# ANTIRETROVIRAL ELUTING INTRAVAGINAL RINGS TO PREVENT THE SEXUAL TRANSMISSION OF HIV

by

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# ABSTRACT

Female-controlled preventative technologies are being developed to decrease HIV sexual transmission rates in resource poor regions of the world where the pandemic is most prevalent. Intravaginal rings (IVRs) comprise extended duration vaginal drug delivery vehicles which may provide sustained release of antiretrovirals at local inhibitory concentrations to prevent initial HIV infection during coitus. Few IVR formulations have been researched for HIV prophylaxis although numerous antiretrovirals are excellent candidates. Poor progress is due in part to limitations of conventional IVR technology in delivering antiretrovirals with diverse physiochemical properties and dosing requirements. Furthermore, there is limited understanding of drug vaginal pharmacokinetics and potential toxicological effects on the local environment. Consequently, in this dissertation several candidate antiretrovirals were formulated in new IVR platforms and animal models were developed to characterize the IVR formulations *in vivo*.

In the first part of the dissertation, polyurethane IVRs were designed that delivered the potent and dual-acting pyrimidinedione congeners for up to one month. The pyrimidinediones attained concentrations throughout the nonhuman primate vaginal tract that were expected to be inhibitory against HIV, with no observed detrimental effects to the vaginal environment. In the second part of the dissertation, an IVR was developed to

simultaneously deliver dapivirine and tenofovir which possess contrasting hydrophilicity and differing mechanisms of action against HIV. A two segment ring design was utilized which independently optimized tenofovir and dapivirine release by using compositionally different polyurethanes. In the final two parts of the dissertation, a hydrophilic polyurethane reservoir IVR was engineered and tested in a new sheep model, whereby tenofovir vaginal concentrations from the IVR were similar to the clinically effective tenofovir vaginal gel but for 90 day duration. The tunable IVR platform allowed for achievement of desired drug release rates and ring mechanical stiffness which were time-independent. No major toxicological effects were observed in sheep, and extensive IVR *in vitro* characterization was performed to ensure that a chemically and physically stabilized product was achieved.

The work reported herein describes the design and characterization of antiretroviral-eluting intravaginal rings which each hold promise as preventative technologies to prevent the sexual transmission of HIV.

To my best friend and companion, Dorthyann

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# NOMENCLATURE

| | |
|---|---|
| HIV | Human immunodeficiency virus |
| STI | Sexually transmitted infection |
| RT | Reverse transcriptase |
| RTI | Reverse transcriptase inhibitor |
| NtRTI | Nucleotide analogue reverse transcriptase inhibitor |
| NNRTI | Nonnucleoside reverse transcriptase inhibitor |
| HRT | Hormone replacement therapy |
| IVR | Intravaginal ring |
| API | Active pharmaceutical ingredient |
| ARV | Antiretroviral |
| HME | Hot melt extrusion |
| PYD | Pyrimidinedione |
| PYD1 | IQP-0528 pyrimidinedione |
| PYD2 | IQP-0532 pyrimidinedione |
| TFV | Tenofovir |
| DPV | Dapivirine |
| PU | Polyurethane |
| NWS-PU | Nonwater swellable polyurethane |

| | |
|---|---|
| WS-PU | Water swellable polyurethane |
| HPU | Hydrophilic polyurethane |
| HPU-35 | HP-60D-35 Tecophilic polyurethane |
| HPU-60 | HP-60D-60 Tecophilic polyurethane |
| HPU-B1 | 75/25 HP-60D-60/HP-93A-100 Tecophilic polyurethane |
| HPU-B2 | 50/50 HP-60D-60/HP-93A-100 Tecophilic polyurethane |
| HPU-B3 | 25/75 HP-60D-60/HP-93A-100 Tecophilic polyurethane |
| EVA | Polyethylene-co-vinyl acetate |
| EVA-R | NuvaRing |
| PTMO | Polytetramethylene oxide |
| $H_{12}$MDI | 4,4'-dicyclohexylmethane diisocyanate |
| PEO | Polyethylene oxide |
| HPLC | High-performance liquid chromatography |
| LC/MS | Liquid chromatography-tandem mass spectrometry |
| DSC | Differential scanning calorimetry |
| TGA | Thermogravimetric analysis |
| WAXS | Wide angle X-ray scattering |
| SAXS | Small angle X-ray scattering |
| FTIR | Fourier transform infrared spectroscopy |
| LOQ | Limit of quantification |
| LLOQ | Lower limit of quantification |
| PK | Pharmacokinetic |

| | |
|---|---|
| AUC | Area under the curve |
| $T_{max}$ | Time of maximum observed concentration |
| $C_{max}$ | Maximum observed concentration |
| C log P | Calculated log partition coefficient |
| $EC_{50}$ | 50% effective concentration |
| $IC_{50}$ | 50% inhibitory concentration |
| CPE | Cytopathic effect |
| SFU | Syncytium-forming unit |
| AZT | Zidovudine |
| XTT | 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide |
| H&E | Haematoxylin and eosin |
| IFN | Interferon |
| IL | Interleukin |
| TNF | Tumor necrosis factor |
| MIP | Macrophage inflammatory protein |
| G-CSF | Granulocyte colony-stimulating factor |
| DAPI | 4',6-diamidino-2-phenylindole |
| PBS | Phosphate-buffered saline |
| DMA | Dimethylacetamide |
| DCM | Dichloromethane |
| THF | Tetrahydrofuran |

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# CHAPTER 1

# INTRODUCTION

This chapter includes excerpts from the manuscript:

STATE OF THE ART IN INTRAVAGINAL RING TECHNOLOGY FOR TOPICAL PROPHYLAXIS OF HIV INFECTION

Patrick F. Kiser, Todd J. Johnson, Justin T. Clark

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## 1.1 Vaginal Drug Delivery

Although the medical device and combination drug-device fields are often perceived as relatively new, intravaginal insertion of devices and therapeutics have existed for centuries with the primary objective of preventing conception (68). Intravaginal drug dosing, while less commonly used compared to oral or transdermal routes of administration, can offer certain advantages. Clearly, women-specific local indications such as vaginal infections (vaginitis) are excellent candidates for vaginal drug delivery. Less known, however, is that many small molecules including hormonal compounds demonstrate high vaginal absorption and thus can achieve effective systemic concentrations for indications including contraception and hormone replacement therapy (HRT) for post-menopausal women. Vaginal drug delivery is also advantageous as it is non-invasive, may be self-administered, avoids first-pass hepatic drug metabolism, often demonstrates high bioavailability with low fluctuation in systemic concentrations (i.e., peaks and troughs), and when intended for local delivery often shows less systemic side effects since a significantly lower dose achieves effectiveness (2, 101). Potential drawbacks of intravaginal drug delivery will be discussed in greater detail below and vary with the type of dosage form, but may include interference with coitus, cultural taboos associated with touching of the reproductive anatomy, and undesirable product attributes such as excess vaginal wetness or leakage.

