An Evaluation of Polycaprolactone Matrices for Vaginal Delivery of the Antiviral, Tenofovir, in Preventing Heterosexual Transmission of HIV

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ABSTRACT: Tenofovir was incorporated in controlled-release polycaprolactone (PCL) matrices designed for production of vaginal inserts for prevention of HIV transmission. Rapid cooling of suspensions of the drug powder in PCL solution resulted in micro-porous matrices with tenofovir loadings up to 12% (w/w) and high incorporation efficiencies in excess of 90%. The release behaviour of tenofovir in simulated vaginal fluid (SVF) demonstrated high delivery efficiency of 85%–99% over 30 days and could be described effectively by a first-order kinetics model giving a mean value of 0.126 day-1 for the release constant (k_1). Tenofovir released from PCL matrices into SVF exhibited high relative activity ranging from 70 to 90%, against pseudo-typed HIV-1-infected HeLa cells. The inhibitory activity of tenofovir standard solutions in SVF provided an IC_{50} value of 2.38 μM. Besides confirming high levels of *in vitro* antiviral activity, the predicted concentrations of tenofovir, which would be released from a PCL intra-vaginal ring *in vivo*, exceeded the IC50 value for HIV-1 by a factor of 35–200 and clinically protective concentrations by a factor of 50. These findings recommend further investigations of antiviral-loaded PCL matrices for controlling heterosexual transmission of HIV. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:3725–3735, 2013

Keywords: intra-vaginal inserts; microbicides; tenofovir; HIV; polycaprolactone matrices; drug release; drug delivery system; HIV/AIDS; mathematical model; in vitro model

INTRODUCTION

The requirement for novel approaches to limit the spread of sexually transmitted infections is highlighted by the continuing HIV/AIDS pandemic.[1] Highly active anti-retroviral therapy suppresses virus replication in host cells and has greatly reduced the spread of AIDS in developed countries. However, 90% of infected people live in developing countries and have limited access to this treatment. Furthermore, the number of new diagnoses attributed to heterosexual contact is on the increase. In sub-Saharan Africa, for example, women are disproportionately affected with those between the ages of 15 and 24 being eight times more likely to be HIV positive than men. The scale of the problem, the lack of progress in developing a vaccine against HIV and the urgent need for improved HIV prevention options that can be controlled by women has intensified research on vaginal delivery of microbicides that kill or inactivate viruses at the mucosal barrier.[2,3]

First-generation microbicides included non-specific agents such as nonoxynol-9 surfactant and buffering agents, which functioned respectively by solubilising virus envelope proteins and maintaining the natural defensive acidic pH of the vagina (3.5–4.5). A number of anionic polymers including cellulose acetate phthalate and naphthalene disulphonate have been widely investigated as virus entry/fusion inhibitors.[4–6] These substances are considered to bind to virus envelope proteins (gp120 and gp141 in HIV) by electrostatic interaction, thereby preventing virus entry into target cells ($CD4^+$-T cells, macrophages and dendritic cells) in the genital sub-mucosal region. Vaginal formulations of galactose-linked polysaccharides (carrageenan) have also been reported to protect mice from *Herpes simplex* virus infection,[7] block HIV infection of cervical epithelial cells and trafficking of HIV-infected macrophages from the vagina to lymph nodes.[8] Although these first generation microbicides showed promise *in vitro* and in clinical trials, none met with clinical success because of safety issues or lack of efficacy.[9]

Second-generation microbicides based on HIV-specific antiretrovirals[10] function as post-entry inhibitors, preventing virus release from cells by disrupting essential steps in the replication cycle. Several compounds, widely used in systemic oral treatments of HIV, are currently being evaluated as topical vaginal microbicides. Application of the non-nucleoside reverse transcriptase inhibitor dapivirine (TMC-120) in a Carbopol 940/hydroxyethylcellulose gel formulation in (hu-SCID) mice prevented systemic infection by cell-associated HIV.[11] A limited study in HIV^- women showed that dapivirine released from a vaginal gel applied once daily over 11 days was well distributed throughout the cervico-vaginal area at concentrations several orders of magnitude above the EC_{50}.[12] Recently, the CAPRISA 004 clinical trial reported that the nucleoside reverse transcriptase inhibitor, tenofovir, applied in a hydroxyethylcellulose gel in a pre-exposure prophylactic study, reduced HIV acquisition by an estimated 39% overall and by 54% in women with high adherence to the study protocol.[13] However, the tenofovir gel was found to perform no better than a placebo in the larger-scale VOICE trial.[14]

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Most candidate microbicides undergoing clinical trials are formulated as semi-solid gels or creams which are inherently "messy" to use, prone to leakage and elicit concerns over ineffective coverage of the epithelium. In addition, they must be applied prior to intercourse (often using an applicator) to reduce the risk of virus infection. These factors make it difficult to guarantee adhesion to trial protocols and to ensure protection for women who may be at risk from HIV transmission. As a result, interest in intra-vaginal ring inserts (IVRs) for controlled, long-term, delivery of antivirals is intensifying[3,15,16] as improved user compliance and sustained concentrations of the drug in vaginal fluids at therapeutic levels are expected to reduce the risk of virus transmission. A 7-day clinical study involving HIV$^-$ women revealed that silicone elastomer IVRs releasing dapivirine elevated drug concentrations in vaginal fluid and tissues to levels three orders of magnitude above the EC_{50}.[17] However, conventional IVRs, produced from silicone elastomer or thermoplastic poly(ethylene vinyl acetate) (pEVA), display certain disadvantages for microbicide delivery. They are generally restricted to delivery of low-molecular-weight, hydrophobic drug candidates such as dapivirine and curing of silicone elastomer at 80°C or hot melt extrusion of pEVA at 110°C is required during manufacture, which could deactivate heat-labile compounds. Pod-type IVR designs have been developed to overcome these limitations and to achieve simultaneous delivery of multiple drugs. Independent control of the release rate of hydrophilic tenofovir and acyclovir has been achieved by embedding polymer-coated drug cores in silicone elastomer IVRs, which permit drug release through a delivery channel. Tenofovir release of 144 μg/day may be sustained for 28 days.[18] In addition, tenofovir-loaded pod-type IVRs were found to produce mean tenofovir levels in vaginal secretions of macaques of around 2 mg/mL over 28 days and intracellular tenofovir diphosphate concentrations in the range that may be protective against simian-human immunodeficiency virus infection in non-human primates.[19] The challenge of incorporation and delivery of hydrophilic compounds in IVRs has also been approached by application of thermoplastic polyether urethane elastomers that incorporate covalently bonded polyethylene oxide segments in the copolymer structure. Clark et al.[20] manufactured tenofovir-loaded IVRs by melt extruding drug-loaded pellets at 147°C, followed by injection moulding at 130°C–135°C. The material swells on hydration, resulting in drug release at levels greater than 2 mg/day for 90 days. Johnson et al.[21] recently described a novel reservoir-type IVR consisting of end-sealed polyether urethane tubing filled with a tenofovir/glycerol/water semi-solid paste. Tenofovir release in excess of 10 mg/day was sustained for 90 days, whereas vaginal concentrations of the drug in sheep (10^6 ng/mL) were reported to be 1000× higher than that, which provided significant protection against HIV transmission in human clinical trials using tenofovir-loaded gel. Polyether urethane has also been utilised to deliver hydrophilic tenofovir disoproxil fumarate. Drug released *in vitro* after 24 h exhibited potent anti-HIV activity in TZM-Bl cell culture and ectocervical explant models of infection.[22]

