Stabilization and sustained release of HIV inhibitors by encapsulation in silk fibroin disks

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Abstract

Topical microbicides have the potential to provide effective protection against sexual transmission of HIV. Challenges in developing microbicides include their application in resource-poor settings with high temperatures and a lack of refrigeration, and low user adherence to a rigorous daily regimen. Several protein-based HIV inhibitors show great promise as microbicides, being highly specific and not expected to lead to resistance that would affect the efficacy of current antiretroviral treatments. We show that four potent protein HIV inhibitors, 5P12-RANTES, 5P12-RANTES-L-C37, Grft, and Grft-L-C37 can be formulated into silk fibroin (SF) disks and remain functional for 14 months at 25, 37, and 50 °C. These HIV inhibitor-encapsulated SF disks show excellent inhibition properties in PBMC and in human colorectal and cervical tissue explants, and do not induce inflammatory cytokine secretion. Further, the SF provides a mechanically robust matrix with versatile material formats for this type of application. Finally, a formulation was developed to allow sustained release of functional Grft for 4 weeks at levels sufficient to inhibit HIV transmission. This work establishes the suitability of HIV inhibitor-encapsulated SF disks as topical HIV microbicides that can be further developed to allow easy insertion for extended protection.

Keywords:

Silk fibroin; drug stabilization; sustained release; HIV microbicide; 5P12-RANTES; Griffithsin

1. Introduction

HIV is a devastating global disease that currently infects over 2 million people per year. Most new infections occur in the developing world and disproportionately affect women [1]. Current HIV prevention efforts include attempts to develop a wide range of strategies, including vaccination, oral pre-exposure prophylaxis (oral PrEP), and topical PrEP (also known as microbicides). Vaccination efforts have had only modest success [2]. PrEP is an antiretroviral (ARV)-based method to prevent HIV transmission that involves adherence to oral or topical dosing. When used with a proper schedule, oral PrEP has been proven safe and efficacious in trials to prevent HIV transmission to men who have sex with men (MSM)[3], the HIV-negative partner in heterosexual serodiscordant couples[4], and for injecting drug users[5]. However, efficacy relies heavily on user adherence. Also, the current ARVs tested in clinical trials for PrEP are being used in highly active antiretroviral therapy (HAART), raising concerns about developing viral drug resistance [6]. Furthermore, there are issues regarding the regular availability of these costly treatments in resource-poor settings.

Topical PrEP, also known as microbicides, are ARVs formulated for topical application to the reproductive or colorectal tract, and represent a critical but unrealized component of HIV prevention[7]. A successful microbicide should be inexpensive, easy to apply, highly potent against a variety of HIV strains, as well as accessible in resource-poor environments such as those without refrigeration. It would also be desirable to use an ARV that is not currently included in therapy regimes to block transmission of HAART-resistant isolates. Clinical trials testing oral and topical PrEP have had mixed to negative results. For instance, a vaginal gel containing a reverse transcriptase inhibitor, tenofovir, initially seemed promising in clinical trials[8]. But it has subsequently become clear that the requirement of a rigorous schedule involving repeated application of microbicide significantly decreases user compliance, particularly among younger women, reducing effectiveness [9–11]. Hence, these and other results have emphasized the need to develop drug formulations that allow sustained release over weeks to months rather than requiring daily use [9,10]. Some progress has been made on sustained release, particularly with an insertable vaginal ring containing the small molecule reverse transcriptase inhibitor, dapirivine. This device showed protection related to the level of compliance[12].

Protein-based HIV inhibitors may provide an attractive alternative to existing ARVs for use as microbicides, showing high potency against a wide range of HIV strains *in vitro* and *in vivo*. In particular, 5P12-RANTES and Griffithsin are promising candidates. 5P12-RANTES is a variant of the chemokine RANTES and was discovered by random mutagenesis and selection[13]. This small protein inhibitor has been shown to bind the HIV co-receptor CCR5 and potently inhibit R5-tropic HIV-1 isolates, with its effectiveness demonstrated *in vivo* against SHIV in macaques[14]. *In vitro* studies have shown that 5P12-RANTES has a high genetic barrier for HIV to gain resistance through mutation[15]. Griffithsin (Grft) is a lectin derived from red alga[16] that binds the HIV envelope glycoprotein gp120 and is among the most potent lectin inhibitor of HIV as well as exhibiting effectiveness against other enveloped viruses including SARS and Hepatitis C[17,18]. Both 5P12-RANTES and Grft have been shown to have properties

suitable for microbicidal use, including stability over a wide pH range and inexpensive production in large quantities[19–22]. Furthermore, chimeras formed by fusing 5P12-RANTES or Grft with the HIV gp41-derived C-peptide C37 via a covalent linkage, namely 5P12-RANTES-L-C37 and Grft-L-C37, have consistently shown even higher potency and wider breadth of inhibition than the original proteins[23,24]. These protein HIV inhibitors have excellent microbicidal properties, but their use in resource-poor settings requires that they maintain activity for months at elevated temperatures (up to 50°C) without refrigeration. Both 5P12-RANTES and Grft have demonstrated initial promise in temperature stability, with full biological functionality retained for 5P12-RANTES incubated for 24 hours at 50 °C or 7 days at 40 °C[19]; and for Grft incubated at 37° C for 7 days[22], or stored at 4°C and room temperature for 3 months[25]. In order to pursue these proteins as clinical microbicides, the proteins would need a formulation to keep them active at elevated temperatures for much longer, on the scale of months or even years. Ideally, the formulation would also support sustained release of functional inhibitor(s) over the course of weeks or longer upon application by the user.

Silk fibroin (SF) has emerged as an outstanding material for biomolecule stabilization and delivery[26–30]. SF is biocompatible and biodegradable, eliciting minimal inflammatory response[31,32], and has been used in medical applications including sutures and surgical mesh scaffolds[33]. Recently, SF has been shown to stabilize a wide range of biological agents and has been used to successfully stabilize and release antibodies[30], serum proteins related to diagnostics[34] and as a coating to preserve labile biologics[35], demonstrating its utility for therapeutic and broader potentials. The stabilization effect of SF is believed to be due to the formation of a matrix containing nanoscale pockets that can immobilize and potentially desolvate the encapsulated active molecule[28,30]. Furthermore, SF is highly versatile and can be formulated into gels, films and microneedles, making it easily applicable to implantable, injectable or transdermal administration routes[36,37].

We report here the encapsulation of four highly potent HIV inhibitors (5P12-RANTES, 5P12-RANTES-L-C37, Grft, Grft-L-C37) in a SF disk format. These proteins were selected based on a combination of properties, including high potency to broadly neutralize many strains of HIV, and experimentally determined suitability as HIV microbicides[19,22]. SF disks are expected to be amenable to users, with easy insertion, followed by release of inhibitor in response to the body's own moisture. This SF disks could potentially be used by both men and women, and could be effective in the reproductive and colorectal tracts. Our results show a SF disk formulation that even after storage at elevated temperatures for over a year demonstrates full activity of each tested HIV inhibitor against HIV pseudovirus. The disks were also protective against HIV infection in activated peripheral blood mononuclear cell (PBMC) and human mucosal tissue explant studies. In addition, we demonstrate sustained release of protein inhibitor over the course of one month. Overall, this work demonstrates the feasibility of protein inhibitor-loaded SF disks as HIV microbicides.

2. Materials and Methods

2.1. Production of protein inhibitors

All the protein inhibitors were produced recombinantly as previously described [23,24]. Briefly, genes encoding the proteins Grft and Grft-L-C37 with an N-terminal His_6 tag were subcloned into pET15b vectors; 5P12-RANTES was subcloned into a pET32a vector with an N-terminal His_6 and Thioredoxin fusion tags; and 5P12-RANTES-L-C37 was subcloned into pET28a with an N-terminal His_6 and SUMO fusion tags. The vectors were transformed into *E. coli* BL21(DE3) cells (Novagen) and cultured in M9 medium with ^{15}N ammonium chloride as the sole nitrogen source (Cambridge Isotopes Lab, Cambridge MA). After overexpression driven by addition of IPTG to 1 mM, cells were harvested by centrifugation. The cell pellets were resuspended with lysis buffer (6 M Guanidinium chloride, 200 mM NaCl, 50 mM Tris pH 8), and lysed by French Press (Thermo IEC). After centrifugation, the supernatants of the lysates were collected. Target proteins were purified using Ni-NTA affinity columns, then refolded using conditions modified from the FoldIt Screen (Hampton Research, Aliso Viejo, CA), and dialyzed. During dialysis, enterokinase was added to 5P12-RANTES, and Ulp-1 protease (produced in-house as previously described[38]) was added to 5P12-RANTES-L-C37 to cleave off the fusion tags. After cleavage was complete, the protein solutions were passed through Ni-NTA affinity columns to remove the fusion tags. All proteins were further purified by reversed phase HPLC using C4 columns (GraceVydac) and lyophilized for storage. The purity and integrity of the protein inhibitors were verified by SDS-PAGE and ^{15}N-^{1}H Heteronuclear Single Quantum Coherence (HSQC) NMR spectroscopy. Concentrations were determined by absorption at 280 nM. In order to fully cyclize the N-terminal glutamine residue of 5P12-RANTES and 5P12-RANTES-L-C37, solutions of these two proteins were incubated at 50 °C for at least 22 hours, and the cyclization was verified by their HSQC spectra (see Supplementary data).

2.2. Extraction of SF

Silk fibroin was prepared as previously described[36]. Briefly, silkworm *Bombyx mori* cocoons were cut into approximately 1 cm^2 pieces, and inspected for debris or stains. Clean cocoon pieces were added to boiling 20 mM Na_2CO_3 solution and boiled for 30 minutes to remove the sericin protein (degumming). The degummed fibers were rinsed thoroughly in deionized water and air dried. The dried SF fibers were solubilized using 9.3 M LiBr solution at 60 °C for 4 hr followed by extensive dialysis against deionized water to fully remove LiBr. The resulting SF solution was centrifuged to remove insoluble debris, and its supernatant was collected. The final SF fibroin solution was sterilized by autoclave, and the solution was stored at 4°C until use.