## 1.2 HIV and Microbicides

Virtually all currently marketed vaginal drug products are intended to treat or prevent unwanted pregnancy, menopausal-related symptoms, and vaginal inflammation

due to infection (vaginitis) (100). However, recently there has been considerable effort to expand indications including uterine fibroids and cancer, diabetes, and prevention of diseases via vaccine or chemoprophylactic delivery – primarily sexually transmitted infections including the Human Immunodeficiency Virus (HIV) (3, 8, 28, 34, 40). Although HIV infects both men and women, a strong rationale exists for developing HIV prophylactic intravaginal dosage forms; 22 of the world's 33 million HIV-infected individuals reside in sub-Saharan Africa, where HIV incidence on average is 1.3 times higher in women than men and an even higher disparity is observed with young adults aged 15-24 (82). The startlingly high HIV incidence in sub-Saharan African young women - up to 20% in some regions (82) - is poorly understood and likely complicated by many intertwined biological and social factors (16, 53, 65). Vaccine development has been the focal point of efforts to prevent the spread of HIV since it was discovered in the early 1980s, yet to-date only one clinical trial has shown any significant protective effect against HIV (31). To protect this high risk, sizeable female population against HIV sexual transmission in the absence of an efficacious vaccine, women-controlled prophylactic vaginal products termed microbicides have been under development for approximately two decades (73).

Thus far, vaginal gel dosage forms have been the only microbicides clinically evaluated for HIV prophylactic effectiveness, in part due to their formulation simplicity and low production cost. The first microbicide clinical trial was conducted in 2002 with a Nonoxynol-9 gel, a nonionic surfactant which has been used for years as a contraceptive spermicide and was more recently found to inactivate HIV by disrupting its envelope. Although *in vitro* antiviral results were promising, limited safety data was generated prior

to efficacy studies in women, where Nonoxynol-9 exposure increased the rate of HIV infection in women who frequently applied the product (84). Similarly, the surfactant C31G, composed of alkyl dimethyl glycine and alkyl dimethyl amine oxide, did not prevent HIV infection and may have increased the incidence of adverse events in women (25, 63). The broad consensus from these early microbicide clinical trials was that these surfactants possess relatively low potency against HIV and harm the protective vaginal mucosa due to their nonspecific mechanism of action, allowing passage of virus to underlying immune cells and/or recruiting susceptible immune cells via an inflammatory response. Polyanions were also evaluated for prophylactic HIV effectiveness, but fared no better with cellulose sulfate demonstrating more HIV infections in the active versus placebo arm (83). Carrageenan-based gels PRO2000 and Carraguard showed no harm but were not effective (50, 75). In the Carraguard trial, gel applicators were examined post-use for evidence of user adherence, and it was discovered that women were not using the gel as instructed and were over-reporting adherence which was disturbingly low at 42% on average. More recently, the CAPRISA 004 trial evaluated the reverse transcriptase inhibitor (RTI) tenofovir formulated at 1 wt% in a vaginal gel (the prodrug form of tenofovir is already taken orally as part of highly active antiretroviral therapy for HIV-infected individuals). Although adherence to the gel dosage regimen was still low (less than 75% on average), the product demonstrated 39% overall effectiveness to become the first clinically effective microbicide and validating RTIs for microbicide applications (1). However, the follow-on VOICE trial tested the same tenofovir gel formulation and found no effectiveness, albeit with a different dosing regimen (87). Altogether, two major conclusions have been made from the microbicide trials performed to-date: First,

targeting specific steps in the HIV replication cycle with highly active and target-specific compounds will improve effectiveness over nonspecific and low activity compounds due to improved safety and specificity. Second, delivering these compounds from dosage forms that provide controlled, sustained inhibitory vaginal concentrations and potentially increased user adherence will improve effectiveness over vaginal gels which have demonstrated short duration drug pharmacokinetics and poor user adherence (32, 87).

### 1.3 Intravaginal Rings as Microbicides

Due to the relative infancy of the microbicide field, microbicide formulations have typically leveraged many of the older vaginal drug delivery platforms developed for contraception, HRT, and vaginal infections. To date, intravaginal drug delivery vehicles primarily comprise gels and rings although newer and/or less utilized dosage forms include capsules, creams, foams, films, pessaries, sponges, suppositories, diaphragms, tablets, and tampons (100). Excluding intravaginal rings (IVRs), all of the above dosage forms may need to be frequently applied due to their relatively short therapeutically effective duration (hours to days), often require dosing before or after sex (coitally dependent), and typically provide dynamic vaginal pharmacokinetics. In contrast, IVRs are capable of weeks to months duration and can provide controlled drug release kinetics to more consistently maintain desired vaginal drug concentrations. Furthermore, IVRs possess favorable user attributes such as the potential for discreet use without interference or detection during sex, allowance for sexual spontaneity due to their long duration, and avoidance of product messiness or leakiness. Although not proven, microbicide IVRs are expected to improve both vaginal pharmacokinetics and user adherence over the

commonly used gel dosage form and are increasingly desired by the HIV prevention field in the search for a highly effective HIV prophylactic product (29, 86, 87).