The synthetic polyester, polycaprolactone (PCL), has been investigated extensively for production of biomedical implants and drug delivery devices in the form of films, micro- and nanoparticles.[23] We have previously shown that micro- or macro-porous matrices based on PCL are effective for controlling delivery of small drug molecules (e.g., steroids and anti-bacterials) and macromolecules such as enzymes with retained activity.[24–26] More recently, the potential utility of these materials was demonstrated for vaginal delivery of anti-bacterials (ciprofloxacin) and anti-fungal agents (miconazole) in the treatment of gonorrhea and candida.[27] In this study, we describe investigations of PCL matrices loaded with the hydrophilic antiviral, tenofovir (aqueous solubility 0.6 mg/mL), as part of an ongoing evaluation of the materials for the prevention and treatment of sexually transmitted infections. The *in vitro* release kinetics of tenofovir were determined in simulated vaginal fluid (SVF) and the antiviral activity of released drug was assessed using a luciferase gene reporter assay involving HeLa cell cultures infected with pseudo-typed HIV-1.

MATERIALS AND METHODS

Materials

Polycaprolactone (Mw 115,000 Da, Capa 650) was obtained from Solvay Interox (Warrington, UK). Tenofovir was supplied by Taizhou Crene Chempharm Company (Zhejiang, China). Sodium chloride, potassium hydroxide, calcium chloride, bovine serum albumin, glucose, glycerol, urea, lactic acid and acetic acid were purchased from Sigma–Aldrich (New South Wales, Australia). All solvents (acetone, methanol, ethanol, dichloromethane) were of analytical grade and were obtained from Sigma–Aldrich. SVF (pH 4.2) was prepared following the method of Owen and Katz[28] and consisted of 3.51 g NaCl; 1.40 g KOH; 0.222 g $Ca(OH)_2$; 0.018 g bovine serum albumin; 2.00 g lactic acid; 1.00 g acetic acid; 0.16 g glycerol; 0.40 g urea and 5.00 g glucose in 1 L of distilled water.

Preparation of Tenofovir-Loaded PCL Matrices

Polycaprolactone solution (15% or 20%, w/v) was prepared by dissolving the polymer in acetone at 50°C for 30 min. Tenofovir powder was dispersed in the PCL solution to produce loadings equivalent to 5%, 10% or 15% (w/w) of the PCL content. The resulting suspensions were poured into a polypropylene syringe body (3 mL) and rapidly cooled in ethanol at −80°C for 24 h to allow crystallisation of PCL. Following the hardening process, the matrices were removed from the moulds and immersed in methanol for 24 h to extract acetone. Residual solvents (acetone and methanol) in the matrices were evaporated under ambient conditions for 24 h prior to testing.

Determination of the Tenofovir Content of PCL Matrices

Sections (approximately 100 mg) were cut from each end of the matrix sample, weighed and dissolved in dichloromethane (2 mL). Precipitation of PCL was induced by adding 5 mL of SVF, followed by vortexing (Vibrax; IKA, Werke Staufen, Germany) at 1000 rpm overnight to allow dichloromethane to evaporate and the drug content to partition into the SVF phase. The residue was washed twice with SVF (10 mL) to extract residual drug and the washings were combined. Tenofovir concentrations in SVF were assayed using UV spectrophotometry at 260 nm and obtained by comparison with a calibration curve constructed using a series dilution of tenofovir in SVF. The linearity ranged from 3 to 20 μg/mL ($R^2 = 0.9996$). The measured drug loading (w/w) of the matrices was compared with the theoretical loading and expressed as incorporation efficiency (%). The experiment was conducted using triplicate samples.

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In Vitro Tenofovir Release

Cylindrical segments of tenfovir-loaded PCL matrices (approximately 15 mm in length, 0.15 g in weight) taken from the middle of the moulding were subjected to a release study. Both ends of the samples were coated with a thin film of PCL by dipping the ends of the samples in 5% (w/v) solution of PCL in acetone followed by drying in air at room temperature. The release study was performed on triplicate samples for each drug loading. Individual samples were placed in 10 mL SVF at 37°C, and the release media were generally collected and replaced with fresh SVF every day for a period of 30 days. The drug concentration in the release media was analysed using UV spectrophotometry at 260 nm, by comparison with a calibration curve constructed using a series dilution of tenofovir in SVF. Drug release behaviour was expressed as cumulative release (%) versus time. Separate release samples were stored at 0°C prior to anti-HIV testing.

Morphology of PCL Matrices

The morphology of the surface and interior of drug-free and tenofovir-loaded PCL matrices before and after drug release was examined using a JEOL JSM-6610LV scanning electron microscope (SEM) (Jeol Ltd., Tokyo, Japan). Specimens were mounted on SEM stubs using carbon tabs and sputter coated with platinum using an Eiko-Sputter coater automatic mounting press (Eiko Ltd., Tokyo, Japan) prior to examination in the SEM at a voltage of 15 kV.

Hardness Testing

Hardness testing was performed on samples of drug-free and drug-loaded matrices using a CT3 Texture Analyzer (Brookfield Engineering Laboratories Inc., Middleboro, MA). As-moulded cylinders were mounted horizontally and compressed locally at a speed of 0.1 mm/min over a distance of 2.0 mm using a flat cylindrical probe (TA39, 2 mm in diameter). The hardness (or indentation resistance) of each sample was calculated from the applied force measured at a depth of travel of 2 mm. A pEVA IVR (Nuvaring®; Schering-Plough Pty limited, New South Wales, Australia) was subjected to the same test procedure for comparison. The outer diameter of the IVR was 54 mm and the cross-sectional diameter was 4 mm.