2.3. Production of inhibitor-infused SF disks

Aliquots were taken from the sterilized SF solution, and their dry weights were determined to calculate the weight-to-volume percentage of the SF stock. The four protein inhibitors were made into solution with 20 mM HEPES pH 8 buffer and sterile

filtered, and their concentrations were determined by absorbance at 280 nm. For the temperature stability study, protein inhibitors were mixed with the SF to make the final solutions that contained 3 μM of an inhibitor with 7% (w/v) of SF. As a control a PBS solution of each protein inhibitor was prepared. Except for the PBS solution control set, the inhibitor-SF solutions were aliquoted 200 μL/well into sterile 96-well plates, frozen and lyophilized. All samples were then incubated in forced air incubators at either 25, 37, or 50 °C. For the sustained release study using Grft, the final solutions were prepared to contain 10 μM Grft, and 1-5% SF, then aliquoted 1 mL/well into sterile 24-well plates, frozen and lyophilized.

2.4. Scanning electron microscopy

SEM was used to evaluate the morphology of the SF disks using a Zeiss EVO MA10 electron microscope (Carl Zeiss AG, Germany). The SF disks were cut to exposure the cross-sections, mounted onto SEM stubs and sputter coated with gold.

2.5. Fourier transform infrared spectroscopy analysis

FTIR was performed with a Jasco FT/IR6200 spectrometer (JASCO, Tokyo, Japan) equipped with a MIRacle™ attenuated total reflection (ATR) Ge crystal cell in reflection mode. For each sample, 32 scans of 4 cm^{-1} resolution were co-added and Fourier transformed using a Blackman-Harris apodization function. The amide I region (1585 to 1720 cm^{-1}) was deconvoluted and peak fitted using Opus 5.0 software (Bruker, Billerica, MA) to characterize the secondary structure content (side chains, β-sheet, random coil, α-helix and β-turns) as previously described [39–41]. The relative contributions of the secondary structure to the C=O stretch were quantified. Briefly, the FTIR spectra obtained from the instrument were cut and baselined between 1750 cm^{-1} and 1150 cm^{-1}, Fourier self-deconvoluted between 1720 and 1585 cm^{-1} using a bandwidth of 27.5 cm^{-1}, noise reduction of 0.3 and a Lorentzian line shape, then baselined again between 1710 and 1585 cm^{-1} and the peaks corresponding to a local minimum in the second derivative were curve fitted using a Levenberg Marquardt algorithm and local least square analysis. The relative peak areas were assigned to different secondary structure contributions based on the peak locations and reported as a percentage of the total peak area.

2.6. Water vapor annealing of SF disks

SF disks prepared for sustained release were incubated in a humidity and temperature controlled chamber, and annealed at 37 °C with ≥ 75% relative humidity for various periods of time. Afterwards, the SF plates were transferred to a 37 °C forced air incubator to allow the disks to dry. The dried disks were stored in desiccators at room temperature.

2.7. Stability study

All four proteins were tested for their stability over extended period at various temperatures. Each protein inhibitor was formulated into SF disks or dissolved in PBS solution as a control set. The inhibitors were stored at three temperatures: 25, 37 and

50 °C. Time points were taken on day 4, 25, 46, 74, 102, 130, 158, 186, 312 and 431. At each time point, triplicate samples of each inhibitor from each format at the three temperatures were taken out, and then the SF disk was dissolved with 200 μL of PBS. The resulting solutions along with their corresponding "inhibitor in PBS" control set were diluted by 10-fold with PBS and tested for their HIV inhibitory effect in TZM-bl cells as described below.

2.8. Sustained release of Grft

The water vapor annealed SF disks containing Grft for sustained release were stored in 24-well plates, with 1 mL of PBS or SVF added, and incubated in a 37 °C incubator. To account for the possible initial "burst" effect of release, the solutions were removed after the first hour, and fresh PBS or SVF was added. This burst was observed to be minimal, accounting for less than 0.2% of total Grft loaded. Later time points were taken daily for the first week, then every two days until day 31. At each time point, the soaking solutions were extracted with their volume measured, and fresh buffers were added to continue the incubation. SF-only disks were used as a control, with timepoints prepared in the same way. Grft concentrations in the time point samples were determined by ELISA. Briefly, time point sample solutions were added 100 μL/well into a 96-well plate (Nunc, Thermofisher) and incubated at 4 °C overnight. Subsequently the solution was removed and the plate was blocked with 3% BSA in PBS. Ni-NTA-conjugated horse radish peroxidase (Qiagen) was added to bind the His_6 tag on the Grft N-terminus. After washing steps, the substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, Thermo Fisher Scientific) was added for signal development. After addition of 1% SDS as a stop solution, absorbance at 405 nm was measured. To accurately measure concentration of released Grft, standardization was carried out as follows. A 26uM Grft stock was used to construct an 8 point concentration ladder, starting with 200nM followed by 2-fold serial dilution. The readings from each concentration point were fitted to a quadratic equation to make a standard curve, showing an $R^2 \geq 0.99$ in each case. Concentrations of 3 nM Grft can be readily detected in this manner., For functional validation of the inhibitors, the potency of samples collected at various time points were tested in single-round HIV assays in TZM-bl cells as described below. Sustained release samples were assayed for endotoxin levels using the ToxinSensor™ Gel Clot Endotoxin Assay Kit (Genscript, Piscataway, NJ) and showed less than 0.25 EU/mL.

2.9. Viral plasmids

All viral and pseudoviral DNA were obtained from the NIH AIDS Research & Reference Reagent Program (http://www.aidsreagent.org/). These include full-length, replication and infection-competent proviral HIV-1 clone, pYU.2 [42,43]; $pSG3^{\Delta env}$ proviral clone containing a defective *vpu* gene and truncated, nonfunctional *env* from Drs. John C Kappes and Xiaoyun Wu[44,45]; the plasmid containing full length *env* and *rev* genes of PVO, clone 4 SVPB11 from Drs. David Montefiori, Feng Gao and Ming Li [46]; the plasmid containing full length *env* and *rev* genes of CAP210.2.00.E8, SVPC17 from Drs. L. Morris, K. Mlisana, and David Montefiori[47].

2.10. Cell and virus culture conditions

All cell cultures were maintained at 37 °C in an atmosphere containing 5% CO_2. HEK-293T cells and TZM-bl cells[44,48,49] were grown in Dulbecco's Minimal Essential Medium (DMEM; Sigma-Aldrich, Inc., St. Louis, MO) containing 10% fetal calf serum (FCS), 10 mM HEPES, and antibiotics (100 U of penicillin/ml, 100 μg of streptomycin /ml). PBMC were isolated from multi-donor buffy coats from healthy HIV-seronegative donors, by centrifugation onto Ficoll-Hypaque, mitogen stimulated as previously described[50], and maintained in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, antibiotics (100 U of penicillin/ml, 100 μg of streptomycin/ml), and 100 U of interleukin-2/ml. The laboratory-adapted isolate HIV-1 YU.2 was passaged through activated PBMCs for 11 days. Pseudovirus stocks of PVO4 and CAP210 were obtained by co-transfection of HEK-293FT cells with $pSG3^{\Delta env}$ and either PVO4 or CAP210 plasmid, and subsequently the culture media supernatants containing the viral particles were harvested 48 hr post-transfection. The viral solutions were sterile filtered and stored in -80 °C until use.

2.11. Patients and tissue explants

Surgically-resected specimens of cervical and colorectal tissues were collected at St Mary's Hospital, Imperial College, London, UK. All tissues were collected after receiving signed informed consent from all patients and under protocols approved by the Local Research Ethics Committee. All patients were HIV negative. On arrival in the laboratory, resected tissue was cut into 2-3 mm^3 explants comprising both epithelial and muscularis mucosae as described previously[51,52]. Tissue explants were maintained with DMEM containing 10% fetal calf serum, 2mM L-glutamine and antibiotics (100 U of penicillin/ml, 100 μg of streptomycin/ml, 80 μg of gentamicin/ml) at 37ºC in an atmosphere containing 5% CO_2.

2.12. Infectivity and inhibition assays

For pseudoviral studies, inhibitors were tested for their activity against PVO4 and CAP210 infection of TZM-bl cells[44,49]. Briefly, TZM-bl cells were harvested and resuspended to 1 x 10^5 cells/mL, then seeded at 1 x 10^4 cells/well in 96-well plates (Nunc, Thermofisher) 24 hr prior to infection with HIV pseudovirus. 4 hr before the assay, the medium from each well was removed, and 50 μL fresh medium was added. From all three temperatures, triplicate SF disks containing individual inhibitors were retrieved, and these SF disks were solubilized with 200 μL of sterile PBS. The corresponding inhibitor sample sets in PBS solution format were retrieved. For all the samples, a 10-fold dilution set with sterile PBS was made. The inhibitor sets were then added to the TZM-bl cell plates, 20 μL/well. For positive control wells, 20 μL/well of cell medium were added. Afterwards, frozen stocks of CAP210 and PVO4 pseudovirus were thawed from -80 °C, diluted with TZM-bl medium, and added to the cell plates, 30 μL/well. For negative control wells, 30 μL/well of cell medium without virus was added. After 20 hr infection, the medium was changed with fresh medium, incubated for 36 hr, then the medium was removed, and the cells were lysed with addition of 30 μL/well of 0.5% NP40 in PBS. After 15 min of incubation at room temperature, 30 μL/well of 8 mM

chlorophenol red-β-D-galactopyranoside (CPRG, Calbiochem) in PBS was added. The plates were incubated at room temperature for signal development, then read by an ELx800 plate reader (BioTek) for absorbance at 570 nm and 630 nm.

