation. Fig. 6 illustrates the results of 1 and 24 hr eluate serial dilutions on infectivity. Both PLGA and PLCL fiber eluates completely inhibited HSV-2 infection, and exhibited decreased protection with increased dilution. Eluates from 24 hr showed greater efficacy against HSV-2 infection due to the increased amount of released TDF.

The IC₅₀s of PLGA and PLCL TDF fibers were assessed using these eluate dilutions. Table 5 shows that the IC₅₀s of PLGA and PLCL 1 and 24 hr eluates were comparable to the IC₅₀ of free TDF (8.9 μ g/mL). The plaque assays showed a trend of enhanced protection across dilutions provided by PLGA EFs after 1 and 24 hr, compared with PLCL fibers. However, no statistical significance was observed between formulations or compared to free TDF. Fiber eluates were also collected to assess HSV-2 protection after 1, 2, and 3 wk. For these time points and fiber concentration (5 mg/mL) tested, no virus inhibition was observed (data not shown). Similarly to the HIV studies, both 20% EF formulations demonstrate similar short-term protection compared with free TDF.

3.5 *In vitro* and EpiVaginal cytotoxicity of PLGA and PLCL fibers

To assess the potential of these fibers to safely interact with epithelial cells, fiber cytotoxicity was assessed in VK2, Ect1, and End1 E6E7 cells, using the MTT assay. As seen in Fig. 7, high cell viability was maintained after administration of TDF fibers for 24, 48, and 72 hr. All cells demonstrated greater than 93, 91, and 96% cell viability at 24, 48, and 72 hr respectively, for all formulations tested. In addition to cell monolayers, EpiVaginal tissue viability after PLGA fiber application was examined using the LDH cytotoxicity assay and H&E staining (Fig. 8). Due to the limited availability of EpiVaginal tissue samples, PLGA EFs were selected for analysis due to their enhanced release profiles and therapeutic potential, relative to PLCL fibers. PLGA EFs incorporating 20% TDF (5 and 50 mg/mL)

were incubated 48 and 72 hours with EpiVaginal tissue. Microscopic examination of the untreated, blank PLGA EF-treated, and TDF PLGA EF-treated tissues, revealed normal full thickness vaginal epithelium, while tissue treated with 0.2% non-oxynol 9 (N-9) exhibited severe degeneration of the epithelium (Fig. 8A). No adverse histological findings were noted in EpiVaginal tissue treated with PLGA or TDF PLGA EFs. Furthermore, tissue incubated with either 5 or 50 mg/mL fiber exhibited comparable viability in the LDH assay relative to untreated tissue controls after 48 and 72 hr (Fig. 8B).

In parallel, cytokine production from EpiVaginal tissue was analyzed after 48 and 72 hr of PLGA fiber administration. Cytokine expression, including GM-CSF, IFN- γ , IL-1 α , IL-1 β , IL-6, IL-8, MCP-1 and TNF- α , was compared to untreated and N-9 treated controls (Fig. 9). After 48 hr administration of TDF EFs, only GM-CSF and IL-6 expression showed statically significant increase in expression (approximately two-fold) compared with untreated tissue. After 72 hr, only the 50 mg/mL TDF fibers showed a slight increase of GM-CSF and MCP-1 cytokine expression (1.3-fold increase for both) relative to untreated tissue. Cytokine expression from tissue samples exposed to blank fibers was comparable with untreated samples at both time points, showing no statistical significance. In contrast, EpiVaginal tissue exposed to N-9 for 48 hr showed a marked decrease in GM-CSF, IFN- γ , IL-6, MCP-1, and TNF- α (0.7, 0.1, 0.12, and 0.4 respectively) while showing a slight increase of both IL-1 α and β (1.7 and 1.2-fold increases, respectively). After 72 hr, the expression of all cytokines following N-9 treatment was lower than observed in untreated samples, which is attributed to the loss of the vaginal epithelium. The negligible increase in cytokine expression (0–2 fold difference) from exposure of TDF EFs demonstrates promising preliminary safety profiles of these fiber formulations [48].

Discussion

There is an urgent need for new topical PrEP technologies that can confer the sustained-release of active agents, while providing discreet and convenient protection against STIs. The emerging application of polymeric electrospun fibers for intravaginal delivery offers the potential to fill this unique role. In these studies, we evaluated two electrospun fiber delivery platforms, comprised of PLGA or PLCL polymers, for their ability to release TDF, and protect against both HIV-1 and HSV-2 infections *in vitro*. Here TDF served as a model antiretroviral drug, as it is only one of two agents approved by the FDA to prevent HIV infection. Furthermore, TDF has demonstrated antiviral activity against both HIV-1 and HSV-2 *in vivo*, establishing its versatility as a multipurpose active agent. The goal of this work was to develop and characterize polymeric electrospun fibers to safely and efficaciously provide protection against both HIV-1 and HSV-2 *in vitro*, as a potential multipurpose prevention platform. For the doses tested in our studies, TDF PLGA and PLCL fibers demonstrated equivalent protection, relative to free TDF, against both HIV-1 and HSV-2 infections upon exposure to short-term (24 hr) release eluates. In addition, enhanced efficacy of TDF EFs compared with free TDF against HIV was demonstrated after exposure to release eluates taken through 2 wk. Moreover, this is the first time the safety of PLGA and PLCL TDF fibers has been investigated in EpiVaginal tissue.

The first goal of this study was to determine the formulation of TDF PLGA and PLCL fibers that resulted in the most cohesive and well-defined fiber macro- and microstructures. During the fabrication of blank PLGA and PLCL fibers, several solvents were assessed (Fig. 1). We observed that both HFIP and TFE solvents yielded reproducible PLGA and PLCL fiber morphologies. These solvents also enabled the incorporation of high weight percent polymer to solvent, which is critical to incorporating high concentrations of active agents in polymers. Using PLGA and PLCL fibers electrospun with HFIP as our baseline platforms, we sought to evaluate the effect of TDF incorporation on fiber diameter (Figs. 2 and 3).