Torus shaped IVRs are prepared from "rubbery" polymers that deform elastically when pinched into an oval or figure-eight shape. The use of intravaginal elastomeric ring-shaped devices to deliver active pharmaceutical ingredients (APIs) is now over four decades old (24, 38, 47, 51, 101). It is not widely known that the contraceptive IVR was one of the first controlled release devices studied in the 1970s at the dawn of modern drug delivery technology (6). These revolutionary drug delivery devices were the first to take advantage of the new mechanistic understanding of controlled drug release from solid implants (9-12, 72). When the IVR is placed in the vaginal lumen, the drug concentration initially is homogeneous throughout the IVR, but immediately upon contact with vaginal tissue a spatial concentration gradient ensues. The drug present on the ring surface (at the polymer/tissue interface) is the first to diffuse from the IVR into the contacting tissue, transiting through a thin conducting layer of vaginal fluid or directly into tissue (Figure 1.1). The rate of drug release is interdependent on a number of factors including the solubility of the drug in the elastomer, the diffusion coefficient of the drug in the elastomer, the solubility of the drug in vaginal fluid, the volume of the vaginal fluid, the partition coefficient of the drug between the IVR and the vaginal fluid and tissue, the rate of diffusion and elimination of the drug through the vaginal tissue, and the rate of anterior to posterior advection of the vaginal fluid. To date, the contribution of each effect to vaginal pharmacokinetics and biodistribution is poorly understood.

## 1.4 IVR Manufacturing

Four of the five commercial IVRs available to women are based on the silicone technology with indications for contraception (Progering® and Fertiring®) and HRT (Femring® and Estring®) (6, 20). Silicone IVR manufacturing is strictly limited to reaction injection molding wherein silicone elastomer base, cross-linking catalyst, and drug are mixed and subsequently injected under high pressure into a mold where the silicone addition-cure or condensation-cure reaction occurs under elevated temperature (48). The most recent commercially available IVR, marketed in 2002, is the dual hormone contraceptive IVR NuvaRing® (88-91), which was one of the first marketed thermoplastic hot melt extruded combination drug-device products. Polymers employed for microbicide IVR applications similarly include thermoset silicones (46, 49, 100), thermoplastic ethylene-co-vinyl acetate (EVA) (43), and more recently a variety of thermoplastic polyurethanes (14, 33, 39). Hot melt extrusion (HME) (30) along with injection molding (103) are two common thermoplastic processing methods that can be used to fabricate IVRs. HME is a continuous process whereby a screw or screws forces a molten material through an opening in an enclosed barrel under elevated temperature and pressure. Depending on the application, twin-screw extruders may be used to facilitate mixing of additives (i.e., excipients or API) or a single screw extruder may be used to provide more precise pressure and tighter control of shaped-product dimensions. Although HME has been used for decades in the plastics and medical device industries, its potential has only recently begun to be realized in the pharmaceutical industry (23). Over the last decade the number of patents, publications, and marketed pharmaceutical products has exponentially increased (67). There are several advantages of HME for

pharmaceutical and combination drug-device products including simultaneous API/carrier compounding and shaping, increased API bioavailability due to formation of a solid state solution (molecularly dissolved API), solvent-free processing to negate use of volatile and/or toxic solvents, and potentially reduced manufacturing costs since it is a continuous process in contrast to conventional pharmaceutical batch-processing (18). Furthermore, due in part to its extensive development in the plastics and medical device industries, HME is a highly controlled and monitored process that enables quality by design and process analytical technology - concepts strongly favored by the Food and Drug Administration (67).

HME is an extremely versatile process and a variety of shaped and multilayered products may be made depending on equipment and accessory configuration. For example, the reservoir IVR NuvaRing® is made via coaxial extrusion which involves simultaneously using two extruders to feed a drug-loaded EVA core and an EVA rate-controlling membrane into a crosshead, resulting in a continuously extruded layered reservoir type product. Two significant drawbacks of HME for pharmaceutical applications are API chemical and physical stability; APIs that are thermally labile may be poor HME candidates although steps can be taken to minimize the extent of thermal degradation. Also, APIs that are formulated in the polymeric carrier above their maximum solubility at storage temperature can achieve API supersaturation during the elevated processing temperatures, resulting in a thermodynamically physically unstable system where API may bulk- or surface-crystallize during storage (7, 14, 90). Therefore, chemical and physical API stability must be considered in the potential development of a HME drug product.

### 1.5 IVR Design and Classification

IVRs are generally categorized as either matrix or reservoir type and offer different advantages. A matrix IVR contains drug which is homogenously dissolved or dispersed throughout the entirety of the elastomeric polymer, whereas a reservoir IVR typically utilizes a drug loaded polymeric core surrounded by a thin layer of polymer of the same or different composition (termed a rate-controlling membrane) to impede and thus regulate drug diffusion (66) (Figure 1.2). Matrix IVRs, owing to their simpler design and construction, are often cheaper to fabricate yet typically provide time-dependent release kinetics proportional to the square root of time with less flexibility in modulating the release kinetics (46). Conversely, reservoir IVRs are generally more costly to fabricate but offer near time-independent drug release kinetics and easier modulation of the release rate (91). The literature provides general equations describing drug release from matrix- and reservoir-type drug delivery devices (Equation 1.1 and 1.2, respectively) based on the Higuchi equation (26):

$$Q = [(2A - C)CDt]^{0.5} \qquad \text{Equation (1.1)}$$

$$Q = \frac{DCt}{\mathrm{h}} \qquad \text{Equation (1.2)}$$

where $Q$ is the cumulative amount of drug released per unit surface area, $A$ is the drug loading per unit volume, $C$ is the solubility of drug in the polymer per unit volume, $D$ is the diffusion coefficient in the polymer, $t$ is time, and $h$ is the rate controlling membrane thickness (for reservoir devices, $C$ and $D$ refer to the solubility and diffusion coefficient

in the outer polymer). An additional consideration beyond the desired drug release profile is the drug release rate; reservoir IVRs of a given polymer will typically demonstrate decreased release rates at early time compared to matrix IVRs due to the rate-controlling membrane which impedes drug diffusion (56). Therefore, selection of a matrix-type or reservoir-type IVR design will depend on the clinical needs and which drug release specifications are most critical to achieve. For example, delivery of contraceptive hormones requires low but consistent daily release rates (i.e., micrograms per day) due to their relatively narrow therapeutic window and high activity; therefore reservoir IVRs are more suitable than matrix IVRs in this instance. An interesting exception to IVR classification exists when matrix IVRs demonstrate time-independent drug release. In the case termed partition controlled release, the drug demonstrates elevated solubility in the polymer and significantly lower solubility in the vaginal fluid and furthermore is formulated at high weight percent in the polymer with a fairly large polymer diffusion coefficient (9). If these conditions are satisfied, time-independent release has been observed since dissolution into the vaginal fluid is the rate limiting step, in contrast to the more typical matrix controlled release discussed above where diffusion through the polymer to the IVR surface is the rate limiting step (69).