For inhibition assays in PBMC and human tissue explants, the infectivity of virus preparations was estimated in TZM-bl cells (by β-galactosidase quantitation of cell lysates, Promega, Madison, WI) and PBMCs (by measure of p24 antigen content in cell culture supernatant). Experiments were performed using a standardized amount of virus culture supernatant normalized for infectivity. Cells or tissue explants were incubated with serial dilutions of inhibitors for 1 hr at 37ºC. Virus was added to cells and left for the time of the experiment. HIV-1 infection was determined by measurement of luciferase expression in TZM-bl cells or p24 levels in PBMC culture supernatants by ELISA (HIV-1 p24 ELISA, Zeptometrix Corporation, Buffalo, NY). Alternatively, tissue explants were incubated with drug for 1 hr before virus was added for 2 hr. Explants were then washed 4 times with PBS to remove unbound virus and inhibitor. Ecto-cervical explants were transferred to a fresh tissue culture plate and colorectal explants were then transferred onto gelfoam rafts (Welbeck Pharmaceuticals, UK). Explants were cultured for 15 days as previously described[51,52] in the absence of inhibitor and approximately 50 % of the supernatants were harvested every 2 to 3 days and explants were re-fed with fresh media. The extent of virus replication in tissue explants was determined by measuring the p24 antigen concentration in supernatants (HIV-1 p24 ELISA, Zeptometrix Corporation, Buffalo, NY).

2.13. Cytokine measurement

A total of thirty-three soluble immune proteins were quantified in four panels by in house multiplex bead immunoassay using a Luminex 100 System (Bio-Rad, Hercules, CA) as previously described[53]. Cytokines measured included IL- 6, G-CSF, IL-8, MCP-1, MIP-3α, IL-7, IL-15, IL-1α, IL-1β, RANTES, TGF-β, IL-12, IP-10,IL-16,GM-CSF, IL-4, IL-2, IFN-γ, IFN-β, TNF-α, MCP-2, SDF-1β, MIG, MIP-1β, human beta defensins (HBD)3, HBD4, IL-10, IL-17, L-selectin, P-selectin, secretory leukocyte protease inhibitor 1 (SLP1), elafin and α-defensin/human neutrophil peptide (HNP) 1– 3.

2.14. Statistical and mathematical analysis

IC_{50} values were calculated from sigmoid curve fitted (Prism, GraphPad) fulfilling the criterion of $R^2 > 0.7$. For FTIR, a one-way ANOVA was used to determine if differences between groups existed for the five different structural contents analyzed. No differences between groups were observed. Therefore, the data for each structural content (i.e. β-sheet, α-helix, random coil, β-turns or side chains) was combined and compared using a student's t-test.

3. Results and Discussion

3.1. Encapsulation and characterization of HIV-1 entry inhibitors in SF disks

The four proteins were produced recombinantly from *E. coli*. Structural integrity was monitored by nuclear magnetic resonance (NMR). A cyclization step was performed in the preparation of the RANTES derivatives, because 5P12-RANTES contains an N-terminal Glutamine residue that is expected to spontaneously cyclize in solution and convert to a pyroglutamate moiety[54]. To ensure homogeneity, purified recombinant 5P12-RANTES and 5P12-RANTES-L-C37 were dissolved in acidic solution and incubated at elevated temperature of 50 °C to promote cyclization. The cyclization of these proteins was monitored by NMR, with the cyclized version being considered as the mature form. As shown in Figure S1A and S1B, 5P12-RANTES is virtually fully cyclized after incubation at pH 2.5 for 22 hours, and Figure S1C and S1D similarly shows 5P12-RANTES-L-C37 in its uncyclized and cyclized forms. Purified Grft is shown in Figure S1E, and Grft-linker-C37 is shown in S1F. Each spectrum shows a homogeneous, pure, folded protein.

SF was prepared as previously described[36], resulting in a concentrated solution of fibroin that was then combined with each HIV inhibitor. For temperature stability studies, each protein was dissolved and then thoroughly mixed with a solution of SF stock. The final solutions were cast into round, disc-shaped materials, frozen and lyophilized. The resulting SF disks were cut to expose the internal structure and visualized via scanning electron microscopy (SEM; Figure 1 A-E). No obvious visual differences were observed among inhibitor-loaded SF disks. Additionally, FTIR was performed to characterize the protein secondary structure of the inhibitor-loaded SF disks (Figure 1F). Increases in β-sheet content have been previously associated with loss of material solubility[41]. The resulting secondary structure content of all the five SF disks (control and the four inhibitor-loaded groups) was not statistically different (Figure 1G). All materials had statistically higher random coil content compared to β-sheet and were found to completely dissolve over the experimental time course.

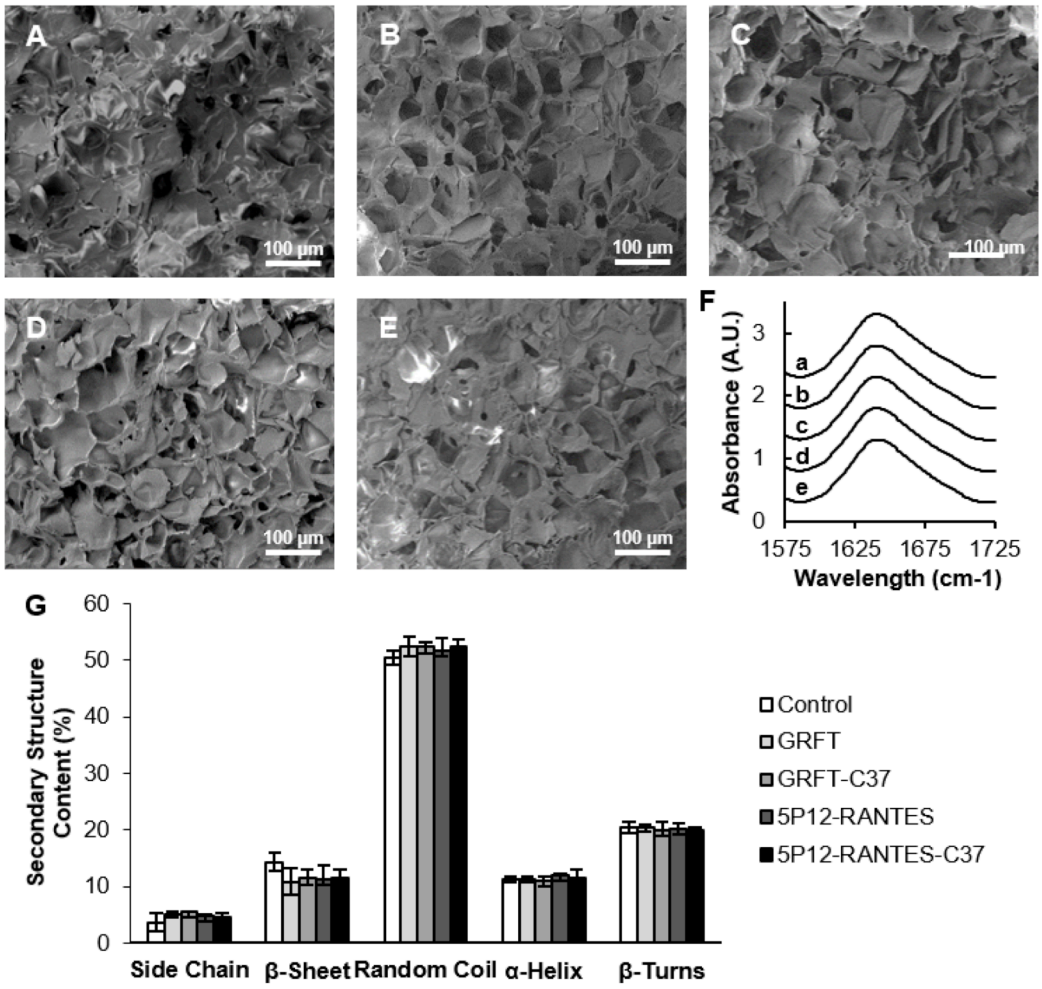


Figure 1. Characterization of HIV-1 entry inhibitor loaded-SF disks for stability studies. SEM images of SF disks containing (A) no HIV-1 entry inhibitor (control SF disks), (B) Grft, (C) Grft-C37, (D) 5P12-RANTES and (E) 5P12-RANTES-C37. (F) FTIR spectra of the Amide I region of (a) control SF disks, and SF disks containing (b) Grft, (c) Grft-C37, (d) 5P12-RANTES and (e) 5P12-RANTES-C37. (G) The deconvoluted FTIR spectra were peak-fitted to quantify the contributions of the protein secondary structure content. No statistical differences were observed between groups within the same secondary structure type. Since the secondary structures for each group were not statistically different, statistical analysis was performed between each combined secondary structure. The β-sheet and α-helix content were not statistically different while all other secondary structure comparisons were statistically different.