Porosity Measurement

The porosity (ε) of PCL matrices was calculated based on the ratio between the apparent matrix density (ρ*) and the bulk density of PCL pellets ($\rho = 1.145$ g/cm^3).[29] The apparent density of the PCL matrix ρ* was defined as the weight of matrix (m) divided by the volume (V) which was calculated from its diameter and length. The porosity of the matrix $\varepsilon = 1 - \rho^*/\rho \times 100\%$.

Anti-HIV Activity Testing

The antiviral activity of tenofovir released from PCL matrices into SVF at 37°C against pseudo-typed HIV-1 was assessed using a luciferase reporter gene assay.

Exogenous Activation of the HIV *Promoter*

Lypoplysaccharide (LPS) is known to be a very potent stimulator of HIV-1 replication and is a major component of the outer membrane of gram-negative bacteria.[30,31] The presence of LPS or other activators of the HIV long terminal repeat (LTR) promoter in the SVF release medium would potentially compromise assay of luciferase expression by HIV. Thus, the possibility of unintentional activation of the HIV LTR promoter by release media was assessed prior to studies involving virus infection of cells. Plasmid pGL3-LTR,[32] which contains the HIV LTR promoter sequence, was used to report any unintentional stimulation by release media. Plasmid pRL-SV40 (Promega Corporation, New South Wales, Australia) was used to control for differences in transfection efficiencies. HeLa cells (ATCC number CCL-2) were cultured in RPMI 1640 (Life Technologies Australia Pty Ltd, Victoria, Australlia) supplemented with 10% newborn bovine serum (Life Technologies), 100 mg/mL streptomycin and 100 U/mL penicillin at 37°C and 5% CO_2. HeLa cells (10^5 cells/mL) were seeded in 24-well plates and transfected the following day with 100 ng pGL3-LTR and 50 ng pRL-SV40. Two wells received an additional plasmid [pcDNA3.1/Tat-FLAG (a gift from Monsef Benkirane, Institut Génétique Humaine, France)] as a positive control for LTR promoter activation. SVF and release media, which had contained samples of drug-free or tenofovir-loaded matrices, were filtered through a 0.22-μm membrane and added to the wells (100 μL/well). After 24 h of transfection, the HeLa cells were lysed in 100 μL Glo Lysis Buffer (Promega Corporation). The amount of luciferase activity [expressed in relative light units (RLU) in 20 μL of each lysate] was determined using the Steady-Glo Luciferase Assay System (Promega Corporation) for pGL3-LTR expression and BioLux Gaussia Luficerase Flex Assay Kit (New England Biolabs, MA) for pRL-SV40 expression, detecting with a luminescence-compatible spectrophotometer (Synergy H4 Hybrid Reader; Biotek, Victoria, Australia). The RLU of pGL3-LTR expression was normalised against the RLU of pRL-SV40 expression. The figure obtained for the samples was compared with that of the negative control (wells without pGL3-LTR or pRL-SV40) and the resulting ratio was defined as the relative-normalised luciferase (RNL) value.

HIV *Infection Assay*

HIV-1 virus pseudo-typed with VSV-G envelope glycoprotein was used to assess the antiviral activity of tenofovir released from PCL matrices because the virus can enter cells independently of CD4 cell surface receptors. The firefly luciferase reporter virus, pNL4–3.Luc.R$^-$E$^-$ (NIH Aids Reagent Program) is based on the HIV-1 proviral clone pNL4–3. Thus, a reduction of luciferase expression can be correlated with increased antiviral activity. HeLa cells (10^5 cells/mL) were seeded in 24-well plates to achieve confluence above 50% after 24 h. Aliquots (100 μL) of the SVF release medium collected at 7-day intervals, containing drug-free or tenofovir-loaded matrices, were filtered through a 0.22-μm membrane, and fivefold dilutions were added to each well. Cells exposed to the release media were incubated at 37°C for 24 h to allow tenofovir to enter the cells and metabolise to an active form.[33] The following day, HIV (10 ng of CAp24 equivalent per well) and hexadimethrine bromide (0.8 μg/mL, for enhancing HIV infection) were added. At 1 day post-infection, the culture was harvested, the cells were lysed in 100 μL Glo Lysis Buffer and the amount of luciferase activity (expressed in RLU) in 20 μL of each lysate was determined using the Steady-Glo Luciferase Assay System (Promega Corporation) and detecting with a luminescence-compatible spectrophotometer (Synergy H4 Hybrid Reader; Biotek).

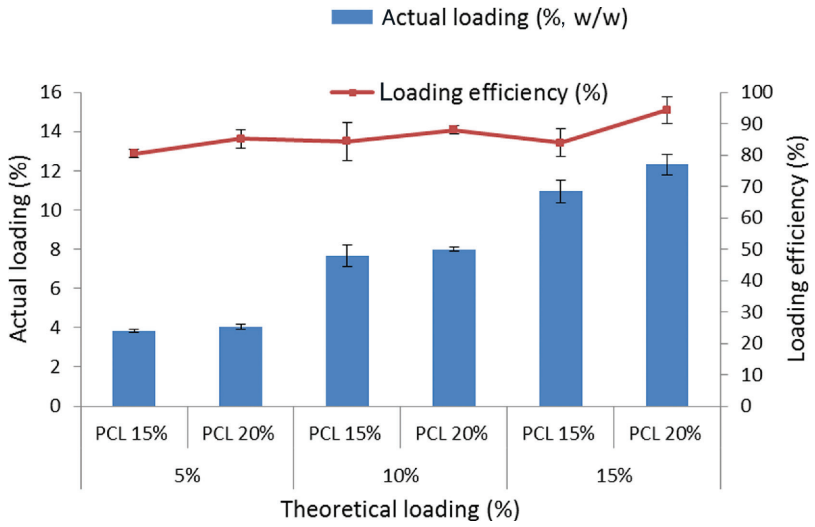

Figure 1. Actual and theoretical loading of tenofovir in PCL matrices (column) and drug incorporation efficiency (%) (line).

The percentage reduction in luciferase activity correlating with the antiviral activity of tenofovir was determined using Eq. (1):

$$\text{Reduction of RLU} = 1 - \frac{\text{RLU}_{\text{test}} - \text{RLU}_{\text{cell}}}{\text{RLU}_{\text{virus}} - \text{RLU}_{\text{cell}}} \quad (1)$$

where RLU_{test} is the RLU of wells containing infected HeLa cells and tenofovir released in SVF, $\text{RLU}_{\text{virus}}$ is the RLU of control wells containing infected HeLa cells and drug-free SVF and RLU_{cell} is the RLU of control wells containing uninfected HeLa cells.