Fiber diameter has a critical role in the release properties of active agents. Previous research has shown that decreasing fiber diameter can enhance the release of active agents. This is attributed to the increased surface-to-volume ratio, and decreased distance necessary for encapsulate diffusion [49, 50]. In our studies, the incorporation of TDF resulted in decreased fiber diameters relative to blank fibers (Fig. 3). The diameters of blank fibers ranged from 1.7 to 2.0 μm ; whereas TDF fiber diameters ranged from 0.7 to 1.1 μm . Even for the lowest concentration (1%) TDF fibers tested, a 50% decrease in fiber diameter was observed. This decrease in fiber diameter may be attributed to the charge of the active agent, and/or increased polymer jet instability resulting from these charge effects. Correspondingly, this jet instability may result in the polymer traveling longer distance/duration during the electrospinning process prior to reaching the mandrel, promoting elongation and decreased fiber diameter [51, 52]. Thus, incorporated active agents can affect the microstructural morphologies and diameters of electrospun fibers.

The incorporation of antiviral or biological agents has been shown to affect fiber diameter in a number of ways. In previous studies, Tenofovir (TFV), a compound less hydrophobic (solubility = 1.87 mg/mL) than TDF (the phosphorylated fumaric salt form of TFV, used in this study 0.71 mg/mL [53, 54]), was shown to slightly increase fiber diameter, though the differences were not statistically significant [55]. In other studies, the incorporation of antivirals resulted in the opposite effect on fiber diameter. Incorporation of TFV in polyvinyl alcohol polymers resulted in slightly smaller diameters, attributed to the increased instability described above [56]. Finally, some experiments show no change in fiber diameter after active agent incorporation. Fibers comprised of the pH-responsive CAP polymer, incorporating TDF, showed no change in fiber diameter relative to blank fibers [30]. These studies highlight that a variety of parameters including solvent choice, polymer selection, solvent-polymer/polymer-drug interactions, active agent characteristics, and solvent viscosity all contribute to the microstructural properties of electrospun fibers.

After obtaining well-defined and reproducible EFs, we next assessed the loading of TDF in PLGA and PLCL fibers as a function of solvent type (HFIP vs. TFE) used in the electrospinning process. From these loading studies (Table 2), we observed that fibers electrospun with HFIP showed ~30% higher encapsulation efficiency, relative to fibers electrospun with TFE. Furthermore, higher polymer concentrations were needed to obtain well-defined fiber microstructure, based on solvent type (15 and 12% w/w for PLGA and PLCL in HFIP; 20% PLGA and PLCL in TFE). Despite both HFIP and TFE sharing many characteristics such as high polarity and similar molecular structure, there are several key differences that may impact fiber properties. As previously described [35], TFE has a higher

dielectric constant (26.1 F/m) compared to HFIP (16.7 F/m) [57]. This increased charge capacity may confer additional instability to TFE solvents during electrospinning, requiring more polymer to produce well-defined fiber morphology [58]. Additionally, this increase in dielectric constant may result in decreased TDF incorporation, and even TDF localization on or near the fiber surface. HFIP also possesses a much lower boiling point (58.2°C) relative to TFE (73.6°C) [59, 60]. Solvents with lower boiling points tend to produce more stable fiber morphology due to complete evaporation during electrospinning; whereas less volatile (higher boiling point) solvents may not fully evaporate from the polymer, causing beaded morphologies [27]. These undesirable properties, in addition to the higher polymer concentrations required to produce TFE fibers with well-defined fiber microstructures and lower loading efficiencies, prompted us to fabricate subsequent formulations with HFIP.

Controlled release studies using TDF EFs yielded several interesting results (Fig. 4). First, as expected with most polymeric delivery vehicles [61], PLGA and PLCL formulations demonstrated a burst release of TDF during the first 24 hr. The exception was 1% TDF fibers, which released TDF quantities near our limit of detection. While the 10% and 20% TDF PLCL fibers showed a higher burst release relative to 10 and 20% PLGA fibers within the first hour, after 24 hr, all PLGA formulations released more TDF. While burst release is a common phenomenon in polymer drug delivery [61], here TDF surface localization may be exacerbated during the electrospinning process, due to charge effects between the incorporated drug, polymers, and solvent. Solution instability during electrospinning due to these charge effects, as well as hydrophilic interactions between the solvent and drug can also result in agent localization near the fiber surface [52, 62]. In the case of PLCL EFs, due to the increased hydrophobicity, more TDF may have accumulated on the fiber surface, increasing burst release within the first 1 hr. Another observation in the release studies was that all PLGA fibers released higher percentages of TDF compared to PLCL fibers, after ~1 wk in SVF. We attribute this increased TDF release to the hydrophilicity of PLGA, enabling enhanced wettability of the fiber, resulting in increased diffusion of TDF from the fiber into the surrounding eluate [28, 63].

Other studies using similar polymers yielded similar release results. In one recent study, TFV (relative to TDF) was incorporated in PLGA and PCL polymers and polymer blends, and controlled release was evaluated for 10 days [32]. Similar to our work, PLGA demonstrated greater overall release of drug while showing an initial lower burst release. In contrast, PCL released all incorporated TFV after 24 hr, while PLGA released only ~20% of incorporated drug during this time. While TDF was not extensively evaluated, similar burst release of TDF was also observed with 20:80 PCL:PLGA fibers, a trend that differed from the prolonged release observed from polymer blends that incorporated TFV [32]. These studies highlighted the effect that small molecular changes in drug design can have on the release kinetics from polymeric delivery vehicles. Furthermore, several formulations of PCL/PLGA blends were fabricated, demonstrating decreased burst release of TFV with increasing PLGA concentration [32]. We expect that in future work, similar blends will prove favorable to tailor the release of TDF, despite its increased lipophilicity.