3.2. Stability of HIV inhibitors in SF disks

Stabilization of the four HIV inhibitors by SF was tested at various temperatures. First, each inhibitor was encapsulated in SF disks, such that upon dissolution and

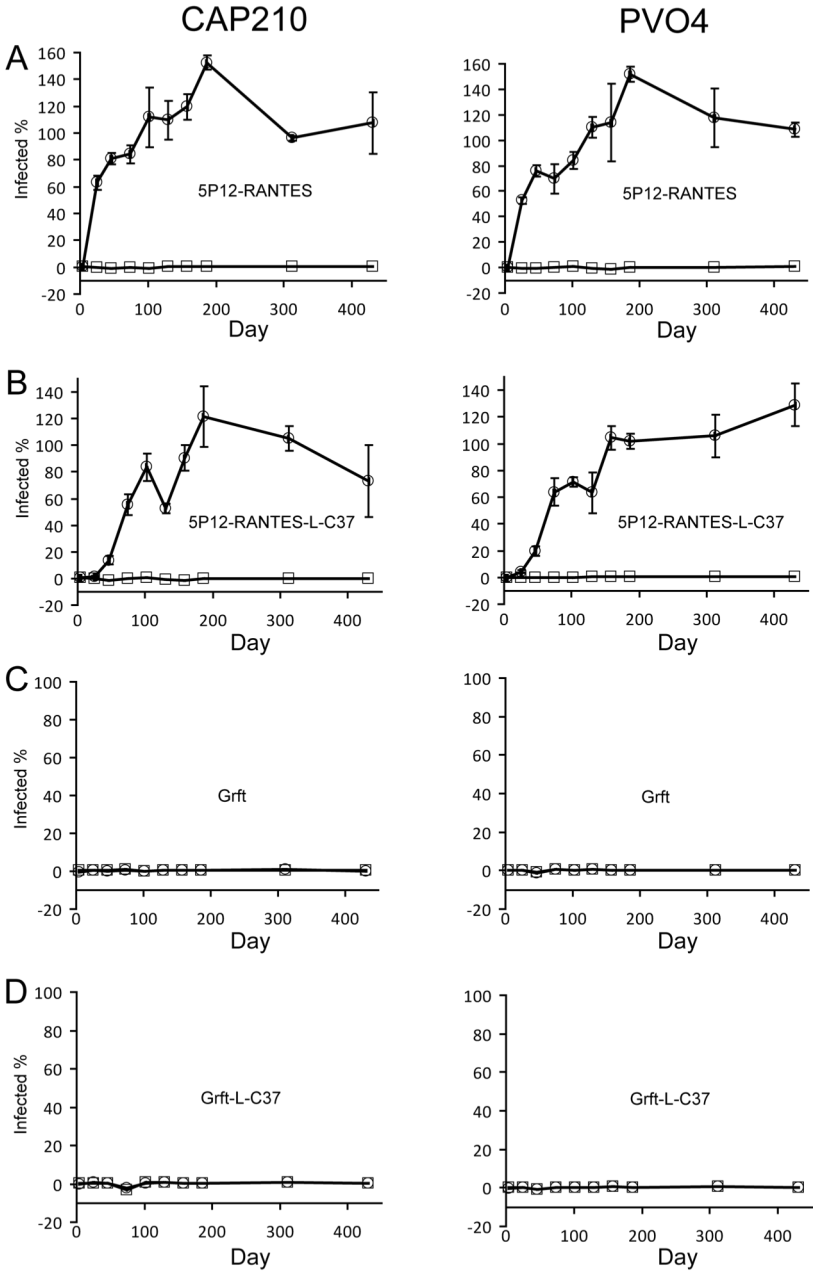


Figure 2. SF formulated inhibitors are stable at 50 °C. Inhibitors formulated in SF (□) or PBS (○) were incubated at 50 °C. At various time points samples were solubilized and tested against pseudoviral strains CAP210 (clade C, left) and PVO4 (clade B, right), with percent infection shown as compared to control without inhibitor. Some points show more than "100% infection" due to comparison with the control (SF alone), which can provide some protection, likely due to a barrier effect. **A**. 5P12-RANTES. **B.** 5P12-RANTES-L-C37. **C.** Grft. **D.** Grft-L-C37. Data are the mean ± SD in triplicate by using three individual SF disks at each time point.

complete release the concentration of inhibitor would be 3 μM, which in turn would be diluted by 10 fold, and further diluted by 5 fold as part of the assay protocol, corresponding to a final concentration of 60 nM in the pseudovirus assay. A solution of each inhibitor at 3 μM was made using PBS. Each group was incubated at three different temperatures: 25 °C, 37 °C, and 50 °C. At various time points, each of the four HIV inhibitors along with an SF disk control was tested for its ability to inhibit HIV single-round pseudoviruses from two different R5-tropic HIV isolates, namely clade B PVO4 and clade C CAP210. Figure 2 shows the level of protection provided by each inhibitor in various formats at 50 °C.

As can be seen in Figure 2 and Supp Figure S2, 5P12-RANTES encapsulated in an SF disk provided full protection against both HIV pseudoviruses *in vitro*, even after 14 months of storage at 50 °C. This is in contrast to the protein incubated in PBS, which gradually lost its inhibitory potency over time (Figure 2A and Supp Figure S2). . Figure 2B and Supp Figure S2 shows equally high levels of retained potency for 5P12-L-C37 in the SF disks. This protein shows no loss of inhibition after 14 months at 25 °C, 37 °C or 50°C in the SF-encapsulated format. In contrast, the inhibitor solution in PBS gradually lost activity when incubated at elevated temperatures. The loss in inhibitory potency is unlikely due to passive adsorption of the proteins to the vials during the incubation time, as passive adsorption is a fast process that tends to occur at low concentrations of protein; also, the loss of activity did not occur for Grft-based inhibitors.

Grft and Grft-L-C37 encapsulated in SF disks are fully protective against both pseudoviruses tested upon incubation at all temperatures for 14 months (Figure 2C, 2D and Supp Figure S2). These proteins show no loss of activity in any format tested. The stability of Grft has been reported previously for incubations up to three months at room temperature[25], and the current work demonstrates that much longer term incubations at high temperatures do not affect the activity of these proteins. This confirms that Grft and its variants could be suitable as microbicides in a variety of formulations.

3.3. Inhibitory activity of non-formulated and SF-encapsulated HIV inhibitors in PBMCs

The potency of the four inhibitors, 5P12-RANTES, 5P12-RANTES-L-C37, Grft and Grft-L-C37, was tested against an R5-tropic isolate, HIV-1 YU.2, in activated PBMCs. A dose-response curve was obtained for all four proteins within the range of concentrations tested (Figure 3A). All four inhibitors exhibited sub-nanomolar activity. The anti-viral activity of non-formulated 5P12-RANTES and Grft increased with conjugation to the C37 peptide (Figure 3A) with a reduction in the IC_{50} value (Supplementary Table 1). Formulation with SF generally reduced the IC_{50} values compared to the unformulated inhibitors, likely due to the solubilized SF being viscous and retaining the inhibitors, potentially resulting in higher local concentrations (Figure 3A, Supplementary Table 1). The SF control had no inhibitory activity (Figure 3A) and importantly, the SF-formulated proteins showed no cytotoxic effect by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) viability assay at the concentrations tested (data not shown).

3.4. SF-encapsulated HIV inhibitors are functional in human tissue explant assays

As candidate microbicides, the inhibitory activity of the four inhibitors was next assessed in mucosal tissue explants. SF-encapsulated proteins and the same proteins unformulated (as lyophilized powder) were tested in non-polarized colorectal and ecto-cervical tissue explants against HIV-1 YU.2, and showed inhibition in the nanomolar range. In both models, the SF-formulated inhibitors were more potent than the corresponding base compounds (Figure 3B, C) in colorectal explants (Supplementary Table 1). Similarly to the results obtained in PBMCs, conjugation of GRFT or 5P12-RANTES to C37 resulted in an increase in potency. SF-encapsulated 5P12-RANTES-L-C37 was the most potent inhibitor in both mucosal models. No inhibition was observed with the SF control (Figure 3B, C).

The safety profile of the SF-encapsulated inhibitors was pre-clinically evaluated in mucosal tissue explant models. Patterns of cytokine release were measured following exposure of tissue explants to SF control or to SF-formulated inhibitors for 3 hr (mimicking a pulse exposure) or 24 hr (mimicking a sustained exposure to the drug) (Figure 4). Pulse exposure of ecto-cervical and colorectal explants to SF or SF-formulated compounds did not induce a significant change in the levels of cytokines measured in culture supernatants compared to baseline levels of non-treated explants. With sustained exposure, no up-regulation of pro-inflammatory markers was observed in either explant model. In colorectal explants, the levels of adaptive cytokine IL-2 were up-regulated by 5P12-RANTES and 5P12-RANTES-L-C37 after sustained exposure. In ecto-cervical explants, sustained treatment with 5P12-RANTES resulted in a statistically significant increase secretion of adaptive cytokine IL-4 and antimicrobial protein P-selectin. Regarding Grft and Grft-L-C37, in colorectal explants, sustained exposure of Grft induced a statistically significant increase of antimicrobial proteins SLP-1, IL-2, and Human β-defensin 3. Meanwhile, sustained exposure of Grft-L-C37 induced a statistically significant decrease in the levels of inflammatory cytokine IL-6, chemokines (MCP-1, MCP-2, MIP-1β, SDF-1β and IP-10), growth factor GM-CSF, and significant increase of antimicrobial protein SLP-1 in culture supernatants. In ecto-cervical explant cultures, exposure to Grft-L-C37 for 24h induced some down-regulation of the chemokine IL-8 and up-regulation of antimicrobial protein L-selectin. The modulation of certain cytokines in this *ex vivo* model should be interpreted with caution and will be analyzed in planned *in vivo* non-human primate studies to further assess the safety profile of these formulations. No pro-inflammatory effects such as those described by others for Nonoxynol-9[55–58] were observed. Hence, our results indicate preliminary suitability of SF materials in this application.