The correlation between percentage reduction in RLU (virus inhibition) and the logarithm of tenofovir concentrations was investigated using Eq. (2).[34]

$$y = y_{\min} + \frac{y_{\max} - y_{\min}}{1 + 10\,(lg\,\text{IC}_{50} - lg\,X) \times k} \quad (2)$$

Where y is the reduction in RLU, X is the tenofovir concentration in the sample, IC_{50} is the concentration of tenofovir at which the percentage reduction of RLU is 50 and k is the slope of the line of RLU reduction versus tenofovir concentration.

IC_{50} and k were determined by plotting the reduction in RLU versus tenofovir standard concentrations using GraphPad Prism 5 (Graphpad Software, San Diego, California). Each concentration of tenofovir was tested in duplicate samples.

The expected reduction in RLU because of exposure of infected HeLa cells to tenofovir released from PCL matrices was calculated by substituting the concentrations of tenofovir in the release medium determined by UV analysis (In vitro Tenofovir release)) into Eq. (2). This value was subsequently compared with the actual percentage reduction in RLU measured for the corresponding well Eq. (1). The ratio between percentage reduction in RLU from Eq. (1) and Eq. (2) was defined as the relative anti-HIV activity (%) of released tenofovir.

Statistical Analysis. The data of diameter, density and porosity of the matrices were subjected to analysis of variance (ANOVA), and then the means were compared by Tukey's test (5%). All statistical analysis was performed with Statista10 (StatSoft Inc., Tulsa, Oklahoma).

RESULTS AND DISCUSSION

Drug Loading of Matrices

The loading of tenofovir in the PCL matrices and drug incorporation efficiency (%) are depicted in Figure 1. Matrices produced using 15% PCL solution resulted in actual tenofovir loadings of 3.8%, 7.7% and 11.0% (w/w), as compared with the theoretical loading of 5%, 10% and 15% (w/w), respectively, resulting in relatively high incorporation efficiencies of 81–84%. A significant increase in drug loading efficiency ($p < 0.005$) from 85 to 94% was achieved when matrices were prepared using 20% PCL solution. In this case, actual tenofovir loadings of 4.1, 8.0 and 12.3% were obtained compared with the theoretical content of 5, 10 and 15%, respectively. The high incorporation efficiency may be explained by the limited solubility of tenofovir in methanol (9.7 μg/mL), which limits partition of tenofovir during the solvent extraction phase of matrix production.

Matrix Morphology and Hardness

The diameter, density and porosity of tenofovir-loaded PCL matrices are presented in Table 1. The mean diameter of matrices prepared using 15% (w/v) PCL solutions tended to be lower than those produced using 20% solutions (6.7 mm compared with 7.3 mm), indicating greater shrinkage of the PCL phase during hardening. The density of matrices prepared from 15% PCL solutions was significantly lower than those prepared from 20% solutions ($p < 0.05$) and correlated with increasing matrix porosity. Drug loading had no significant effect on diameter, density or porosity of the matrices ($p > 0.05$).

Rapid cooling of PCL solutions in acetone containing dispersed particles of tenofovir results in uniform, flexible mouldings on drying which are free of cracks and voids in the surface and interior. SEM examination revealed the irregular porous morphology of matrices formed by crystallisation and hardening of the PCL phase. The surface of drug-free and tenofovir-loaded matrices exhibits a nodular morphology with pore dimensions of 3–5 and 5 –10 μm, respectively (Figs. 2a and 2b).

Table 1. Density and Porosity of PCL Matrices

PCL Solution Concentration (%)	Actual Tenofovir Loading (%)	Diameter (mm)	Density (g/cm^3)	Porosity (%)
15	3.8	6.8 (0.75)	0.29 (0.007)	75 (0.6)
	7.7	6.6 (0.72)	0.32 (0.022)	72 (0.2)
	11.0	6.7 (0.12)	0.30 (0.019)	74 (1.6)
20	4.1	7.2 (0.15)	0.34 (0.01)	71 (0.9)
	8.0	7.2 (0.71)	0.32 (0.01)	72 (0.9)
	12.3	7.5 (0.72)	0.32 (0.07)	72 (0.1)

Values in parentheses represent the standard deviation.

The flattened areas visible in Figure 2a are probably formed by contact of the material with the mould surface. The internal structure of drug-free and drug-loaded matrices (Figs. 2c and 2d) exhibited a more angular and elongated morphology. Particles of tenofovir (Fig. 2e) were not observed on the exterior but were detected in the interior of samples (arrowed in Fig. 2d).

Figure 3 shows hardness values for drug-free PCL matrices, tenofovir-loaded matrices and pEVA (Nuvaring®). Drug-free PCL matrices were fairly soft as reflected by the low indentation resistance of around 2500 –2700 mN/mm^2. The inclusion of increasing amounts of tenofovir particles in the PCL phase provides an increasing reinforcing effect, resulting in a slightly harder material compared with drug-free matrices. However, the hardness of the matrices is far lower than that of pEVA (Nuvaring®), which reaches 9280 mN/mm^2. Thus, PCL matrices could offer potential advantages of improved comfort *in situ* compared with conventional devices. An increase in tenofovir loading of the matrix from approximately 4%–12% resulted in an increase in indentation resistance of around 25% (Fig. 3). However, the effect of increasing the PCL solution concentration appears to have a greater influence. For example, the hardness of matrices loaded with around 4% tenofovir particles was increased by 60% from 2500 to 4000 mN/mm^2 on raising the solution concentration from 15% to 20%. This finding could simply be explained by increased molecular inter-connections in the more concentrated solution leading to a higher density of the PCL structural network (Table 1). The reduction in hardness for 7.7% tenofovir-loaded matrices produced using 15% PCL solution suggests that localisation of particles has occurred in the core of the moulding.