Polymer hydrophobicity is another important consideration for providing sustained-release. In another study relevant to microbicide delivery, the antiviral compounds, MVC

(Maraviroc) and AZT, were encapsulated in PCL, polyethylene oxide (PEO), and poly-L-lactic acid (PLLA) polymer blends. Sustained-release from 70:30 PEO/PLLA blends showed almost complete release of hydrophilic compounds after 1 hr, due to the hydrophilicity and quick degradation of PEO. In contrast, 30:70 PEO:PLLA blends exhibited lower burst release and higher sustained-release relative to the more hydrophilic 70:30 PEO:PLLA blends. Additionally, the moderately hydrophobic 30:70 PEO:PLLA fibers demonstrated better release profiles relative to pure PCL fibers, which released around 95% of incorporated drugs after 1 hr. This more efficacious release profile was attributed to the intermediate hydrophobicity and crystallinity of PLLA compared to PEO and PCL [33]. Whereas the hydrophilicity of PEO confers quick degradation in aqueous solutions, resulting in burst release; PCL is highly hydrophobic, causing incorporated compounds to localize on the fiber surface, thereby significantly contributing to high burst release. The results from these studies are in agreement with our observations that polymers comprised of lactic and glycolic acid, relative to the more hydrophobic PLCL (or PCL), exhibit less burst release of moderately hydrophilic compounds. Moreover they emphasize the advantages of fabricating blended formulation to tune release properties.

After characterizing these fibers, TDF EFs were evaluated for their potential to protect against HIV-1 and HSV-2 infections *in vitro* (Figs. 5 and 6). In these studies, TDF EF eluates collected for up to 2 wk, conferred protection against HIV, particularly for the 10 and 20% formulations (Fig. 5); whereas HSV-2 inhibition was only achieved using the 1 and 24 hr release eluates (Fig. 6). The most evident factor that contributes to this lack of efficacy associated with longer release times is the difference in TDF potency against HSV-2 and HIV-1. While TDF is efficacious against both HSV-2 and HIV-1, TDF is much less efficacious against HSV-2 ($IC_{50} = 8.9 \mu\text{g/mL}$) relative to HIV ($0.0053 \mu\text{g/mL}$). Based on the release profiles of the 20% TDF PLGA and PLCL polymers, we expect that we would need approximately 15–20 mg fiber to provide 3–4 mg of TDF release (over one month), and corresponding efficacy after 2 wk release. This dose corresponds to previous studies indicating that concentrations ranging from 100 to 500 $\mu\text{g/mL}$ are needed to completely prevent HSV-2 replication *in vitro* [36]. These estimates are within the dosing we envision for *in vivo* studies, in which similar studies have delivered a range of 0.2 to 0.7 mg/mL TDF per day within the murine reproductive tract to prevent HIV/HSV-2 infections [36, 37].

In addition to the increased amount of TDF needed to prevent HSV-2 relative to HIV-1 infection, the duration of fiber exposure to eluate likely impacts the potency of TDF released from the fibers. This is clearly observed in our efficacy studies where the administration of 1 and 24 hr TDF fiber eluates demonstrated similar efficacy to free TDF; whereas, after 1 wk of release, TDF fiber eluates exhibited greater efficacy against HIV-1, relative to free TDF (Tables 3 and 4).

One factor that supports the improved IC_{50} s of TDF fibers, relative to free TDF with respect to time, is that TDF is known to hydrolyze to the monoester derivative (mPTFV) in aqueous environments both *in vitro* and *in vivo* [36, 63–65]. While increasing the stability of active agents is a benefit of utilizing delivery platforms such as fibers, we acknowledge that even TDF fiber eluate exhibited decreased efficacy against HIV-1 after 1 to 2 wk in media (Table 4). HPLC analysis showed that fiber-incorporated TDF was protected from hydrolysis, with

no indication of mPTFV accumulation for samples collected during the first week of release. This lack of mPTFV measured in loading and early release samples indicates that the monoester derivative was formed subsequent to fiber release, and that EFs function as an appropriate delivery vehicle to provide drug stability in solution. However, drug that is released and exposed to surrounding fluid for longer durations (here > 1 wk), will be less efficacious. Additionally, for long-term applications, lactic acid release may enhance TDF hydrolysis and contribute to the decreased efficacy of TDF [65]. Thus, as expected, the longer an incorporated drug remains within the polymer under physiological pH, the longer it will retain efficacy. To modulate release in future work, utilization of a different polymer or polymer blends may more optimally maintain active agent activity against HIV-1 and HSV-2 for durations exceeding 1 to 2 weeks.

Finally, the safety of both TDF PLGA and PLCL fibers was assessed after administration to vaginal and cervical cells, and to EpiVaginal tissue. In VK2, Ect1, and End1 E6E7 cells, all cell lines demonstrated strong viability after fiber administration for 1 to 3 days (Fig 7). This is in agreement with our expectations; given that both polymers and TDF are FDA-approved (PLCL is a derivative of the FDA-approved polymer PCL). Similarly, after 2 and 3 days exposure to blank or TDF PLGA fibers, EpiVaginal tissue exhibited comparable viability relative to untreated tissue controls. Furthermore, blank PLGA EF- and TDF PLGA EF-treated tissues, revealed normal full thickness vaginal epithelium, with no apparent adverse histological findings. Based on these *in vitro* results, we expect to see similar safety profiles in *in vivo* studies.

Another critical aspect of intravaginal delivery is the assessment of inflammatory response. Studies have shown that increased expression of cytokines such as IL-6, IL-8, as well as IL-1 α and β is strongly associated with increased susceptibility to HIV infection [66, 67]. Nonoxynol-9, once a promising microbicide candidate against HIV, was shown to increase the rate of HIV infection in clinical studies due to its pro-inflammatory properties and disruption of the reproductive epithelium [68, 69]. Therefore, it is critical that any active agents or delivery vehicles used as a microbicide must minimally induce pro-inflammatory cytokines. For these experiments, PLGA was selected for examination due to the polymer exhibiting both decreased burst release and longer sustained-release properties relative to PLCL fibers. Despite analyzing a plethora of cytokines, TDF EFs were found only to weakly induce (0–2 fold increase) the expression of GM-CSF, IL-1 α , and IL-6. This cytokine expression was not observed after 72 hr, and may have been the result of the actual application of EF onto the EpiVaginal tissue [46]. Finally, the nonoxynol-9 control showed a marked decrease of cytokine expression after 72 hr, which we attribute to epithelial necrosis and shedding. Previous studies have shown that concentrations of N-9 as low as 0.03% can induce epithelial disruption and necrosis after 24 hr [70]. However, no epithelial disruption was observed from TDF EF exposure, indicating that these fibers are non-inflammatory and will not elicit a cytokine response. As in previous studies with microbicides, antiviral compounds may induce cytokine production as high as 3–10 fold, which is still considered reliably safe [48].