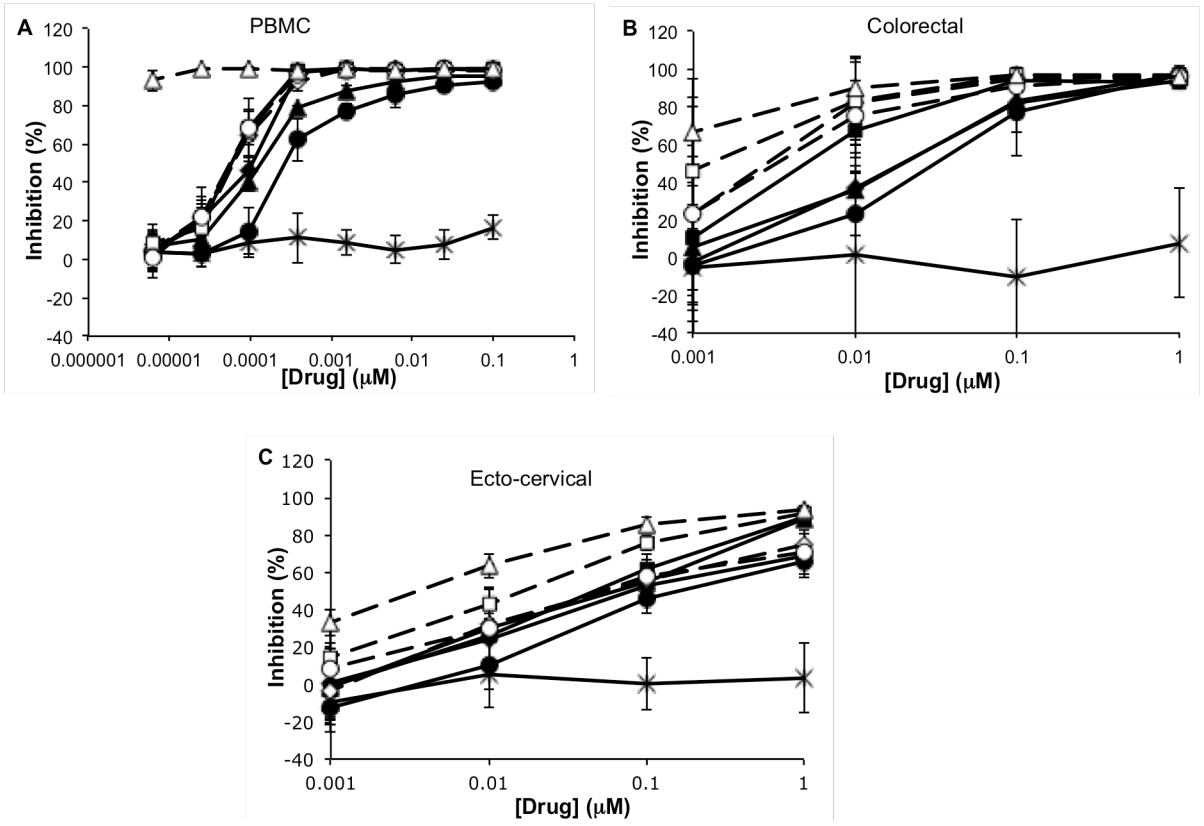
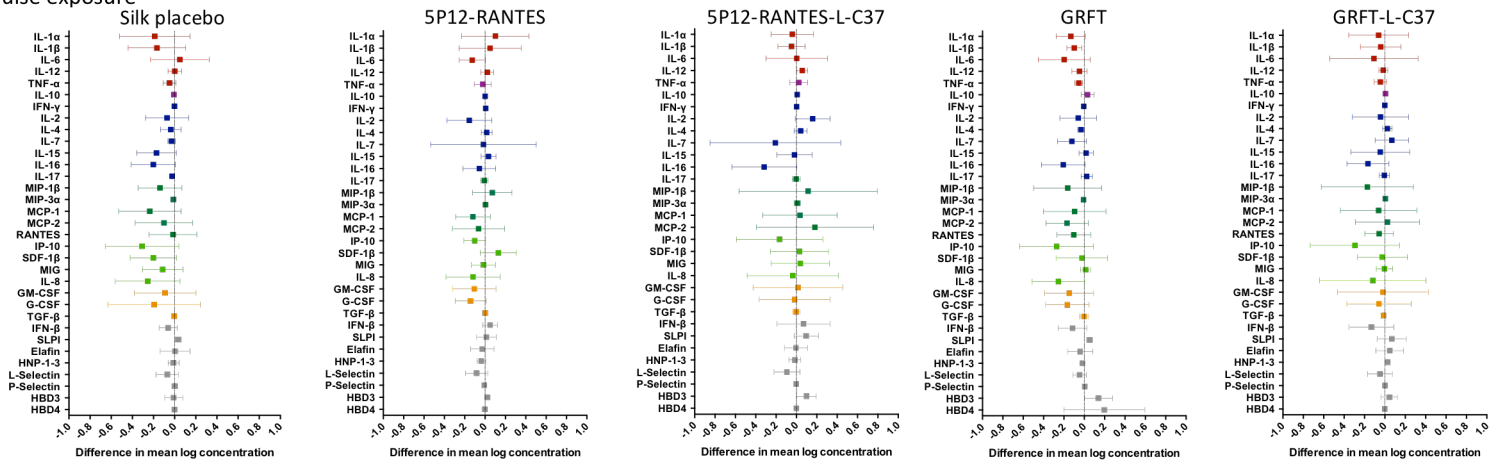


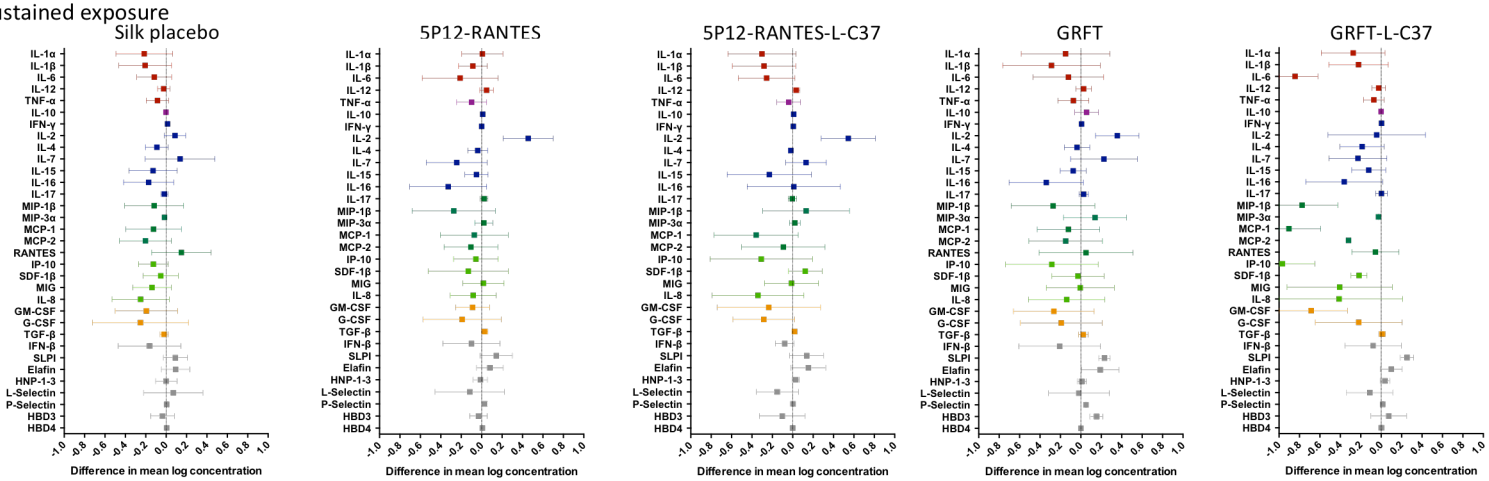
Figure 3. **Dose-response curves of non-formulated and SF-encapsulated inhibitors against HIV-1 YU.2 in PBMCs and human mucosal tissue explants. A)** Activated PBMCs, **B)** colorectal tissue explants or **C)** ecto-cervical explants were treated for 1h with or without non-formulated or SF-encapsulated Grft (◆,◇), Grft-L-C37 (■,□), 5P12-RANTES (●,○), or 5P12-RANTES-L-C37 (▲, △), or left untreated (X) prior to addition of virus. PBMCs were cultured for 6 days post-infection. Tissue explants were exposed to virus for 2 h, washed with PBS and cultured for 15 days. The levels of p24 in the harvested supernatants were quantified by ELISA and the extent of inhibition by each compound was calculated. The percentage of inhibition was normalized relative to the p24 values obtained for cells or explants not exposed to virus (0% infectivity, curves depicted with X) and for cells or explants infected with virus in the absence of compound (100% infectivity). Data are the mean ± SD of three independent experiments performed in triplicate.

A) Colorectal explants

Pulse exposure



Sustained exposure



Inflammatory cytokines
Adaptive cytokines
CXC chemokines
Antimicrobial proteins
Anti-inflammatory cytokines
CC chemokines
Growth factors