In Vitro Release Behaviour

The cumulative release profiles of tenofovir from PCL matrices in SVF at 37°C are presented in Figure 4 and provide an indication of delivery efficiency. Overall, the release behaviour followed a similar pattern. A small burst release phase of 4%–8% in day 1 was followed by a phase of gradual, low drug release from day 1 to day 8, suggesting that a zone of reduced drug concentration exists near the matrix surface. Rapid release occurred from day 9 to the end of the experiment at day 30. The two-phase release process may also be influenced by morphological factors. Initially, drug diffusion occurs predominantly through the 5–10 μm micro-porous structure inherent in the PCL phase. However, as drug release progresses, larger pores and channels are created by dissolution of drug particles, resulting in greater porosity and subsequently higher drug release as observed from day 9. Highly efficient drug delivery in

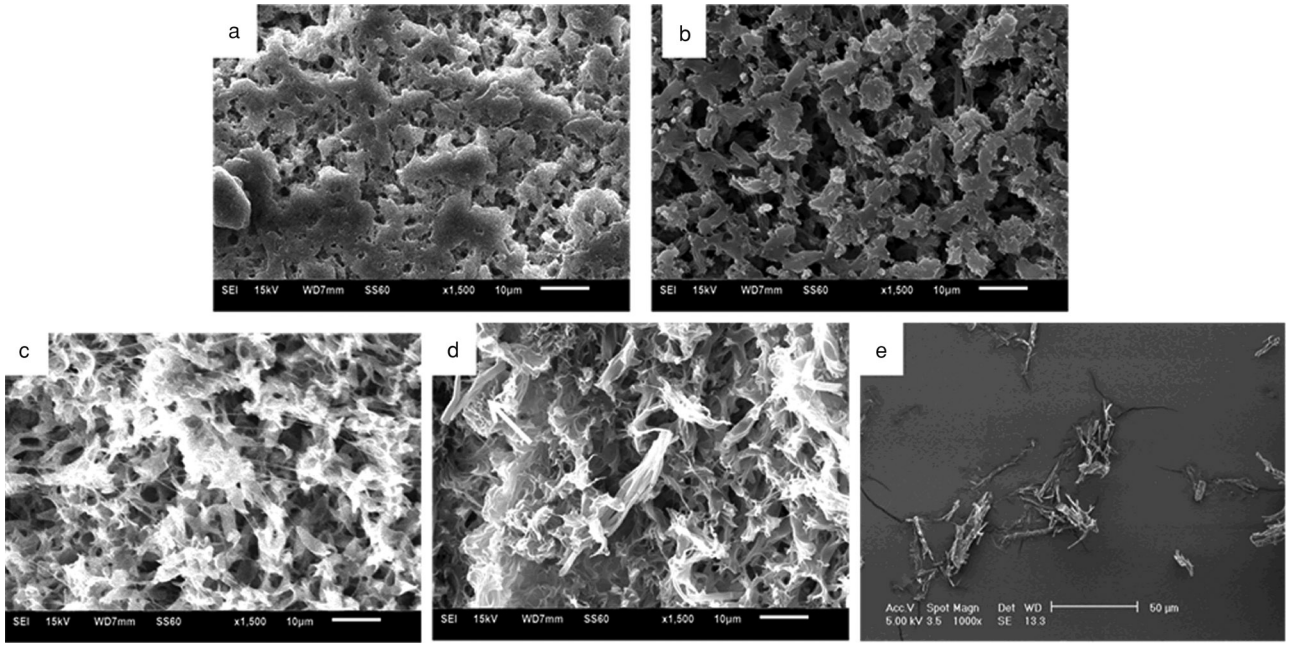

Figure 2. Scanning electron micrographs of PCL matrices before release testing: (a) external surface of drug-free PCL matrix, (b) external surface of 3.8% tenofovir-loaded matrix, (c) interior of drug-free PCL matrix, (d) interior of 3.8% tenofovir-loaded matrix and (e) tenofovir powder.

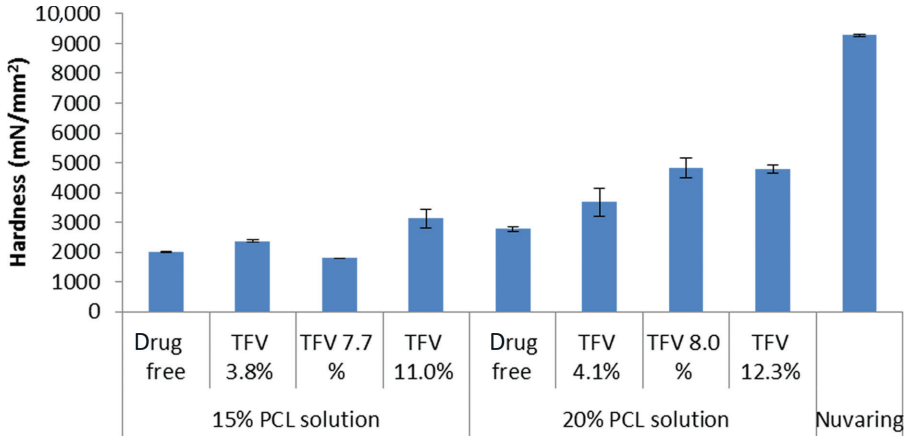

Figure 3. Hardness of drug-free PCL matrices, tenofovir (TFV)-loaded matrices and poly(ethylene vinyl acetate) (Nuvaring®).

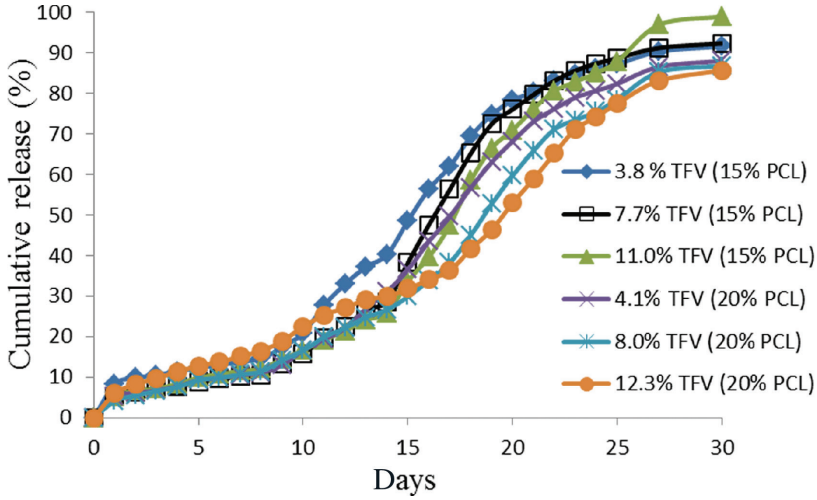

Figure 4. Cumulative release (%) of tenofovir (TFV) from PCL matrices in SVF at 37°C.

excess of 85% was also apparent, indicating the presence of an interconnected, fluid-filled pore-channel structure within the matrix, which facilitates drug dissolution and elution. After 30 days, matrices prepared using 15% PCL solution released from 91% to 99% of the initial tenofovir content, whereas samples prepared from 20% PCL solution released from 85% to 88%, probably reflecting the lower matrix porosity (Table 1). Figure 5 depicts the cumulative weight of tenofovir released versus time and provides an indication of the dose of tenofovir delivered into vaginal fluid over time. Examination of the data in Figure 5 suggests that drug concentration in the matrix is more influential than porosity factors in controlling drug release, for example drug loadings of 3.8% and 4.1% resulted in similar release rates of tenofovir despite a decrease in mean matrix porosity for 4.1% loaded samples (Table 1). The higher drug release with increasing drug content is conveniently explained by the steeper concentration gradient for Fickian diffusion but may also involve micro-cracking effects[35] which facilitate entry of release medium and dissolution of drug particles.