Although the primary function of an IVR is to deliver therapeutics to the vaginal tract, IVRs must also possess certain dimensions and mechanical properties in order to be inserted and retained in the vaginal tract - both from an ease-of-use and device functionality perspective. Vaginal rings should exhibit an elastic nature since the IVR must be compressed to be inserted, yet have sufficient recoil force once in place so as to be retained in the vaginal tract. A force balance thus exists between the elastic recoil of

the IVR and the musculature of the vaginal wall, with ring dimensions and material elastic modulus determining the final conformation and retention of the IVR. Marketed vaginal ring outer diameters and cross-sectional diameters range from 54 to 58 and 4 to 9 mm, respectively (2). The four marketed silicone rings (Estring®, Femring®, Progering®, and Fertiring®) possess a significantly lower elastic modulus than the marketed EVA ring (NuvaRing®). As a result, the silicone rings encompass the larger dimensions to increase ring rigidity whereas the EVA ring is the smallest with a 54 by 4 mm outer diameter and cross-sectional diameter, respectively. To arrive at these finalized ring dimensions during product development, several clinical studies evaluated silicone or EVA rings of various cross-sectional diameters and elastic moduli in women who used them for extended durations up to a year, and user acceptability, functionality, and/or effects to the vaginal epithelium were monitored. It was discovered that rings with relatively lower cross-sectional diameters (resulting in a mechanically softer ring) had a higher rate of vaginal expulsion or ring movement due to their lower elastic recoil force (41, 70). However, if the ring elastic recoil force was too great, there was some evidence that users had greater difficulty inserting the ring and that the ring may have caused trauma to the vaginal epithelium (96). Logically there exists an optimum range of IVR stiffness keeping in mind that there will be a wide range of vaginal shapes and sizes among the female user population (60-62). In one particularly large clinical study, four rings of wide-ranging stiffness were evaluated and all were observed to provide no vaginal trauma compared to the naïve arm (27). Since there is no quantitative model to determine the ideal mechanical properties of a vaginal ring, early microbicide IVR development may benefit by demonstrating similar ring stiffness to the above mentioned marketed products

which have exhibited high user acceptability and safety in large user populations. Also, a mechanical model relating the force required for the point compression of thin elastic rings may be useful for IVR research to better understand the influence of variables such as ring dimensions and elastic modulus on ring stiffness (Equation 1.3) (37).

$$F = \frac{\pi^2 E {r_o}^4}{(\pi^2 - 8)(R - r_o)^3} Y \qquad \text{Equation (1.3)}$$

where $F$ is force, $E$ is the elastic modulus, $r_o$ is the cross-sectional outer radius, $R$ is the ring outer radius, and $Y$ is the reduction in ring outer diameter. The fourth-power force dependence yet third-power volume dependence on the cross-sectional ring diameter (assuming a fixed ring outer diameter) allows for flexibility when designing an IVR. Also, IVR compression force is linearly related to the elastic modulus of the IVR matrix, which can be affected by the class or grade of polymer used and by incorporation of drugs or excipients. Although this mechanical model ignores polymer viscoelasticity and assumes linear stress-strain behavior, which is invalid for high ring deformations such as during ring insertion where polymer, the model can still be a useful tool for ring design, especially when considering small ring deformations such as those seen in a magnetic resonance imaging assessment of NuvaRing® retention in the female vaginal tract (5).

## 1.6 Acceptability and Adherence

In the context of microbicides, the terms "acceptability" and "adherence" are often misused interchangeably and therefore should be defined and distinguished. Acceptability refers to the extent to which a product is desirable to the target user population while

adherence refers to the extent to which users obey specified dosing instructions. Although acceptability and adherence will often correlate, the specific relationship between the two has not been well defined in the context of microbicides. Clearly, however, an efficacious microbicide product must be desirable to women (i.e., possess high acceptability) or the product will not be used (i.e., demonstrate low adherence) and its real world effectiveness will be limited (52). Few microbicide IVR acceptability studies have been performed but it is hypothesized that microbicide IVR acceptability will be similar to contraceptive IVRs, which have been extensively evaluated. In a multinational clinical trial, 66% of the women initially preferred oral contraceptives but after NuvaRing® use 81% preferred the vaginal ring (57). The change in contraceptive preference was mainly attributed to ease of use and not having to remember to take a pill each day, and overall acceptability of the IVR was approximately 96% (71). However, an important consideration is that IVR acceptability in developed countries - where NuvaRing® has been well studied - may be different than in developing countries where most microbicide IVRs will be used and where women's perceptions and cultural practices will be diverse. A study in Brazil concluded that women found IVRs to be more acceptable than other microbicide dosage forms such as gels (35), with long duration and spontaneity being offered as the primary reason. Another relevant study for the microbicide field examined African women sex workers' acceptability of IVRs for HIV prevention (76). Most women favored an IVR over a gel due to its long duration and covert use - although women were concerned that men might detect the ring. Women's opinions are likely coupled to men's opinion in the many patriarchal societies in the developing world; therefore, men's concerns and opinions on IVRs should not be discounted. In the study, men's primary concerns were

feeling the ring during intercourse, yet overall thought it more acceptable than a gel that may give undesired vaginal lubrication. Since both sexes were concerned about the possibility of male contact with the ring during coitus, the study suggests that IVR dimensions should be minimized to decrease the probability of this event. Most recently, an extensive acceptability study was performed where 157 young African women inserted a placebo silicone ring for 12 weeks (86). In general, women found the IVR favorable and were attracted by its potential for discreetness, noninterference with coitus, and continuous use capability which could allow for spontaneity or protection against HIV in the case of rape. 67% of female participants considered it important for their male partner to not detect the ring during intercourse, and 63% and 13% feared that their partner may become angry or physically abusive, respectively, if the ring was discovered. It is worth noting that both African acceptability studies evaluated silicone IVRs with roughly twice the diameter of the contraceptive NuvaRing®. Although the majority of men did not feel the ring during intercourse, minimizing IVR dimensions will likely increase women's confidence in the discreet capability of the IVR and decrease the probability of undesired male contact with the ring during coitus.