While our preliminary work with EpiVaginal tissue demonstrates promising biocompatibility, future mechanical testing with respect to the interactions between the

fibers, vaginal mucosa, and virus will need to be investigated *in vivo*. Importantly, tissue contact studies will need to assess how mechanical properties (e.g., flexibility, rigidity, size) impact host tissue interactions. Additionally, while we envision that electrospun fibers may be administered similarly to vaginal films, appropriate studies will need to assess the retention time and distribution of fibers within the vaginal cavity and relate these to the structural properties and mechanical durability after different durations of administration *in vivo*.

More broadly, the user preference and feasibility of different dosage forms and administration methods must be considered for subsequent clinical development. A variety of studies have highlighted the lack of correlation and reporting of user adherence in clinical trials [71, 72]. Hence, there is a need for more accurate reporting and adherence, to fully achieve the prophylactic and/or therapeutic potential of intravaginal delivery vehicles. In particular, if women experience user adherence challenges (resulting in unadministered doses), or feel uncomfortable using the dosage form, lower adherence (and efficacy) may result. Given these factors, women's input is critical to the development of microbicide dosage forms that women not only want to use, but are able to use correctly and consistently [73]. Interestingly, recent work that investigated user preferences in vaginal films (for more "on-demand" applications) identified factors – such as the opacity and size of films – that most significantly impacted user preference [74]. As we further develop electrospun fibers for intravaginal applications, we are aware that addressing similar considerations will be necessary to ensure vehicle success in subsequent development stages.

In parallel with these long-term goals of advancing fiber formulations for *in vivo* studies and clinical applications, in the near-term our laboratory seeks to improve the release profiles, and enhance the efficacy of our electrospun fibers against multiple STIs. The use of different polymers or polymer blends will likely reduce the initial burst release of incorporated products while simultaneously providing for prolonged (> 1 wk) release. Additionally while these fibers were not specifically formulated for mucoadhesion, surface-modification or a different polymer choice/blend (e.g., chitosan, acrylic acid polymers) may be considered to improve mucoadhesivity. Furthermore, the development of multilayered and coaxially-spun fibers may also provide a more suitable platform for the delivery of multiple compounds with sustained-release profiles.

Conclusions

There is an urgent need to develop new and alternative sustained-release technologies to prevent HIV-1 and HSV-2 infections. To address these needs, we fabricated PLGA and PLCL electrospun fibers, and compared the loading and release properties of these fibers, using TDF as a model antiviral. Both PLGA and PLCL fibers provided complete protection against both HIV-1 and HSV-2 infections *in vitro*. Both short- (1 and 24 hr) and long-term release eluates (1 and 2 wk) provided protection against HIV-1; whereas short-term protection (attributed to fiber dosing and difference in IC₅₀) was achieved against HSV-2 *in vitro*. Additionally, TDF fibers demonstrated significantly enhanced efficacy against HIV-1, relative to free TDF, after long-term release of 1 wk. Vaginal and cervical cells exposed to TDF PLGA and PLCL fibers showed high viability, after up to 3 days post-administration,

demonstrating their safety *in vitro*. Finally, PLGA fibers induced negligible and temporary increases (0–2 fold) in cytokine expression, suggesting their potential for *in vivo* applications.

Comparing the attributes of PLGA and PLCL TDF EFs, PLGA appears to be a more promising candidate compared to PLCL, based on its improved release profile. However, as both formulations demonstrated efficacy against HIV and HSV-2 *in vitro*, future testing may reveal both fiber formulations to be equally efficacious *in vivo*.

Using the information obtained from this work, we seek to further enhance the efficacy and delivery duration of small molecule antivirals and biologics from EFs by utilizing a variety of encapsulants and polymer blends. In particular, future plans include fabricating formulations that co-deliver multiple active agents. Our hope is that these future fibers will provide more potent protection. We predict that these, or similar electrospun fibers will confer long lasting and sustained protection against both HIV and HSV-2 infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We sincerely thank Dr. Stuart Williams III for the generous use of his electrospinning equipment.

Funding Information

We acknowledge and appreciate our laboratory start-up funds provided by the University of Louisville Bioengineering Department to fund this work. We also thank the Jewish Heritage Fund for Excellence and the University of Louisville Foundation for funding provided to JMSR and KMT.

References

1. Geneva, S.W.H.O. Sexually Transmitted Infections (STI) Fact Sheet. World Health Organization; 2013. p. 110(<http://www.who.int/mediacentre/factsheets/fs110/en/>)
2. Freeman EE, et al. Herpes simplex virus 2 infection increases HIV acquisition in men and women: systematic review and meta-analysis of longitudinal studies. *AIDS*. 2006; 20(1):73–83. [PubMed: 16327322]
3. Corey L, et al. The effects of herpes simplex virus-2 on HIV-1 acquisition and transmission: a review of two overlapping epidemics. *J Acquir Immune Defic Syndr*. 2004; 35(5):435–45. [PubMed: 15021308]
4. Galvin SR, Cohen MS. The role of sexually transmitted diseases in HIV transmission. *Nat Rev Microbiol*. 2004; 2(1):33–42. [PubMed: 15035007]
5. Beyrer C, et al. Global epidemiology of HIV infection in men who have sex with men. *Lancet*. 2012; 380(9839):367–77. [PubMed: 22819660]
6. Beyrer C, Abdool Karim Q. The changing epidemiology of HIV in 2013. *Curr Opin HIV AIDS*. 2013; 8(4):306–10. [PubMed: 23743721]
7. FDA approves first drug for reducing the risk of sexually acquired HIV infection. *FDA NEWS RELEASE*. 2012
8. Baeten JM, et al. Antiretroviral prophylaxis for HIV prevention in heterosexual men and women. *N Engl J Med*. 2012; 367(5):399–410. [PubMed: 22784037]
9. Heneine W, Kashuba A. HIV prevention by oral preexposure prophylaxis. *Cold Spring Harb Perspect Med*. 2012; 2(3):a007419. [PubMed: 22393535]