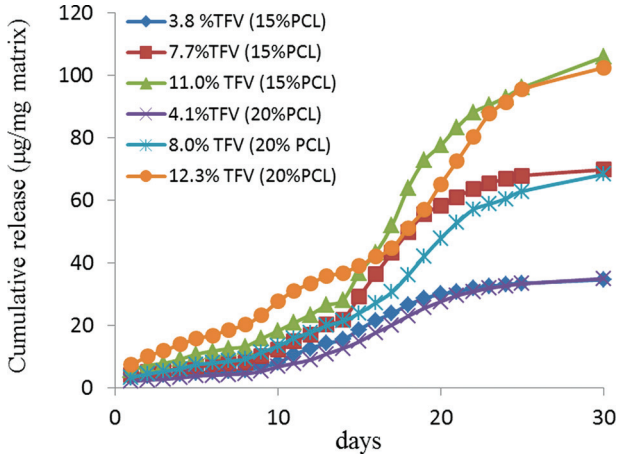

Figure 5. Cumulative release of tenofovir (TFV) (µg/mg matrix) from PCL matrices in SVF at 37°C.

Zero-order, first-order, Higuchi square root of time and Korsmeyer–Peppas models are most often applied to describe and predict the kinetics of drug release from matrix-type devices containing dispersed drug particles.[36] A curve fitting analysis conducted on the release profiles of tenofovir-loaded matrices is shown in Table 2. The release kinetics over 30 days were found to be described most satisfactorily by the first-order model with high correlation coefficients of 0.98 to 0.99. A value of 0.126 day^{-1} was obtained for the first-order release constant

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Table 2. Curve Fitting Analysis of Tenofovir Released from PCL Matrices in SVF at 37°C

			Zero-Order Kinetics		First-Order Kinetics		Higuchi Model		Korsmeyer–Peppas		
	PCL solution concentration (%)	Actual loading (%, w/w)	k_0	R^2	K_1	R^2	k_d	R^2	k	n	R^2
	15	3.8	0.283	0.952	0.1198	0.975	1.707	0.891	0.042	0.903	0.823
		7.7	0.360	0.922	0.1405	0.983	3.187	0.854	0.024	1.002	0.849
		11.0	0.456	0.934	0.1320	0.992	4.527	0.847	0.023	1.053	0.821
	20	4.1	0.528	0.923	0.1376	0.990	1.863	0.870	0.046	0.808	0.926
		8.0	0.557	0.927	0.1351	0.990	3.390	0.859	0.022	1.048	0.852
		12.3	0.603	0.913	0.1013	0.981	4.692	0.870	0.025	1.029	0.853
Phase 1 (day 1–8)	15	3.8	0.065	0.988	0.0323	0.956	0.242	0.994	0.089	0.258	0.991
		7.7	0.105	0.992	0.0537	0.960	0.387	0.990	0.055	0.329	0.982
		11.0	0.201	0.991	0.0414	0.963	0.737	0.978	0.048	0.428	0.975
	20	4.1	0.076	0.987	0.0599	0.937	0.279	0.969	0.047	0.446	0.944
		8.0	0.178	0.988	0.0568	0.965	0.660	0.989	0.053	0.393	0.954
		12.3	0.368	0.989	0.0502	0.967	1.367	0.998	0.067	0.489	0.999
Phase 2 (day 9–30)	15	3.8	0.343	0.974	0.051	0.935	2.241	0.946	0.004	1.826	0.985
		7.7	0.690	0.97	0.063	0.961	4.372	0.927	0.001	2.176	0.984
		11.0	0.958	0.967	0.060	0.980	6.342	0.936	0.002	1.997	0.978
	20	4.1	0.392	0.984	0.058	0.971	2.544	0.947	0.001	2.067	0.981
		8.0	0.697	0.970	0.061	0.971	4.686	0.941	0.002	2.002	0.995
		12.3	0.900	0.948	0.038	0.982	6.327	0.932	0.012	1.297	0.961

Zero order: $M = k_0 t$, k_0, zero-order release constant (μg/mg/day), M, amount of drug released at time (t).
First order: $M_t = M_0\ e^{-k_1 t}$, k_1, first-order release constant (day^{-1}), M_t, amount of drug released at time (t).
Higuchi: $M = K_d t^{0.5}$; K_d, release rate constant (μg/mg/$day^{0.5}$). M, amount of drug released at time (t).
Korsmeyer–Peppas: $M_t / M_\infty = kt^n$, M_t, the amount of drug release at time (t), M_∞, the total drug released over a long-time period, k, kinetic constant, n, release exponent.

(k_1). First-order release kinetics shows a linear dependence of the log of the concentration of drug released from the matrix versus time and will be governed by a complex relationship between several factors including drug content in the matrix, drug solubility, the rate of drug diffusion in fluid-filled pores of the matrix, porosity, pore structure and tortuosity and the rate of ingress of release medium.[37]

The kinetics of drug release from matrix-type devices containing dispersed drug particles, where release occurs through fluid-filled pores in the matrix, are frequently controlled by Fickian diffusion and often interpreted using the simplified Higuchi equation[36]

$$M = K_d\, t^{0.5}$$

where M is the amount of drug released in time (t) and K_d is a release rate constant.

A dependency of drug release is found on the square root of time, along with a decrease in drug release rate over time, because of drug depletion in the matrix and a corresponding increase in the diffusion path length. A curve fitting analysis conducted on the tenofovir release data obtained from day 1 to 8 (phase 1) and day 9 to 30 (phase 2) is shown in Table 2. The release kinetics were found to be described satisfactorily by the simplified Higuchi model for the initial phase only. The Korsmeyer–Peppas model was applied in a further attempt to correlate the release data with underlying controlling factors and to identify release kinetics arising from multiple phenomena

$$M_t/M_\infty = kt^n$$

where M_t is the amount of drug released at time t, M_∞ is the total amount of drug in the matrices, k is a kinetic constant characteristic of the drug/polymer system and n is an exponent which characterises the mechanism of drug release.[36]

Drug delivery from a cylindrical matrix is considered to be governed by Fickian diffusion when $n = 0.45$, whereas relaxation behaviour (zero-order release) is suggested when $n = 1$. Values of n between 0.5 and 1 indicate anomalous (non-Fickian) kinetics. When the release data were analysed using the Korsmeyer–Peppas model, the exponent value (n) was close to 0.45 (0.3–0.5) in phase 1, suggesting that drug release was governed mainly by Fickian diffusion. In phase 2, the high exponent values indicate that anomalous, non-Fickan kinetics are dominant.