An excellent review article by Heise *et al.* discusses microbicide prevention trials and considerations when interpreting success of user-controlled methods (36). The authors define efficacy as an improvement in outcome under ideal conditions (perfect use) whereas effectiveness is the improvement in outcome achieved in practice (imperfect or inconsistent use). The microbicide field is concerned with adherence in clinical trials, especially following the Carraguard vaginal gel clinical trial where women adhered to the dosing regimen only 42% of the time (75). This result brought the

realization that product efficacy is meaningless for prospective microbicide products demonstrating low user adherence, as the overall effectiveness is so low. In the CAPRISA 004 trial with the TFV 1% gel, user adherence positively correlated with HIV prophylaxis. The hope is that a long-lasting, coitally-independent IVR will increase adherence and therefore effectiveness, shortening the gap between efficacy and effectiveness. We warn that there are little data supporting this hope. In fact, the 21 day NuvaRing® is only as good at preventing unplanned pregnancy as the once daily oral contraceptive (6), and only a 9-12% increase in adherence is achieved for various pharmaceutical products when going from daily to weekly frequency with an overall trend of intermittent dosing having higher adherence than frequent dosing (42, 58). However, the messy, nondiscreet, and burdensome gel dosage form may not be a fair comparison to the oral dosage form which generally demonstrates good user adherence. Little data exists on nonuser reported once per month administration, but methods to accurately measure microbicide adherence are being developed (79, 81).

### 1.7 Microbicide IVR Limitations and Unknowns

As discussed previously, an advantage of the EVA polymer technology over the silicone polymer technology is the ability for continuous and controlled manufacturing. Equally important, however, is the ability to more easily modulate ring stiffness (since silicone possesses a relatively narrow elastic modulus range) so that IVR cross-sectional diameter can be reduced to potentially increase user acceptability. Although NuvaRing® development has advanced IVR technology, both silicone and EVA IVR platforms have only demonstrated success to deliver hydrophobic small molecules at micrograms/day

levels. Numerous microbicide IVRs have been developed using these platforms to deliver hydrophobic small molecule antiretrovirals and one product is advancing for clinical efficacy evaluation (55). Clearly, however, the vaginal drug delivery field would benefit from IVR platforms which 1) can provide controlled and sustained delivery of doses higher than micrograms/day and 2) can deliver APIs besides hydrophobic small molecules. Unfortunately, many hydrophilic APIs are minimally soluble in the silicone and EVA polymers and therefore often cannot be delivered by simple diffusion at desired release rates for sustained duration. The hydrophilic small molecule tenofovir is a key example of current IVR technology inadequacy; Tenofovir was recently proven effective against HIV when formulated in a vaginal gel yet lacks a suitable IVR delivery system due to insufficient release rates from conventional EVA and silicone polymers (77). In addition to hydrophilic small molecules, there are a diverse set of macromolecular and biopharmaceutical APIs (including nucleic acids, peptides, and proteins) (97-99) that may be potential topical microbicides. However, biologics will require unconventional IVR designs as they often cannot withstand conventional IVR processing temperatures required for injection molding and hot melt extrusion, and/or have limited diffusivity in elastomeric polymers due to their size and limited polymer solubility (98). Therefore, designing IVRs for long-term delivery of drugs with wide ranging physicochemical properties and desired release kinetics is challenging and will require new and innovative technology.

The primary objective of microbicide IVRs is to deliver antiretrovirals locally to prevent initial infection of susceptible immune cells in the vaginal tract, and therefore knowledge of spatiotemporal vaginal drug concentrations is deemed crucial to device

success. Since all of the currently marketed IVRs target systemic hormonal delivery, there is very limited knowledge about the vaginal biodistribution and pharmacokinetics of drugs that are delivered from IVRs. Prior to initiation of the dissertation work reported herein, there were no peer-reviewed *in vivo* studies in women or large animals to examine safety, vaginal biodistribution and pharmacokinetics, or potential efficacy of microbicide IVR formulations. To evaluate some of these critical unknowns prior to testing in women, several animal models offering unique advantages and disadvantages are now being utilized by the field (92, 94). A comparison of vaginal mucosal models is presented in literature (17, 78). The cheapest yet least relevant animal models for HIV prophylaxis are rodents and rabbits. Their vaginal anatomy and physiology are quite different from women, but clearly are a step forward from *in vitro* studies and may offer a quick, low cost option for screening potential formulations prior to testing in more relevant animal models. The rabbit vaginal irritation model is widely used for preliminary microbicide safety studies as it is currently a required preclinical test by the Food and Drug Administration (21, 22), and more recently pilot pharmacokinetic studies have been performed with rabbits (13, 15). A major drawback of testing microbicide IVRs in rabbits has been the need to suture devices in place to avoid expulsion from the vaginal tract. The invasive suturing procedure causes tissue trauma at the implant site and may likely impact safety and pharmacokinetic outcomes. To improve the relevancy and usefulness of this model, a shape memory polyurethane fixture was recently invented in our laboratory which allows for nonsurgical device insertion and retention of the test article in the vagina.

Amongst large animal models, the sheep model has only recently gained traction and greater use for microbicide safety and pharmacokinetic evaluation. The sheep vaginal anatomy and stratified squamous epithelium are similar to humans and thus offer two significant improvements over small animal models. Due to their anatomical similarity to women, human-sized IVRs may be tested in sheep although significant deviations include different native vaginal microflora and a vaginal fluid pH of approximately 8 as opposed to 4.5 in women (78, 95). The pig animal model, particularly the minipig species, has remarkably similar anatomy and physiology to women and also shows promise for microbicide safety and pharmacokinetic testing (85). Pigs display a similar acidic pH to women although their microflora is generally dissimilar (19, 44, 54). Depending on the species utilized, a human-sized IVR could potentially be tested in pigs.

Although the preceding animal models each have unique advantages and may be appropriate for certain stages and objectives during microbicide IVR development, nonhuman primates are considered the most relevant animals for HIV prophylaxis research since they exhibit the most similar anatomy and physiology to humans (80, 94). One particular advantage of pigtail and rhesus macaques for microbicide testing is that their vaginal microflora is most similar to women, with the presence of lactic acid secreting lactobacillus which maintains an acidic vaginal pH to provide an innate defense against pathogens (59, 102). As a result, the potential negative effects of microbicide formulations on endogenous microflora, in addition to the vaginal epithelium, can be evaluated in the pigtail and rhesus models. Another potential advantage of the pigtail and rhesus models is the capability to perform efficacy studies of microbicide formulations using the related simian immunodeficiency and simian-human immunodeficiency

viruses, although the ability of these viral challenge studies to accurately predict HIV infection and prophylaxis in women is uncertain and the subject of debate (80).