10. Thigpen MC, et al. Antiretroviral preexposure prophylaxis for heterosexual HIV transmission in Botswana. *N Engl J Med*. 2012; 367(5):423–34. [PubMed: 22784038]
11. McMahon JM, et al. Oral pre-exposure prophylaxis (PrEP) for prevention of HIV in serodiscordant heterosexual couples in the United States: opportunities and challenges. *AIDS Patient Care STDS*. 2014; 28(9):462–74. [PubMed: 25045996]
12. Mastro TD, Sista N, Abdool-Karim Q. ARV-based HIV prevention for women -where we are in 2014. *J Int AIDS Soc*. 2014; 17(3 Suppl 2):19154. [PubMed: 25224614]
13. Marrazzo JM, et al. Tenofovir-based preexposure prophylaxis for HIV infection among African women. *N Engl J Med*. 2015; 372(6):509–18. [PubMed: 25651245]
14. Abdool, Karim Q., et al. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. *Science*. 2010; 329(5996):1168–74. [PubMed: 20643915]
15. Boonstra H. Multipurpose Prevention Technologies for the Developing World: U.S. Investment Is Critical. *Guttmacher Policy Review*. 2015; 18(3)
16. Hankins CA, Dybul MR. The promise of pre-exposure prophylaxis with antiretroviral drugs to prevent HIV transmission: a review. *Curr Opin HIV AIDS*. 2013; 8(1):50–8. [PubMed: 23201856]
17. Akil A, et al. Formulation and characterization of polymeric films containing combinations of antiretrovirals (ARVs) for HIV prevention. *Pharm Res*. 2015; 32(2):458–68. [PubMed: 25079391]
18. Akil A, et al. Increased Dapivirine tissue accumulation through vaginal film codelivery of dapivirine and Tenofovir. *Mol Pharm*. 2014; 11(5):1533–41. [PubMed: 24693866]
19. Bunge KE, et al. A Phase 1 Trial to Assess the Safety, Acceptability, Pharmacokinetics, and Pharmacodynamics of a Novel Dapivirine Vaginal Film. *J Acquir Immune Defic Syndr*. 2016; 71(5):498–505. [PubMed: 26565716]
20. Clark MR, et al. Evaluation of Rapidly Disintegrating Vaginal Tablets of Tenofovir, Emtricitabine and Their Combination for HIV-1 Prevention. *Pharmaceutics*. 2014; 6(4):616–631. [PubMed: 25494201]
21. Baeten JM, et al. Use of a Vaginal Ring Containing Dapivirine for HIV-1 Prevention in Women. *N Engl J Med*. 2016
22. Nel A, et al. Safety and pharmacokinetics of dapivirine delivery from matrix and reservoir intravaginal rings to HIV-negative women. *J Acquir Immune Defic Syndr*. 2009; 51(4):416–23. [PubMed: 19623693]
23. Moss JA, et al. Simultaneous delivery of tenofovir and acyclovir via an intravaginal ring. *Antimicrob Agents Chemother*. 2012; 56(2):875–82. [PubMed: 22123689]
24. Ho EA. Intravaginal rings as a novel platform for mucosal vaccination. *Mol Pharm Orgnic Process Res*. 2013; 1(2)
25. Steinbach JM. Protein and oligonucleotide delivery systems for vaginal microbicides against viral STIs. *Cell Mol Life Sci*. 2015; 72(3):469–503. [PubMed: 25323132]
26. Grooms TN, et al. Griffithsin-Modified Electrospun Fibers as a Delivery Scaffold to Prevent HIV Infection. *Antimicrob Agents Chemother*. 2016
27. Pillay V, et al. A Review of the Effect of Processing Variables on the Fabrication of Electrospun Nanofibers for Drug Delivery Applications. *Journal of Nanomaterials*. 2013
28. Gunatillake PA, Adhikari R. Biodegradable synthetic polymers for tissue engineering. *Eur Cell Mater*. 2003; 5:1–16. discussion 16. [PubMed: 14562275]
29. Makadia HK, Siegel SJ. Poly Lactic-co-Glycolic Acid (PLGA) as Biodegradable Controlled Drug Delivery Carrier. *Polymers (Basel)*. 2011; 3(3):1377–1397. [PubMed: 22577513]
30. Huang C, et al. Electrospun cellulose acetate phthalate fibers for semen induced anti-HIV vaginal drug delivery. *Biomaterials*. 2012; 33(3):962–9. [PubMed: 22018388]
31. Huang C, Soenen SJ, van Gulck E, Rejman J, Vanham G, Lucas B, Geers B, Braeckmans K, Shahin V, Spanoghe P, Demeester J, De Smedt SC. Electrospun polystyrene fibers for HIV entrapment. *Polym Adv Technol*. 2014; 25(8)
32. Carson D, Jiang Y, Woodrow KA. Tunable Release of Multiclass Anti-HIV Drugs that are Water-Soluble and Loaded at High Drug Content in Polyester Blended Electrospun Fibers. *Pharm Res*. 2016; 33(1):125–36. [PubMed: 26286184]