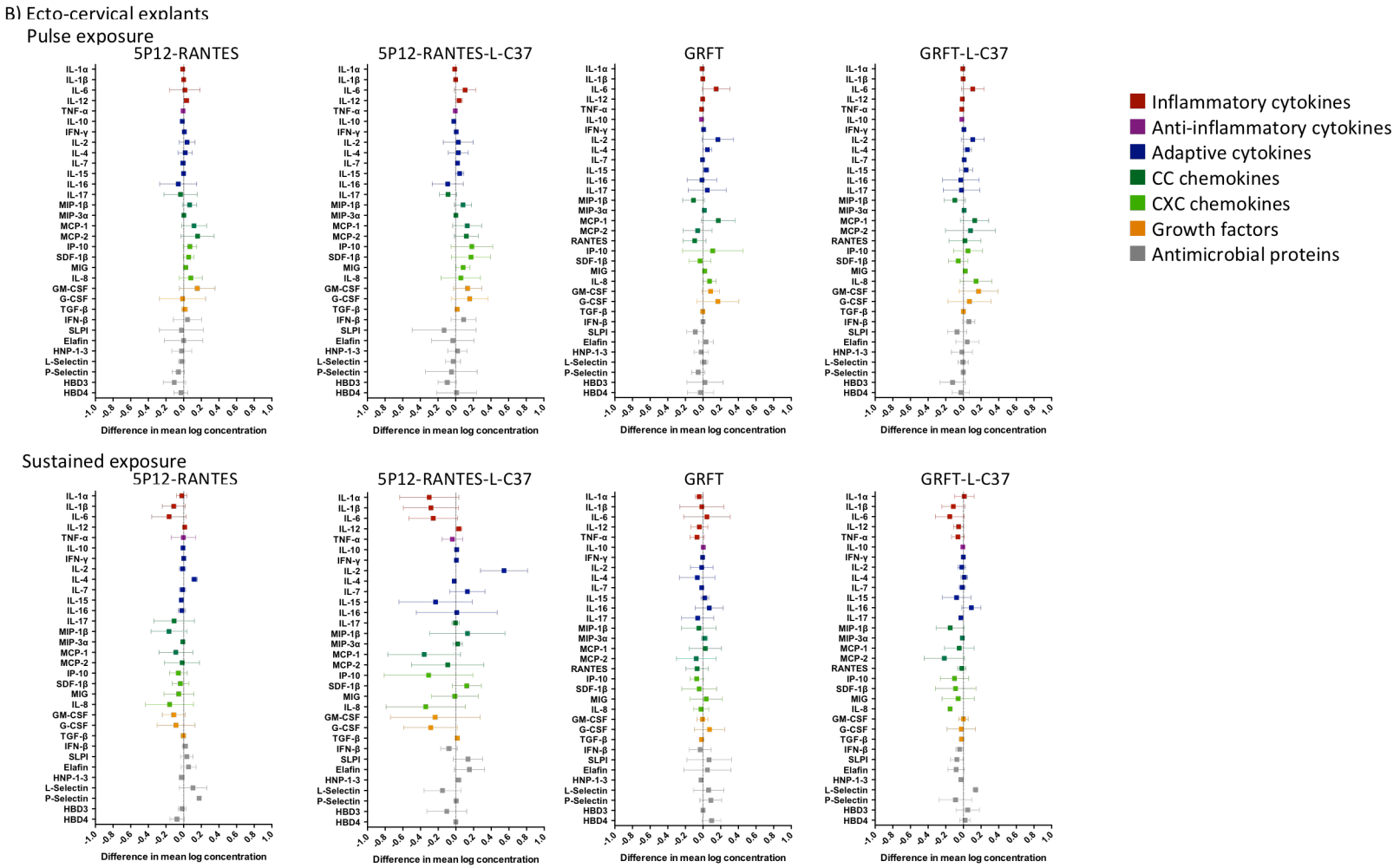

B) Ecto-cervical explants
Pulse exposure
5P12-RANTES
5P12-RANTES-L-C37
GRFT
GRFT-L-C37
Sustained exposure
5P12-RANTES
5P12-RANTES-L-C37
GRFT
GRFT-L-C37
IL-1α
IL-1β
IL-6
IL-12
TNF-α
IL-10
IFN-γ
IL-2
IL-4
IL-7
IL-15
IL-16
IL-17
MIP-1β
MIP-3α
MCP-1
MCP-2
RANTES
IP-10
SDF-1β
MIG
IL-8
GM-CSF
G-CSF
TGF-β
IFN-β
SLPI
Elafin
HNP-1-3
L-Selectin
P-Selectin
HBD3
HBD4
-1.0 -0.8 -0.6 -0.4 -0.2 0.0 0.2 0.4 0.6 0.8 1.0
Difference in mean log concentration
Inflammatory cytokines
Anti-inflammatory cytokines
Adaptive cytokines
CC chemokines
CXC chemokines
Growth factors
Antimicrobial proteins

Figure 4. Analyte concentrations in **A)** colorectal and **B)** ecto-cervical tissue supernatant following pulse or sustained exposure to SF or SF-formulated compounds were compared with control tissue not exposed to drug. Difference in mean log concentration ± SD from two experiments in quadruplicate for colorectal and in duplicate for ecto-cervical tissue are shown.

3.5. Sustained Release of SF-encapsulated HIV inhibitors

Modifications during the SF formulation process were tested to explore the possibility of sustained release of relevant amounts of inhibitor over time. As opposed to the SF disks in the stability studies that were designed to quickly dissolve and fully release all the encapsulated inhibitors, an SF disk for sustained release should stay largely insoluble. In a scenario of sustained inhibitor release, it is envisioned that the user would insert a SF disk and the body's moisture would gradually mediate the release of the inhibitor over the course of days or weeks. In this case, SF disks should act as a scaffold/matrix, while allowing for slow release of the inhibitor in an aqueous/mucosal environment. A process termed "water vapor annealing" (WVA) has been reported to promote β-sheet formation in SF materials, reducing their water solubility. Extensive WVA processing results in a fully insoluble SF scaffold, hindering or even prohibiting drug release. On the other hand, insufficient annealing leads to lack of sustained release capability due to dissolution of the SF[41]. For time release of macromolecules such as HIV inhibitory proteins, it is important to tailor the formulation parameters for a specific molecule to achieve the desired release profile. To demonstrate the feasibility of HIV protein inhibitor time release, Grft was selected as the inhibitor for testing. Various parameters of the SF disk were tested, including the SF percentage, the size of the disk, as well as the temperature, relative humidity and annealing duration used in the WVA process. It was experimentally determined that satisfactory Grft release profiles can be achieved by encapsulating 147 μg Grft (10 μM final in-SF concentration) into a round, disc-shaped material that is 1 mL in volume (2 cm^2 of bottom surface area, 5 mm in thickness), comprised of 1-2% SF, that has been annealed for 3-4 hr at 37°C with ≥ 75% relative humidity. The annealed SF disks were able to maintain their general structure in the presence of buffer solution, and gradually release Grft over time.

To determine the suitability of the time-release SF disks for potential physiological use, WVA-processed SF disks containing Grft were incubated in either PBS or simulated vaginal fluid (SVF[59], pH 4.2), representing colorectal and vaginal conditions, respectively. At each time point, the incubation solution was removed and replaced with fresh buffer solution and tested for the presence of Grft. Grft release was detected in both buffers throughout the experimental duration (Figure 5). During the first three weeks, the amount of released Grft ranged from 550 to 1300 ng in approximately 1 mL fluid (corresponding to 41.3-99.4 nM) in PBS, and from 570 to 1000 ng (corresponding to 43.1- 75.5 nM) in SVF (Figure 5A, C). After three weeks, the amount of release decreased, but was still sustained at levels of around 300-400 ng per mL (20 nM for PBS release, and about 30 nM for SVF release; Figure 5B, D). The reported IC_{50} values for Grft inhibition towards a variety of HIV strains are typically in the subnanomolar range[60]. As such, the amount of Grft released is expected to effectively inhibit HIV infection. No significant difference was observed between the release behavior in PBS and SVF. Cumulatively, a total of 14.8 ± 1.6 μg of Grft was released in PBS, and 13.3 ± 0.8 μg of Grft was released in SVF, representing ~10% and ~9% of loaded Grft, respectively. These amounts are satisfactory as initial proof of concept from a pharmacological perspective. Recent reports describe intravaginal rings that are manufactured with much higher quantities of small molecule and antibody inhibitors,

showing release in the mg range [61–63]. We are also pursuing larger amounts of protein in the context of larger disks, films, and inserts. Further material development is expected to provide various release kinetics, if desired.

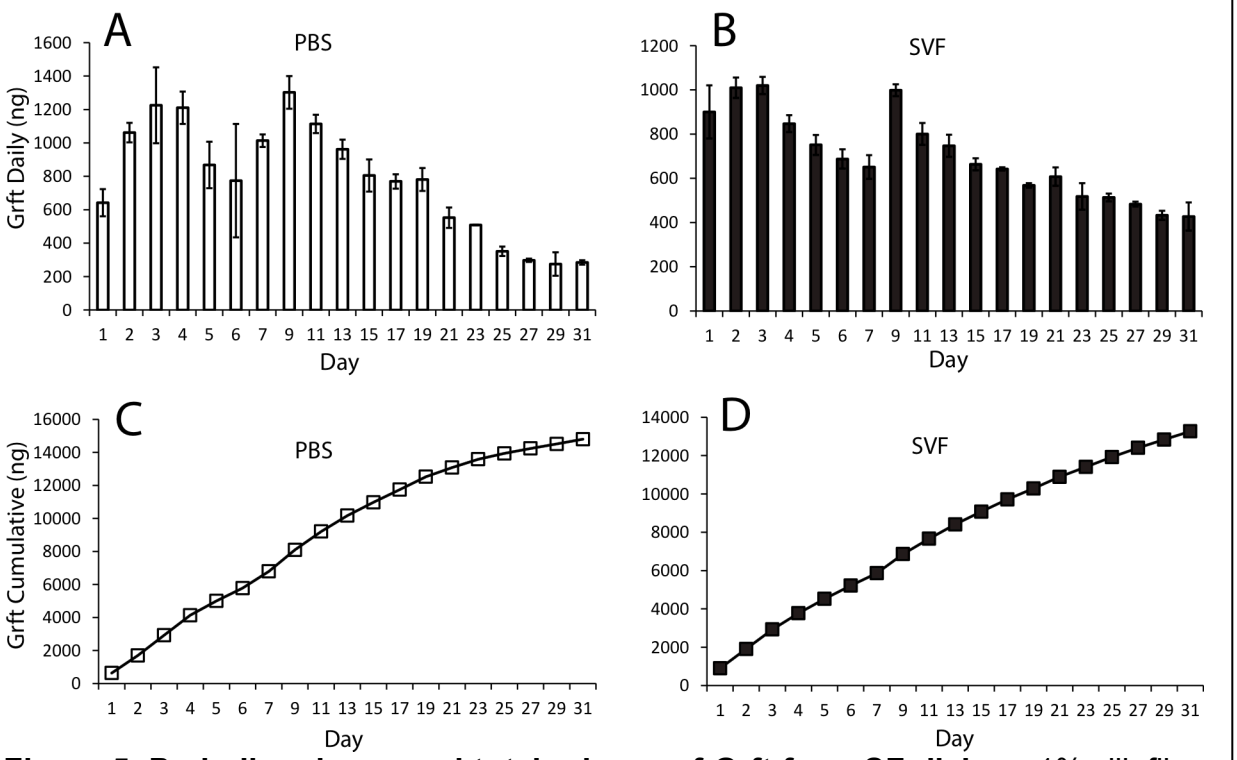


Figure 5. Periodic release and total release of Grft from SF disks. 1% silk films containing Grft were prepared according to Methods. 1 mL PBS (A) or 1 mL SVF (B) was added to the film and incubated at 37°C. The solution was removed and fresh solution added at various time points. Released Grft was quantitated by an ELISA, according to Methods. (C) and (D) show cumulative release of Grft over time when incubated with PBS or SVF, respectively. Data are the mean ± SD in triplicate by using three individual SF disks.