In deciding between rhesus and pigtail macaque models for IVR evaluation, several aspects must be considered. Readers are pointed towards comprehensive reviews of microbicide animal models, which includes rhesus and pigtail macaques (93, 94), but two major differences are highlighted: First, pigtail macaques cycle monthly (akin to women) whereas rhesus are seasonal breeders (64). Therefore, rhesus macaques may require progesterone administration to synchronize their menstrual cycle. Unfortunately, this treatment thins the vaginal epithelium and thereby increases API as well as virus permeability (45, 93). Secondly, the supply of pigtail macaques is quite limited and therefore large studies and/or cost may necessitate the use of rhesus macaques. Altogether, both species still cost significantly more to purchase and house compared to the other animal models discussed, thereby potentially limiting experimental design and statistical analyses.

Another drawback of the macaque model is scaling issues related to their smaller vaginal anatomy compared to women, complicating the correlation of pharmacokinetics between macaques and women and preventing the testing of human-sized vaginal rings. The Centers for Disease Control and Prevention recently evaluated various size rings in both rhesus and pigtail macaques and found that 25 mm outer diameter IVRs fit well with no mucosal inflammation or lesions (64). If ring outer diameter is reduced from the human-sized 55 mm to 25 mm yet the same cross-sectional diameter is maintained, ring mass and surface area is reduced by approximately 60%. Since the drug release rate is proportional to the change in ring surface area (26), the reduction in ring size also

corresponds to a 60% decrease in release rate. The average woman weighs approximately 10 times that of a macaque (59, 74), therefore simply scaling drug release by body mass (weight/weight) results in an approximately 6-fold higher overall exposure of drug in the macaque than in women. Similarly, an approximation of vaginal volume, given mean length and diameter measurements (4, 59), also results in a 6-fold difference between macaques and women. Therefore, these issues should be considered when designing macaque studies and performing pharmacokinetic extrapolations from macaques to women.

Animal models for microbicide IVR evaluation are only now being developed. To date, there are many unknowns related to microbicide IVR *in vivo* performance, particularly vaginal biodistribution and pharmacokinetics, formulation effects on the vaginal epithelium and endogenous microflora, and the required vaginal concentrations for potential effectiveness against HIV. Many unanswered questions exist, such as how long after ring insertion does it take for vaginal fluid and tissue drug concentrations to reach protective levels? Specifically, what are the spatiotemporal drug concentrations proximal to the ring at the endocervix as well as near the introitus which is centimeters away from ring placement? How long after IVR removal are protective concentrations maintained? Drug release kinetics from IVRs have primarily been tested *in vitro* using sink conditions wherein the concentration of the drug in the release media is never allowed to exceed one-tenth its maximum solubility. Whether these *in vitro* conditions are predictive of *in vivo* performance is unclear, especially for hydrophobic antiretrovirals which display poor aqueous solubility and may thus achieve saturated concentrations in the vaginal fluid. The selection of an appropriate animal model should depend on the

main study objectives and questions asked, and several factors should be considered including cost and study size, anatomy and physiology, ring size and scaling, and whether or not efficacy studies are deemed important.

## 1.8 Conclusions

In summary, this work is motivated by an overall desire to develop prophylactic methods and products to prevent HIV transmission. Specifically, the focus of this dissertation work is to develop long-lasting intravaginal ring technologies which can deliver proven antiretrovirals to the vaginal tract to prevent the male-to-female sexual transmission of HIV. Intravaginal rings offer several advantages over other candidate vaginal dosage forms to locally deliver antiretrovirals; First, their extended duration capability can minimize final product cost through amortization. Second, their long-lasting, non-messy, coitally independent, and discreet potential may help increase user adherence. Finally, their inherent ability to provide long-duration, controlled drug release may enable IVRs to better attain and maintain effective prophylactic vaginal concentrations in contrast to less controlled and short-duration dosage forms.

Although intravaginal rings hold promise for microbicide applications, there has been little design innovation since their initial development decades ago for contraceptive and hormone replacement indications. As a result, IVR delivery of therapeutics which are not highly active hydrophobic small molecules is generally suboptimal. Furthermore, as intravaginal ring technologies have historically aimed for systemic delivery of therapeutics, there is little understanding of drug vaginal biodistribution and pharmacokinetics from IVRs, or of IVR effects on the vaginal environment pertaining to

safety. Thus, the primary focus of this dissertation work is to 1) design new intravaginal rings capable of delivering a variety of antiretrovirals and 2) develop new *in vitro* tests and *in vivo* models to increase IVR performance understanding and thereby provide new IVR platforms and products to prevent the sexual transmission of HIV.

## 1.9 Dissertation Chapter Overview

Each following chapter is a separately published manuscript or is pending publication. An overview of each chapter and its publication information is provided below.

### 1.9.1 Chapter 2 overview

In Chapter 2, we sought to design and test *in vivo* a polyurethane intravaginal ring to deliver the promising small molecule antiretroviral agent congeners IQP-0528 and IQP-0532. In particular, this dose-finding and safety study aimed to determine 1) whether loading-dependent drug release is observed *in vivo* as is observed *in vitro*, 2) what IVR drug loading would achieve vaginal concentrations deemed to be sufficient to prevent HIV infection, and 3) whether the formulations cause any harm or irritation to the vaginal mucosa and endogenous microflora. The two congeners were evaluated at various loadings in pigtail macaques and compared to placebo and naïve control arms. The primary study output was vaginal pharmacokinetics and safety. This was the first peer-reviewed publication reporting drug safety and pharmacokinetics of a microbicide IVR in nonhuman primates.

Publication: Johnson T. J., Srinivasan P., Albright T. H., Watson-Buckheit K., Rabe L., Martin A., Pau C.P., Hendry R.M., Otten R., McNicholl J., Buckheit R., Smith J., Kiser P.F. *Safe and Sustained Vaginal Delivery of Pyrimidinedione HIV-1 Inhibitors from Polyurethane Intravaginal Rings*. Antimicrobial Agents and Chemotherapy, 2012. 56(3): p. 1291-1299.