33. Ball C, et al. Drug-eluting fibers for HIV-1 inhibition and contraception. *PLoS One*. 2012; 7(11):e49792. [PubMed: 23209601]
34. Asvadi NH, et al. Evaluation of microporous polycaprolactone matrices for controlled delivery of antiviral microbicides to the female genital tract. *J Mater Sci Mater Med*. 2013; 24(12):2719–27. [PubMed: 23892484]
35. Aniagyei SE, et al. Evaluation of poly(lactic-co-glycolic acid) and poly(dl-lactide-coepsilon-caprolactone) electrospun fibers for the treatment of HSV-2 infection. *Mater Sci Eng C Mater Biol Appl*. 2017; 72:238–251. [PubMed: 28024582]
36. Mesquita PM, et al. Intravaginal ring delivery of tenofovir disoproxil fumarate for prevention of HIV and herpes simplex virus infection. *J Antimicrob Chemother*. 2012; 67(7):1730–8. [PubMed: 22467632]
37. Smith JM, et al. Intravaginal ring eluting tenofovir disoproxil fumarate completely protects macaques from multiple vaginal simian-HIV challenges. *Proc Natl Acad Sci U S A*. 2013; 110(40):16145–50. [PubMed: 24043812]
38. Owen DH, Katz DF. A vaginal fluid simulant. *Contraception*. 1999; 59(2):91–5. [PubMed: 10361623]
39. Wei X, et al. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob Agents Chemother*. 2002; 46(6):1896–905. [PubMed: 12019106]
40. Platt EJ, et al. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J Virol*. 1998; 72(4):2855–64. [PubMed: 9525605]
41. Montefiori DC. Measuring HIV neutralization in a luciferase reporter gene assay. *Methods Mol Biol*. 2009; 485:395–405. [PubMed: 19020839]
42. Ham AS, et al. Development of a combination microbicide gel formulation containing IQP-0528 and tenofovir for the prevention of HIV infection. *J Pharm Sci*. 2012; 101(4):1423–35. [PubMed: 22227864]
43. Ayehunie S, et al. Development of an in vitro alternative assay method for vaginal irritation. *Toxicology*. 2011; 279(1–3):130–8. [PubMed: 20937349]
44. Ayehunie S, et al. Organotypic human vaginal-ectocervical tissue model for irritation studies of spermicides, microbicides, and feminine-care products. *Toxicol In Vitro*. 2006; 20(5):689–98. [PubMed: 16309879]
45. Fichorova RN, et al. A quantitative multiplex nuclease protection assay reveals immunotoxicity gene expression profiles in the rabbit model for vaginal drug safety evaluation. *Toxicol Appl Pharmacol*. 2015; 285(3):198–206. [PubMed: 25818602]
46. Nixon B, et al. Vaginally delivered tenofovir disoproxil fumarate provides greater protection than tenofovir against genital herpes in a murine model of efficacy and safety. *Antimicrob Agents Chemother*. 2014; 58(2):1153–60. [PubMed: 24323471]
47. Kouokam JC, Lasnik AB, Palmer KE. Studies in a Murine Model Confirm the Safety of Griffithsin and Advocate Its Further Development as a Microbicide Targeting HIV-1 and Other Enveloped Viruses. *Viruses*. 2016; 8(11)
48. Kouokam JC, et al. Investigation of griffithsin's interactions with human cells confirms its outstanding safety and efficacy profile as a microbicide candidate. *PLoS One*. 2011; 6(8):e22635. [PubMed: 21829638]
49. Chen SC. The influence of fiber diameter of electrospun poly(lactic acid) on drug delivery. *Fibers and Polymers*. 2012; 13(9):5.
50. Hrib J, et al. Nanofibers for drug delivery - incorporation and release of model molecules, influence of molecular weight and polymer structure. *Beilstein J Nanotechnol*. 2015; 6:1939–45. [PubMed: 26665065]
51. Chew SY, et al. Mechanical properties of single electrospun drug-encapsulated nanofibres. *Nanotechnology*. 2006; 17(15):3880–3891. [PubMed: 19079553]
52. Zong X. Structure and process relationship of electrospun bioabsorbable nanofiber membranes. *Polymer*. 2002; 43(16):10.