The diffusion coefficient (D) of tenofovir in PCL matrices was subsequently calculated (assuming Fickian diffusion in phase 1) using the following relationship applied by Miyajima et al.[37] to describe drug release from cylindrical poly(L-lactic acid) matrices.

$$F = 4\left(Dt/\pi r^2\right)^{1/2}$$

where F is the fractional amount of drug released at time (t) and r is the radius of the cylindrical PCL matrix. A mean value of D_1 of 0.8×10^{-7} cm^2/s was obtained when day 1–8 release data were analyzed. This compares with figures of 0.3–$1.5 \times 10^{-7} cm^2/s$ obtained by Cost et al.[38] for tenofovir release from a 1% drug-loaded intra-vaginal gel prepared from hydroxymethylcellulose and carbomer 940P. Meanwhile, a higher average value of D_2 of 4.5×10^{-7} cm^2/s was obtained for the later stage of tenofovir release from PCL matrices (day 14 to 30)

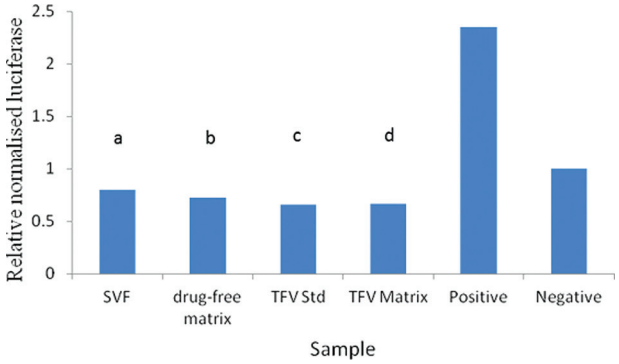

Figure 6. Determination of unintentional activation of the HIV LTR promoter in (a) SVF alone; (b) SVF which had contained drug-free PCL matrices; (c) tenofovir dissolved in SVF and (d) SVF which contained tenofovir-loaded PCL matrices. TFV, Tenofovir.

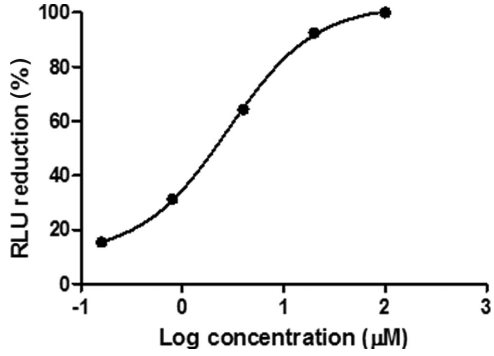

Figure 7. Inhibition of pseudo-typed HIV-1 activity by tenofovir standards in SVF.

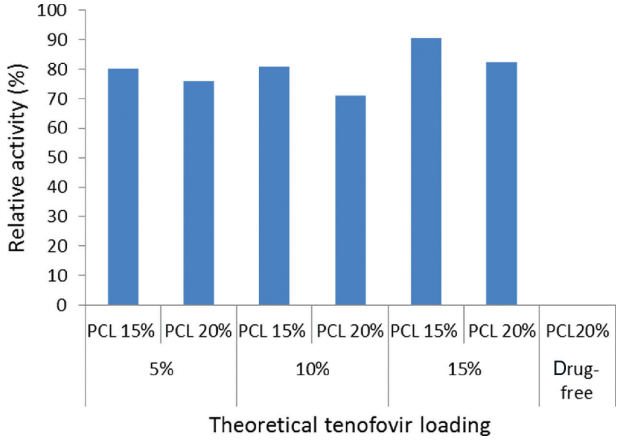

Figure 8. Activity of tenofovir released from PCL matrices into SVF at 37°C.

when analyzed using the Roseman–Higuchi model, which explores log fractional drug release versus time.[37]

$$(1-F)\ln(1-F)+F=\frac{4C_sD_2t}{Ar^2}$$

where C_s is the drug solubility in SVF, A is drug content in the matrix which far exceeds the drug solubility in the polymer.

The value of D_2 compares with figures of 4.9–6.7 $\times 10^{-7}$cm^2/s reported by Clark et al.[20] for tenofovir release from hydrated 21% drug-loaded polyether urethane matrices. Thus, tenofovir diffusivities in PCL matrices and polyether urethane devices are similar despite the known differences in materials, drug loading, release medium and modelling approach used to calculate D and the expected differences in pore structure, drug particle size and spatial distribution.

Anti-HIV Activity of Released Tenofovir

Release media could contain a contaminant, possibly with an endotoxin-like activity, which may stimulate HIV-1 gene expression[39] and thus could confound analysis of the antiviral activity of matrix release media. To address this possibility, we performed a standard trans-activation of HIV-1 gene expression assay to determine whether sterile SVF or SVF that had been incubated with matrices could unintentionally activate transcription from the HIV-1 LTR promoter. HeLa cells were transfected with a pGL3-LTR luciferase reporter plasmid alone or with a plasmid expressing Tat. Tat is a potent activator of the HIV-1 LTR promoter. To control for transfection efficiency, a control plasmid constitutively expressing Renilla luciferase was co-transfected in each experiment. After incubation for 16 h, the transfected cells were treated with release media samples and incubated for a further 24 h. Figure 6 shows the RNL values of samples screened for the unintentional activation of the HIV-1 LTR promoter. The RNL of the negative control, which was untransfected cells lacking any luciferase expression, denotes background levels of the luciferase assay. RNL levels for the positive control, which was cells expressing luciferase via Tat-mediated trans-activation of the LTR promoter, confirmed activation of luciferase gene expression in HeLa cells. Sterile SVF or SVF release medium, which was incubated with either tenofovir-loaded matrices or drug-free matrices, produced RNL values similar to the negative control, indicating that the release media contained no material that could unintentionally activate the HIV LTR promoter. These data indicate that the HIV LTR-luciferase system is appropriate for testing the antiviral activity of matrix release media.