1.9.2 Chapter 3 overview

In Chapter 3, we sought to develop a combination IVR to simultaneously deliver the two promising antiretroviral molecules dapivirine and tenofovir which possess different mechanisms of HIV inhibition and contrasting hydrophilicity. Combination microbicides are under development to reduce the probability of drug-resistant virus which could arise from single agent HIV prophylaxis. The delivery of tenofovir from conventional hydrophobic polymer IVRs has proven inadequate owing to its hydrophilicity and low polymer miscibility. Our main aims were to 1) design and characterize a new IVR platform to improve tenofovir release rates over conventional IVRs, and 2) provide sustained release of both dapivirine and tenofovir for up to 30 days. This was the first peer-reviewed publication of an advanced multi-segment IVR platform to deliver multiple antiretrovirals, and the first report of using hydrophilic polyurethanes for IVR applications.

Publication: Johnson T.J., Gupta K.M., Fabian J., Albright T.H., Kiser P.F. *Segmented Polyurethane Intravaginal Rings for the Sustained Combined Delivery of Antiretroviral Agents Dapivirine and Tenofovir*. European Journal of Pharmaceutical Sciences, 2010. 39: p. 203-212.

1.9.3 Chapter 4 overview

In Chapter 4, we sought to design and evaluate in a sheep animal model a tenofovir (TFV) reservoir IVR which could avoid the time-dependent TFV release kinetics and ring mechanical stiffness observed with the matrix IVR developed in Chapter 3. Our main aims were to develop a ring that could 1) attain similar vaginal TFV concentrations as the clinically effective TFV 1% gel but for 90 day duration, 2) show no harmful vaginal effects, and 3) achieve time-independent ring mechanical stiffness similar to commercially available IVRs. This was the first publication to demonstrate similar or higher TFV vaginal concentrations from an IVR compared to the TFV 1% gel, and furthermore in a time-independent fashion for 90 days with no significant toxicological effects.

Publication: Johnson T.J., Clark M.R., Albright T.H., Nebeker J.S., Tuitupou A.L., Clark J.T., Fabian J., McCabe R.T., Chandra, N., Doncel, G.F., Friend D.R., Kiser P.F. *A Ninety Day Tenofovir Reservoir Intravaginal Ring for Mucosal HIV Prophylaxis*. Antimicrobial Agents and Chemotherapy. Under Review.

1.9.4. Chapter 5 overview

In Chapter 5, we sought to further design and characterize the TFV IVR prototype reported in Chapter 4. Our main aims were to 1) design and test IVRs of various composition and dimension to arrive at an optimized design and better understand the design space, and 2) determine the underlying mechanism behind drug release rate changes as a function of product storage time in order to achieve a thermodynamically stable formulation. This manuscript reported the engineering of an unconventional

reservoir IVR platform which could be used to deliver a spectrum of therapeutics and is the first publication to demonstrate and explain the impact of polyurethane microphase separation kinetics on drug flux. This was also the first IVR platform capable of delivering daily milligram quantifies of an API for greater than 30 days, and moreover while maintaining time-independent ring mechanical stiffness.

Publication: Johnson T.J., Nebeker J.S., Tuitupou A.L., Smith E.M., Clark J.T., Fabian J., McCabe R.T., Clark M.R., Friend D.R., Kiser P.F. *Engineering of a New Intravaginal Ring Technology for HIV Prophylaxis*. Pharmaceutical Research. Manuscript in preparation.

### 1.9.5. Chapter 6 overview

In Chapter 6, brief conclusions of the dissertation work will be drawn and recommendations will be made regarding future research in the microbicide IVR field.

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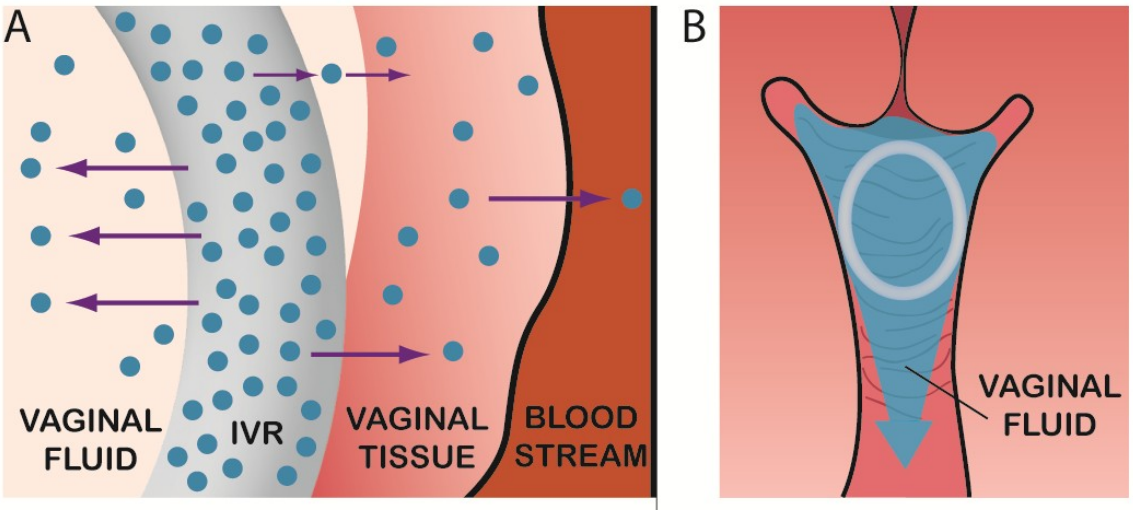


Figure 1.1. Diagram of *in vivo* drug release and transport from an IVR. A. Diffusive transport of drug from an intravaginal ring directly into vaginal tissue or first into a thin conducting layer of vaginal fluid and then into vaginal tissue, with transport into the blood being the ultimate sink. B. Advective transport of the drug in vaginal fluid from the anterior vagina near the IVR (ectocervix) to the posterior vagina (introitus).