53. DrugBank Tenofovir. 2005 Sep 8. 2016 [cited 2016 8Sep16]; Available from: <http://www.drugbank.ca/drugs/DB00300>
54. DrugBank Tenofovir disoproxil fumarate. 2005. [cited 2016 8Sept16]; Available from: <http://www.drugbank.ca/salts/DBSALT000172>
55. Blakney AK, et al. Delivery of multipurpose prevention drug combinations from electrospun nanofibers using composite microarchitectures. *Int J Nanomedicine*. 2014; 9:2967–78. [PubMed: 24971008]
56. Krogstad EA, Woodrow KA. Manufacturing scale-up of electrospun poly(vinyl alcohol) fibers containing tenofovir for vaginal drug delivery. *Int J Pharm*. 2014; 475(1–2):282–91. [PubMed: 25169075]
57. Gu X. Electrospinning of poly(butylene-carbonate):Effect of Solvents on the Properties of the Nanofibers Film. *Int J Electrochem Sci*. 2014; 9:11.
58. Sun ZC, et al. The effect of solvent dielectric properties on the collection of oriented electrospun fibers. *Journal of Applied Polymer Science*. 2012; 125(4):2585–2594.
59. Aldrich, S. 2,2,2-Trifluoroethanol ReagentPlus®, 99%. Available from: <http://www.sigmaaldrich.com/catalog/product/sigald/t63002?lang=en®ion=US>
60. Aldrich, S. 1,1,1,3,3,3-Hexafluoro-2-propanol 99%. Available from: <http://www.sigmaaldrich.com/catalog/product/aldrich/105228?lang=en®ion=US>
61. Yu D-G. Electrospun nanofiber-based drug delivery systems. *Health*. 2009; 1(2):67–75.
62. Huang ZM, et al. Encapsulating drugs in biodegradable ultrafine fibers through coaxial electrospinning. *J Biomed Mater Res A*. 2006; 77(1):169–79. [PubMed: 16392131]
63. Geboers S, et al. Intestinal behavior of the ester prodrug tenofovir DF in humans. *Int J Pharm*. 2015; 485(1–2):131–7. [PubMed: 25747454]
64. Havele S, Dhaneshwar SR. Stress studies of tenofovir disoproxil fumarate by HPTLC in bulk drug and pharmaceutical formulation. *ScientificWorldJournal*. 2012; 2012:894136. [PubMed: 22606065]
65. Agrahari V, et al. Evaluation of degradation kinetics and physicochemical stability of tenofovir. *Drug Test Anal*. 2015; 7(3):207–13. [PubMed: 24817173]
66. Arnold KB, et al. Increased levels of inflammatory cytokines in the female reproductive tract are associated with altered expression of proteases, mucosal barrier proteins, and an influx of HIV-susceptible target cells. *Mucosal Immunol*. 2016; 9(1):194–205. [PubMed: 26104913]
67. Doncel GF, Chandra N, Fichorova RN. Preclinical assessment of the proinflammatory potential of microbicide candidates. *J Acquir Immune Defic Syndr*. 2004; 37(Suppl 3):S174–80. [PubMed: 16419269]
68. Dayal MB, et al. Disruption of the upper female reproductive tract epithelium by nonoxynol-9. *Contraception*. 2003; 68(4):273–9. [PubMed: 14572891]
69. Fichorova RN, Tucker LD, Anderson DJ. The molecular basis of nonoxynol-9-induced vaginal inflammation and its possible relevance to human immunodeficiency virus type 1 transmission. *J Infect Dis*. 2001; 184(4):418–28. [PubMed: 11471099]
70. Jain JK, et al. The effect of nonoxynol-9 on human endometrium. *Contraception*. 2005; 71(2):137–42. [PubMed: 15707564]
71. Montgomery ET, et al. Misreporting of Product Adherence in the MTN-003/VOICE Trial for HIV Prevention in Africa: Participants' Explanations for Dishonesty. *Aids and Behavior*. 2017; 21(2): 481–491. [PubMed: 27858268]
72. Woodsong C, et al. Microbicide clinical trial adherence: insights for introduction. *J Int AIDS Soc*. 2013; 16:18505. [PubMed: 23561044]
73. IPM. Identifying Women's Needs & Preferences. 2017. Available from: <http://www.ipmglobal.org/our-work/access/identifying-women-needs-preferences>
74. Fan MD, et al. Preferred Physical Characteristics of Vaginal Film Microbicides for HIV Prevention in Pittsburgh Women. *Arch Sex Behav*. 2017; 46(4):1111–1119. [PubMed: 27571742]

Fig. 1. SEM images of blank PLGA and PLCL fibers electrospun using different solvents. (A) 15% w/w PLGA in HFIP; (B) 20% w/w PLGA in TFE; (C) 12% w/w PLCL in HFIP; and (D) 20% w/w PLCL in TFE. Scale bars represent 10 μm .

Fig. 2. SEM images of PLGA and PLCL fibers prepared with increasing concentrations of TDF, using HFIP as the solvent. (A) Blank PLGA, (B) 1% TDF, (C) 10% TDF, and (D) 20% TDF PLGA fibers; (E) Blank PLCL, (F) 1% TDF, (G) 10% TDF, and (H) 20% TDF PLCL fibers. Scale bars represent 10 μm .

Fig. 3.

Average diameters of electrospun fibers measured from SEM images, using ImageJ. (A) Diameters of blank PLGA and PLCL fibers electrospun with either HFIP or TFE solvents. Diameters ranged from 1.7 to 2.0 μm. No statistical significance was observed between fiber diameters prepared with either HFIP or TFE. (B) Diameters of TDF EFs electrospun with HFIP were significantly smaller than those of blank fibers, ranging from 0.7 to 1.2 μm. While the PLGA TDF fiber diameters seemed randomly distributed, PLCL TDF fibers demonstrated a trend of decreased diameter with increasing TDF concentration. Statistical significance was observed between blank fibers and all TDF fiber diameters; however, no statistical difference in diameters was observed between the TDF EF formulations.

Fig. 4.

Release profiles of TDF from 1, 10, and 20% TDF PLGA and PLCL fibers in SVF. (A) Cumulative release of TDF per milligram of fiber (μg TDF/mg fiber) and (B) as percent total loading over 4 wk. While PLCL fibers showed a higher burst release after 1 hr, PLGA fibers exhibited greater release, as early as 24 hr, relative to PLCL fibers.

Fig. 5.

PLGA and PLCL fiber eluates inhibit HIV-1 infection *in vitro* after 1 and 24 hr, and 1 and 2 wk of release. Dilutions of release eluate from 10 mg/mL fiber concentrations at different time points were normalized to untreated/infected cell control RLUs to assess percent HIV inhibition *in vitro*. Figures show the percent of cells infected, after incubation with: (A) 1 and (B) 24 hr release eluates from all PLGA and PLCL EF formulations; (C) 1 and 2 wk release eluates from 20% TDF PLGA and PLCL fibers; (D) 3 and 4 wk eluates from 20% TDF PLGA and PLCL fibers; compared to (E) free TDF (200 µg/mL) 1 and 2 wk eluate, and (F) blank fiber eluates at each time point.

Fig. 6.

Both PLGA and PLCL fiber release eluates prevent HSV-2 infection *in vitro*. Plaque assays were conducted to assess the efficacy of 20% TDF PLGA and PLCL fiber eluates (5 mg/mL) against HSV-2 infection *in vitro*. Plaques were counted and normalized to untreated/uninfected cells. Results demonstrate HSV-2 efficacy attained with: (A) 1 and (B) 24 hr fiber eluate dilutions.

Fig. 7.

Cytotoxicity assessed via MTT assay. Vaginal epithelial cell lines: (A) Ect1 E6/E7, (B) VK2 E6/E7, (C) and End1 E6/E7, were incubated with blank or 20% TDF PLGA or PLCL fibers (10 mg/mL) for 24, 48, and 72 hr. All cells demonstrated greater than 93, 91, and 96% cell viability at 24, 48, and 72 hr respectively, for all formulations tested.