Use of the luciferase gene reporter assay of HIV-1 pseudovirus viability[40] has become routine in screening for inhibitory activity. The VSV-G envelope glycoprotein improves the stability and infectivity of the virus population, facilitating testing and improving the sensitivity and reproducibility of the assay.[41] Figure 7 shows the inhibitory activity of tenofovir standards prepared in SVF, fitted to a log dose-response curve (R^2= 0.9998), which yielded an IC_{50} value (the concentration of tenofovir required for 50% virus inhibition) of 2.38 μM. A similar IC_{50} of 2.2 μM was obtained by Van den Berg et al.[42] for tenofovir tested on the same pseudo-type virus with VSV-G. IC_{50} values of 1–6 μM have been reported for tenofovir against wild-type HIV-1-infected MT2 or MT4 cells.[43] The effect of incubation time of tenofovir with HeLa cells on antiviral activity was not found to be significantly different between 24 and 48 h (data not shown). Therefore, 24 h exposure is sufficient for transforming the drug to its active form by cellular metabolism. Relative antiviral activity was expressed as the actual inhibitive activity of tenofovir released from matrices compared with the inhibitive activity of corresponding concentrations of non-formulated drug. Tenofovir released from PCL matrices into SVF at 37°C collected at 7-day intervals exhibited high relative activity, ranging from 70% to 90% (Fig. 8). Neither

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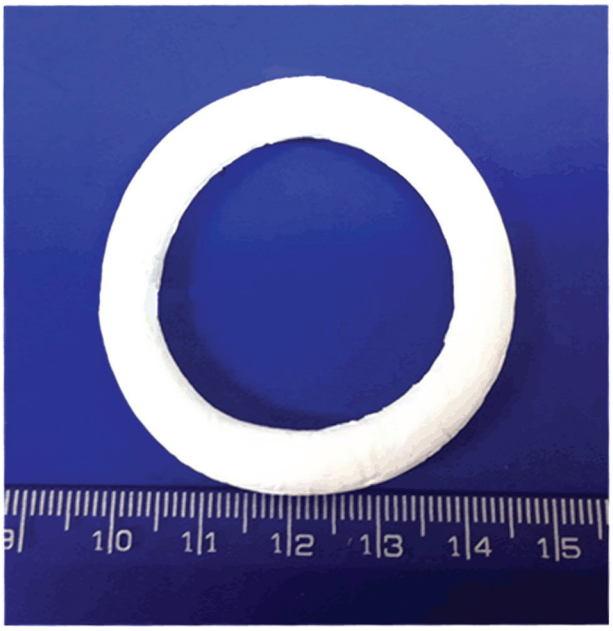

Figure 9. Polycaprolactone IVR (48 mm outer diameter, 7 mm cross-sectional diameter) produced using a split mould and the rapid cooling technique.

the loading of tenofovir in the matrices nor the PCL solution concentration used for matrix production had a statistically significant effect on the relative antiviral activity. In addition, SVF and release medium, which had contained drug-free matrices, did not give rise to anti-HIV activity.

Besides confirming high levels of *in vitro* antiviral activity, the predicted concentrations of tenofovir, which would be released from a PCL intra-vaginal ring (Fig. 9) into vaginal fluid, exceeded the reported IC_{50} value for HIV-1 and the concentration of 1000 ng/mL shown to provide significant protection against HIV transmission in the CAPRISA 004 clinical trial.[21] This assessment is based on the linear length of an intra-vaginal ring of 150 mm (outer diameter 58 mm, inner diameter 38 mm) and weight (1.5 g) being 10 times that of the studied matrices (15 mm, 0.15 g). The minimum release amount of 41 μg tenofovir/day (Fig. 10) corresponds to a drug release rate from a PCL IVR, in excess of 400 μg/day. Assuming that the *in vitro* release rate from PCL matrices is similar to the *in vivo* release rate from a PCL vaginal ring and a maximum vaginal fluid turnover rate of 8 mL/day,[15,28] then the expected minimum concentration of tenofovir in vaginal fluid is approximately 207μM or 50 μg/mL. These estimates do not take into account the complex variations in vaginal fluid volume and biochemical composition over time or the possibility of systemic uptake of drugs. However, the released drug concentrations are much higher (by a factor of 35–200) than the reported IC_{50} of tenofovir against HIV-1 (1–6 μM) and a factor of 50, higher than clinically protective tenofovir concentrations.

CONCLUSION

Polycaprolactone matrices can be loaded with the hydrophilic tenofovir, to a level of 12% (w/w) with high entrapment efficiency of up to 90%. Gradual release of over 85% of the drug content occurs in SVF over 30 days with retention of antiviral activity in excess of 70% of the activity of non-formulated drug. Moreover, the predicted *in vivo* release concentrations from a PCL IVR in vaginal fluid are higher than the IC_{50} against HIV-1 by a factor of more than 30 and higher than clinically protective concentrations by a factor of 50. These findings demonstrate the potential for providing sustained delivery of tenofovir from PCL matrices in the form of an intra-vaginal device for control of heterosexual HIV transmission.

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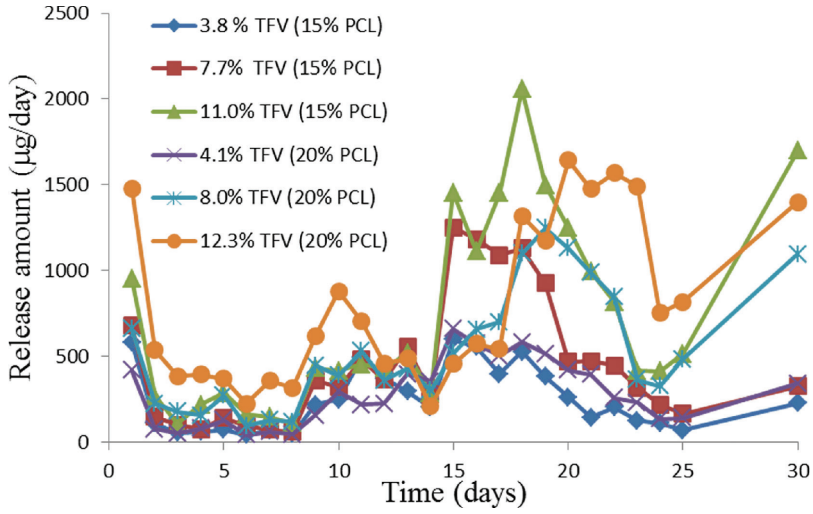

Figure 10. Amount of tenofovir (TFV) release (μg/day) from PCL matrices in SVF at 37°C.

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