3.6. Sustained release Grft inhibits HIV infectivity in vitro

In order to determine whether the Grft from sustained release SF disks is capable of inhibitory function against HIV, the activity of SF disk-released Grft over the course of a month (obtained as described in Methods) was tested against HIV-1 pseudoviruses PVO4 and CAP 210 in TZM-bl cells (Figure 6). Grft released into (Figure 6 A and C) PBS or (Figure 6 B and D) SVF at various time points effectively inhibited both viruses, with all the time point samples from the first three weeks showing full inhibition, and the day 31 samples showing over 90% inhibition. This long-term inhibition property is particularly desirable in situations where the user prefers, and hence would

be more adherent to, a longer-acting inhibitor that does not require daily dosing. Given this and findings from others, SF-mediated sustained release systems could be applicable for a broad range of anti-viral molecules[64].

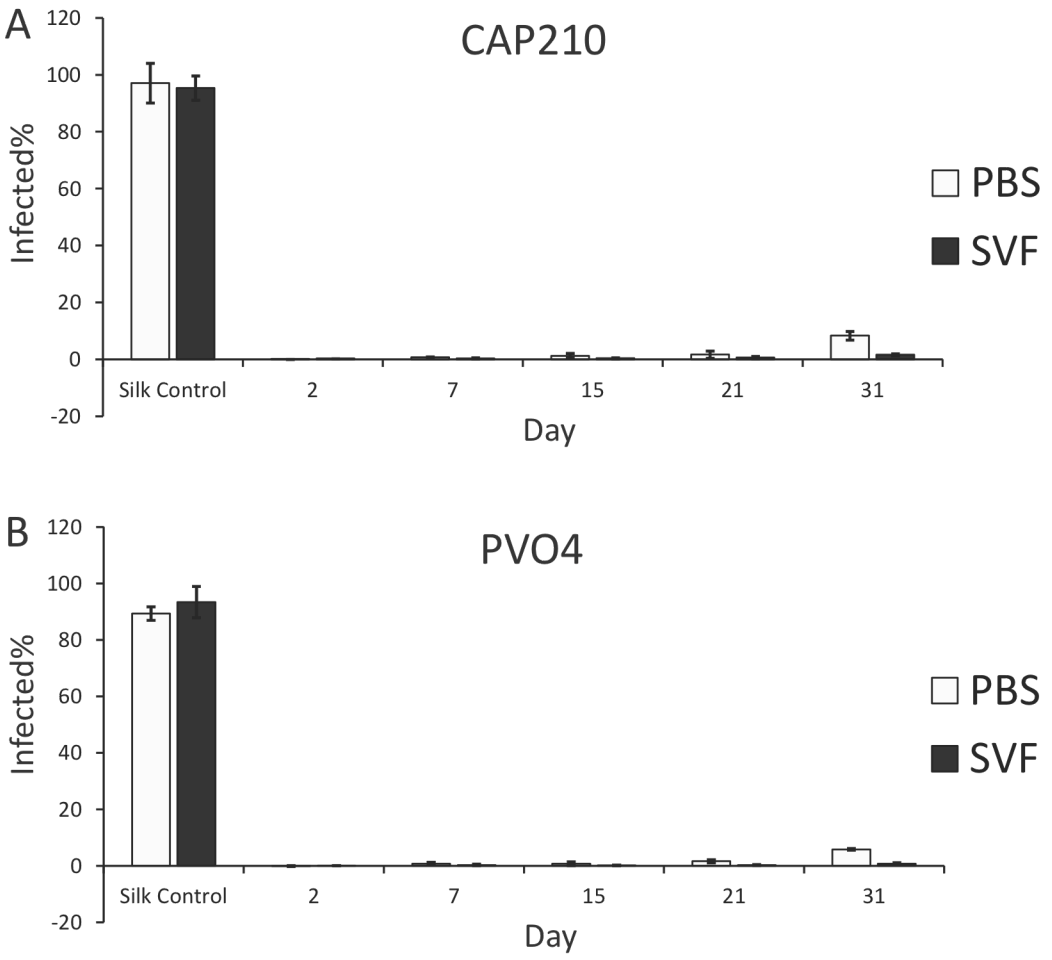


Figure 6. Effective inhibition by sustained release of Grft in both PBS and SVF. 1% SF disks containing Grft were prepared according to Methods with 4 hr WVA. 1 mL PBS (open) or 1 mL SVF (solid) was added to the disk and incubated at 37°C. The solution was removed and fresh solution added at various time points. Released Grft was tested in triplicate against pseudovirus in TZM-bl cells. Bars are shown as mean ± SD of percent infection of (A) CAP210 or (B) PVO4. Controls of SF (without inhibitor) for each corresponding day showed no inhibition and their values are averaged and shown at far left of each graph.

4. Conclusion

In this study, we present a silk fibroin-based inhibitor delivery system that not only shows great capability in stabilizing protein-based HIV inhibitors but also shows the feasibility of being developed for sustained release of these macromolecules. The stability of SF-encapsulated inhibitors was illustrated with four protein inhibitors, which vary in molecular weight, tertiary structure and charge distribution. Each of these retained potent functionality in HIV pseudovirus assays, even after incubation at 50 °C for over 14 months. In comparison, when stored in solution, some protein inhibitors showed decreased activity. Furthermore, formulated inhibitors were shown to be effective against HIV in both colorectal and cervicovaginal tissues, and in PBMC. This demonstrates the potential utility of SF formulations without refrigeration in areas with extreme temperature conditions such as sub-Saharan Africa. Therefore, this pre-clinical study describes the feasibility of a SF disk approach as part of an HIV prevention strategy.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Supporting Information:

Figure S1: NMR HSQC spectra of protein HIV inhibitors used in this study.

Figure S2 and S3: Stability of inhibitor-infused SF disks at 25°C and 37°C, respectively.

Table S1: IC50 of inhibitor-encapsulated SF disks in PBMC and human tissue explants.

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Supporting Information:

Stabilization and sustained release of HIV inhibitors by encapsulation in silk fibroin disks

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Supplementary data

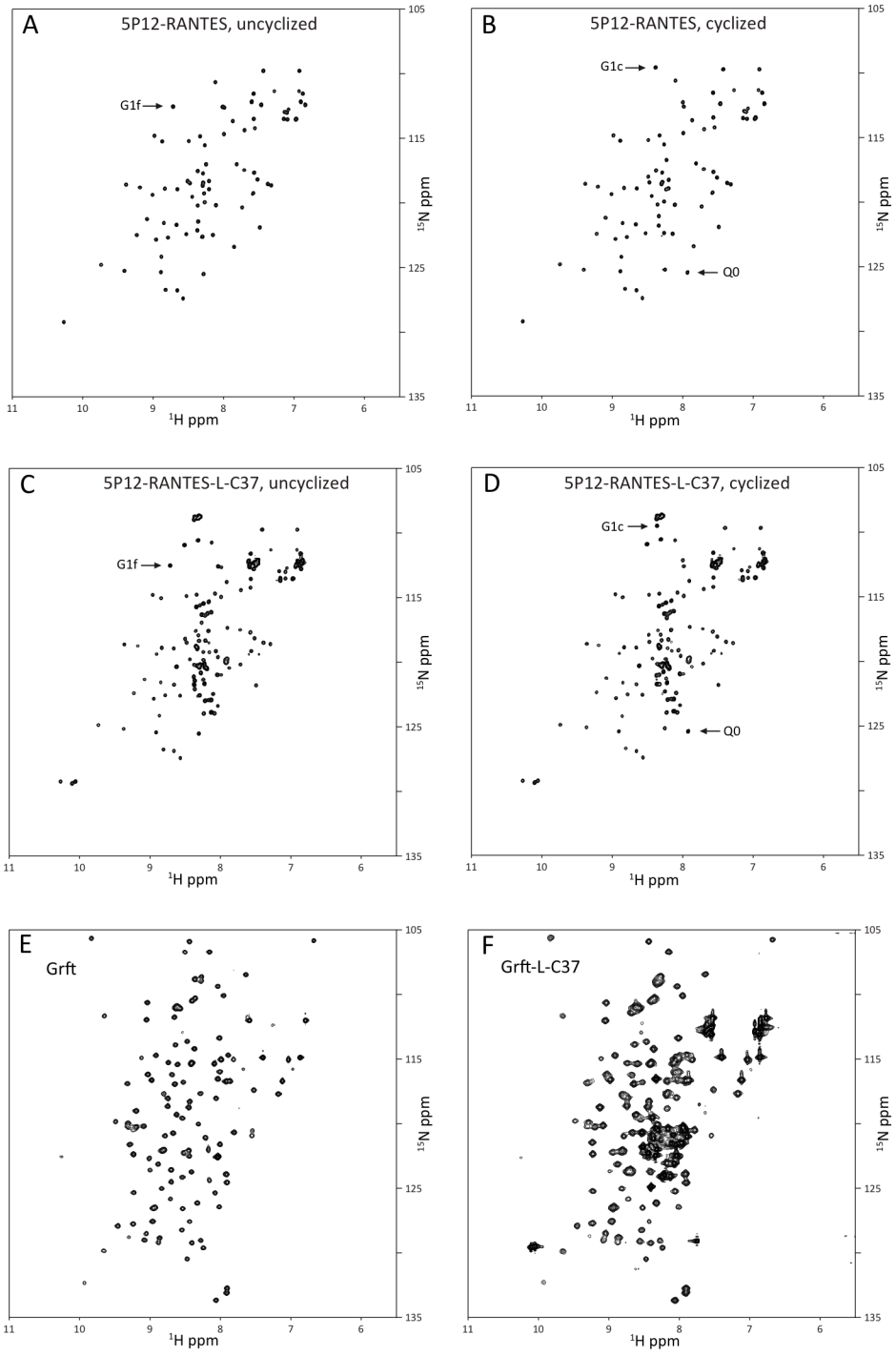

Figure S1. ^{15}N HSQC spectra of HIV inhibitors used in this study. (A) 5P12-RANTES uncyclized at the N-terminal glutamine (Q0) residue. (B) 5P12-RANTES cyclized at the N-terminal glutamine (Q0) residue. (C) 5P12-RANTES-L-C37 uncyclized at the N-terminal glutamine (Q0) residue. (D) 5P12-RANTES cyclized at the N-terminal glutamine (Q0) residue. “G1f” refers to the peak corresponding to the glycine residue adjacent to flexible, uncyclized Q0. “G1c” refers to the peak corresponding to the glycine residue adjacent to the cyclized Q0. (A-D) Spectra were measured in 20 mM NaOP pH 2.5. (E, F) Spectra were measured in 20 mM Tris pH 8.0.