Fig. 8.

EpiVaginal cytotoxicity was assessed via H&E staining (A) and LDH assay (B). (A) H&E stained cross-sections of EpiVaginal VEC-100-FT tissues following 3-day exposure to PLGA TDF fibers, relative to untreated and toxic control (0.2% N-9) groups. Scale bar represents 200 μ m. (B) Tissue viability (LDH) measurements for EpiVaginal VEC-100-FT tissues following two or three day exposure to PLGA TDF fibers, relative to untreated and toxic control (0.2% N-9) groups.

Fig. 9.

Cytokine expression from EpiVaginal studies after (A) 48 and (B) 72 hr administration of fibers. TDF EFs elicited minimal cytokine expression relative to untreated samples after 72 hr. Positive N-9 treated samples failed to induce cytokine expression due to epithelial cell death.

Table 1

Diameters of electrospun fibers. Blank and TDF fibers were fabricated using HFIP or TFE solvents. EFs incorporating TDF exhibited decreased diameters compared with blank fibers.

Fiber Formulation		Average Width (μm)
HFIP 15% PLGA	Blank Fiber	1.7 ± 0.6
	1% TDF	1.1 ± 0.3
	10% TDF	0.8 ± 0.3
	20% TDF	1.1 ± 0.4
HFIP 12% PLCL	Blank Fiber	1.7 ± 0.5
	1% TDF	1.1 ± 0.5
	10% TDF	0.9 ± 0.3
	20% TDF	0.7 ± 0.2
TFE 20% PLGA Blank Fiber		2.0 ± 1.0
TFE 20% PLGA 10% TDF		1.2 ± 0.4
TFE 20% PLCL Blank Fiber		1.9 ± 0.9
TFE 20% PLCL 10% TDF		0.6 ± 0.2

Table 2

Quantification of TDF fiber loading and encapsulation efficiency. PLGA and PLCL fibers electrospun with HFIP demonstrated higher loading and encapsulation efficiency, relative to PLGA and PLCL fibers electrospun with TFE. Increases in encapsulation efficiency were observed based on the amount of TDF added to 10% TDF PLGA and PLCL formulations

Fiber Formulation		Loading TDF/Fiber ($\mu\text{g}/\text{mg}$)	Encapsulation Efficiency (%)
HFIP 15% PLGA	1% TDF	4.9 ± 0.8	80.7 ± 12.4
	10% TDF	45.7 ± 0.8	81.4 ± 1.5
	20% TDF	82.1 ± 2.5	76.3 ± 2.3
HFIP 12% PLCL	1% TDF	5.0 ± 0.4	81.1 ± 5.8
	10% TDF	54.0 ± 0.5	89.7 ± 0.8
	20% TDF	92.5 ± 9.6	80.4 ± 8.3
TFE 20% PLGA 10% TDF		52.3 ± 3.5	62.7 ± 4.2
TFE 20% PLCL 10% TDF		54.6 ± 0.4	60.4 ± 0.4

Table 3

The IC₅₀s of PLGA and PLCL fibers (against HIV-1) after administration of the 1 and 24 hr release eluates. The IC₅₀s of PLGA and PLCL TDF 1 and 24 hr fiber eluates were similar to, or less than free TDF. While no statistical significance was observed between the IC₅₀s of fiber eluates taken at the same time, there was a statistically significant increase in the IC₅₀s of all formulations after 24 hr release ($p > 0.05$). Confidence intervals of the IC₅₀s are shown in parentheses.

Fiber Formulation	IC ₅₀ at 1 Hr (ng/mL)	IC ₅₀ at 24 Hr (ng/mL)
PLGA 10% TDF	2.4 (2.0 to 3.0)	7.2 (6.0 to 8.5)
PLGA 20% TDF	4.6 (4.3 to 5.0)	5.1 (3.9 to 6.7)
PLCL 10% TDF	3.1 (2.5 to 3.7)	7.4 (4.2 to 13.0)
PLCL 20% TDF	3.1 (2.0 to 4.7)	1.9 (1.1 to 3.5)
Free TDF	12.1 (10.9 to 13.3)	5.3 (3.4 to 8.2)

Table 4

The IC₅₀s of PLGA and PLCL fibers (against HIV-1) after administration of 1 and 2 wk release eluates. As exposure time to media increased, the efficacy of TDF fibers decreased. However, both PLGA and PLCL fiber eluates were more efficacious than free TDF after 1 wk exposure to media. Confidence intervals of the IC₅₀s are shown in parentheses.

Fiber Formulation	IC ₅₀ (ng/mL)
PLGA 20% TDF (1 Wk)	1.9 (0.9 to 4.1)
PLGA 20% TDF (2 Wk)	10,181 (3,631 to 25,550)
PLCL 20% TDF (1 Wk)	11.9 (9.0 to 15.9)
PLCL 20% TDF (2 Wk)	7,226 (3,947 to 13,229)
Free TDF (1 Wk)	4,504 (3,858 to 5,259)
Free TDF (2 Wk)	4,927 (4,470 to 5,432)

Table 5

The IC₅₀s of PLGA and PLCL fibers (against HSV-2) after administration of the 1 and 24 hr release eluates. Plaque assays were performed to assess the antiviral activity of 20% TDF fiber eluates against HSV-2 infection. Fiber eluates from 1 and 24 hr showed similar activity, relative to free TDF. Confidence intervals of the IC₅₀s are shown in parentheses.

Fiber Formulation	IC ₅₀ (μg/mL)
PLGA 20% TDF (1 hr)	7.3 (6.6 to 8.1)
PLGA 20% TDF (24 hr)	14.3 (13.4 to 15.3)
PLCL 20% TDF (1 hr)	14.0 (13.5 to 14.5)
PLCL 20% TDF (24 hr)	20.3 (18.3 to 22.5)
Free TDF	8.9 (4.2 to 18.8)