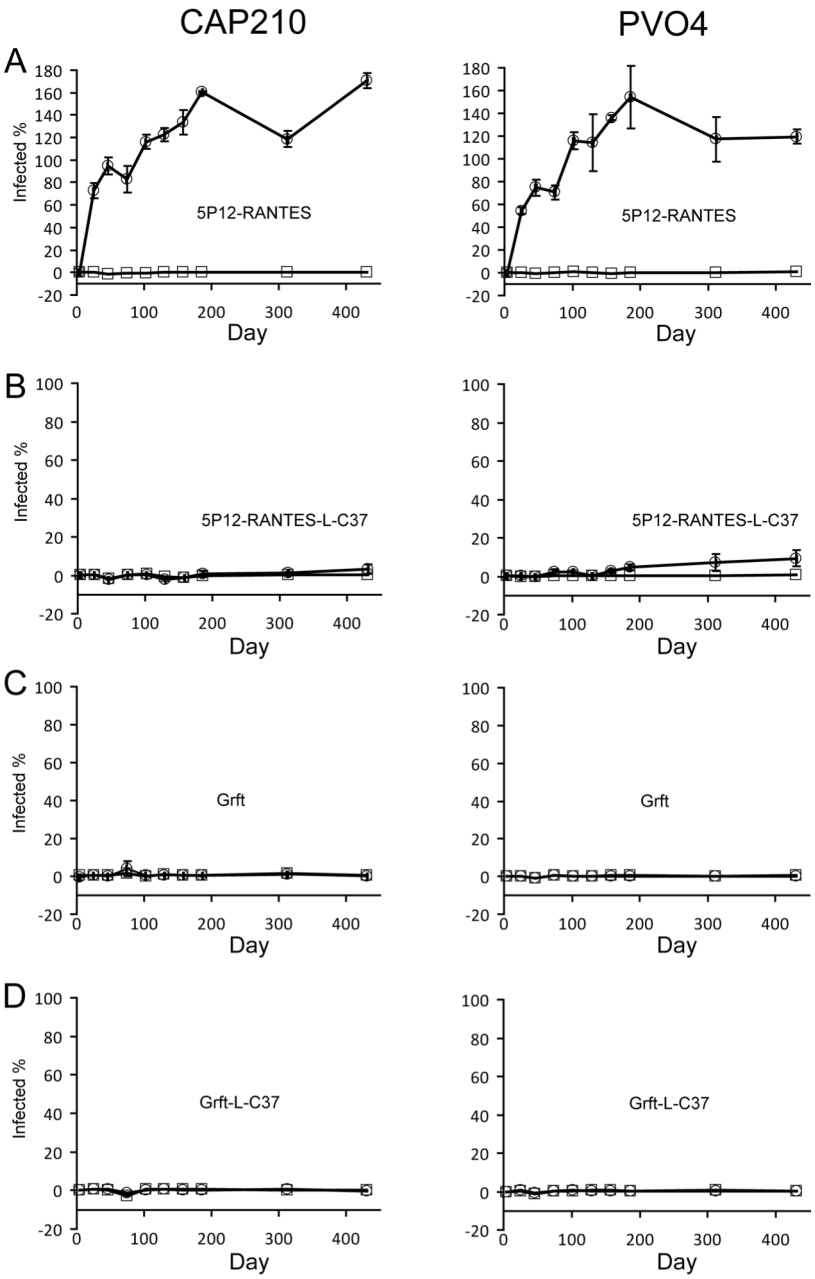

Figure S2: Inhibitors formulated in SF (□) or PBS (○) were incubated at 25 °C. At various time points samples were solubilized and tested against pseudoviral strains CAP210 (clade C, left panels) and PVO4 (clade B, right panels) with percent infection shown as compared to control without inhibitor. Inhibitor proteins evaluated were (A) 5P12-RANTES, (B) 5P12-RANTES-L-C37, (C) Grft and (D) Grft-L-C37. Data are presented as mean ± SD in triplicate by using three individual SF disks at each time point.

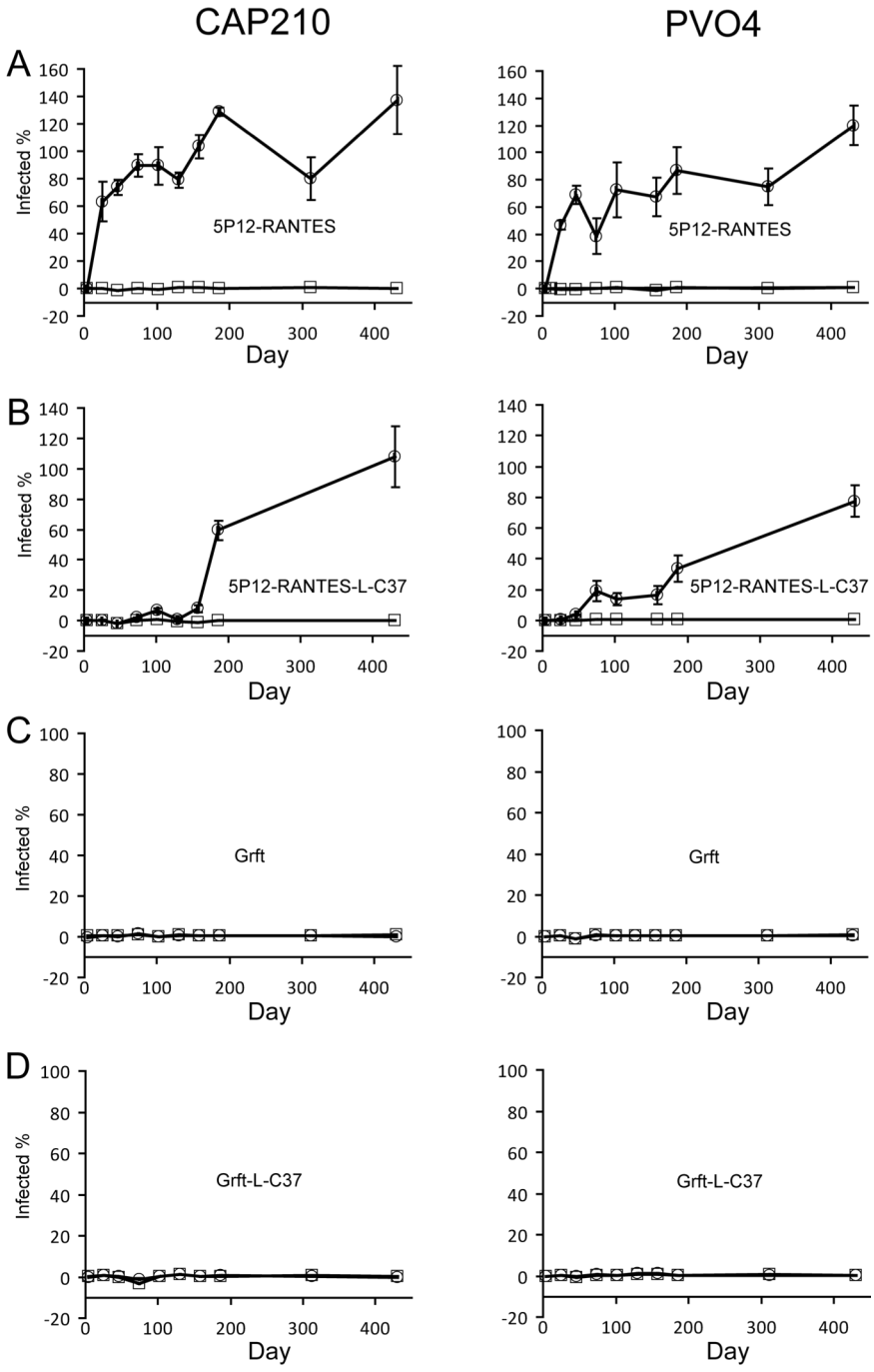

Figure S3: Inhibitors formulated in SF (□) or PBS (○) were incubated at 37 °C. At various time points samples were solubilized and tested against pseudoviral strains CAP210 (clade C, left) and PVO4 (clade B, right) with percent infection shown as compared to control without inhibitor. Inhibitor proteins evaluated were (A) 5P12-RANTES, (B) 5P12-RANTES-L-C37, (C) Grft and (D) Grft-L-C37. Data are presented as mean ± SD in triplicate by using three individual SF disks at each time point.

Supplementary Table 1. IC_{50}s of non-formulated or SF-encapsulated drugs in PBMCs and tissue explants

Model and formulation		IC_{50} for each drug (nM)[a]			
		5P12-RANTES	5P12-RANTES-L-C37	GRFT	GRFT-L-C37
PBMCs	Non-formulated	0.347 ± 0.152	0.135 ± 0.010	0.083 ± 0.005	0.056 ± 0.006
	SF	0.053 ± 0.007	0.001 ± 0.0004	0.059 ± 0.009	0.060 ± 0.005
Colorectal explants	Non-formulated	38.68 ± 16.17	18.36 ± 6.41	18.38 ± 9.61	4.93 ± 2.49
	SF	3.05 ± 0.19	0.47 ± 0.53	3.02 ± 2.73	2.49 ± 3.06
Ecto-cervical explants	Non-formulated	104.95 ± 2.32	55.10 ± 39.76	56.88 ± 10.77	50.71 ± 26.75
	SF	59.74 ± 22.95	4.57 ± 1.06	38.41 ± 33.31	15.54 ± 0.54

[a] The data are means ± SD derived from three independent experiments performed in triplicate.