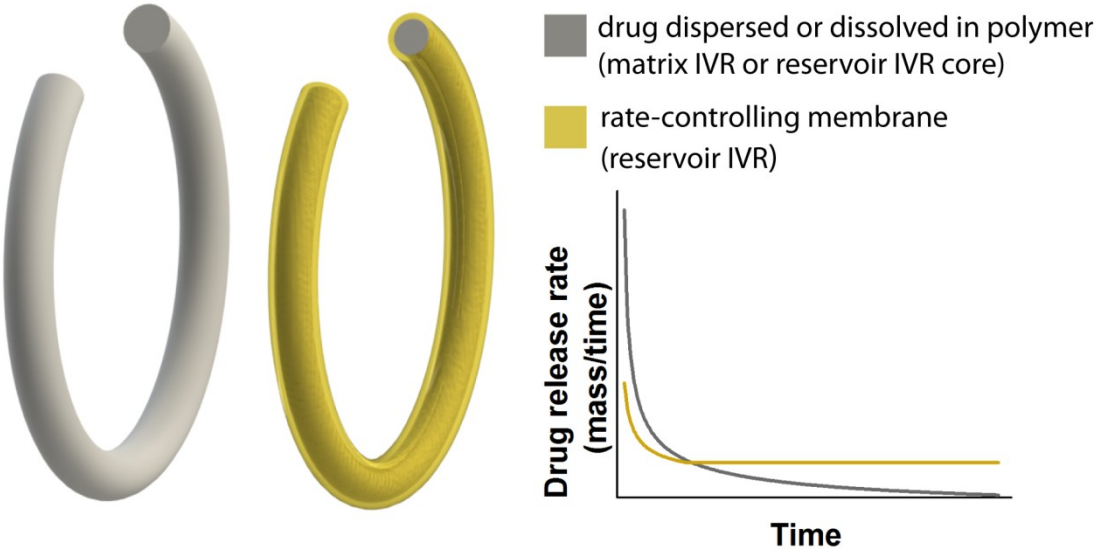


Figure 1.2. Comparison of matrix and reservoir IVR designs and their corresponding drug release kinetics.

CHAPTER 2

# SAFE AND SUSTAINED VAGINAL DELIVERY OF PYRIMIDINEDIONE HIV-1 INHIBITORS FROM POLYURETHANE INTRAVAGINAL RINGS

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# Safe and Sustained Vaginal Delivery of Pyrimidinedione HIV-1 Inhibitors from Polyurethane Intravaginal Rings

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**The potent antiretroviral pyrimidinediones IQP-0528 (PYD1) and IQP-0532 (PYD2) were formulated in polyurethane intravaginal rings (IVRs) as prophylactic drug delivery systems to prevent the sexual transmission of HIV-1. To aid in the selection of a pyrimidinedione candidate and the optimal loading of the drug in the IVR delivery system, four pyrimidinedione IVR formulations (PYD1 at 0.5 wt% [$PYD1_{0.5wt\%}$], $PYD1_{1wt\%}$, $PYD2_{4wt\%}$, and $PYD2_{14wt\%}$) were evaluated in pigtail macaques over 28 days for safety and pyrimidinedione vaginal biodistribution. Kinetic analysis of vaginal proinflammatory cytokines, native microflora, and drug levels suggested that all formulations were safe, but only the high-loaded $PYD2_{14wt\%}$ IVR demonstrated consistently high pyrimidinedione vaginal fluid and tissue levels over the 28-day study. This formulation delivered drug in excess of 10 $\mu$g/ml to vaginal fluid and 1 $\mu$g/g to vaginal tissue, a level over 1,000 times the *in vitro* 50% effective concentration. The *in vitro* release of PYD1 and PYD2 under nonsink conditions correlated well with *in vivo* release, both in amount and in kinetic profile, and therefore may serve as a more biologically relevant means of evaluating release *in vitro* than typically employed sink conditions. Lastly, the pyrimidinediones in the IVR formulation were chemically stable after 90 days of storage at elevated temperature, and the potent nanomolar-level antiviral activity of both molecules was retained after *in vitro* release. Altogether, these results point to the successful IVR formulation and vaginal biodistribution of the pyrimidinediones and demonstrate the usefulness of the pigtail macaque model in evaluating and screening antiretroviral IVR formulations prior to preclinical and clinical evaluation.**

The HIV/AIDS public health crisis urgently needs preventative technologies to protect the 1 million women who contract the virus each year in sub-Saharan Africa, where certain countries report infection rates exceeding 20% in young women (44). Recently, the CAPRISA 004 clinical trial evaluated the antiretroviral tenofovir in a coitally dependent vaginal gel and demonstrated a significant reduction in HIV-1 infections (1). However, user adherence was generally low and declined with time, likely due to the inconvenience of the dosage regimen. Since high rates of user adherence correlated with a reduced HIV infection rate, delivery systems with higher patient adherence may be crucial for maximizing topical microbicide effectiveness. Intravaginal rings (IVRs) offer a number of advantages over vaginal gels, including a preference by women, ease of use, and sustained therapeutic delivery for up to 90 days (15). With all formulations, effectiveness is dependent on user adherence, potency of the active pharmaceutical ingredient (API), and delivery of API to the target site. Therefore, it is anticipated that microbicide IVRs will demonstrate greater effectiveness than frequently applied or coitally dependent dosage forms such as gels or condoms (16, 47).

The significant reduction in HIV infections in the CAPRISA 004 trial validates the use of reverse transcriptase (RT) inhibitors (RTIs) as microbicides and provides the rationale for an RTI-based IVR to enable long-term topical delivery of RTIs (29). The 2,4(1*H*,3*H*)-pyrimidinediones (PYDs) comprise an important RTI class, with several analogs demonstrating both nanomolar-level HIV-1 RT and cell entry inhibition *in vitro* (4). Microbicides that can inhibit HIV-1 at multiple steps in its replication cycle are attractive since they may prevent initial infection and thereby reduce selection of drug-resistant virus (12). Additionally, the PYDs are generally chemically stable, have few or no chiral centers, and are synthesizable in four steps from readily available precursors (5). Of the available PYDs, analogs IQP-0528 (PYD1) and IQP-0532 (PYD2) have been chosen for microbicide formulation due to their favorable calculated log partition coefficient (*C* log *P*), photostability, therapeutic index, and HIV-1 inhibition (Fig. 1 and Table 1) (24).

Comprehensive IVR formulation requires relevant *in vivo* studies to optimize and evaluate API pharmacokinetics, biodistribution, and safety. Two parallel *in vivo* human pharmacokinetic studies with the RTI dapivirine formulated in silicone IVRs have recently been reported (31, 38). Although these first microbicide IVR pharmacokinetic studies demonstrated the feasibility and safety of an IVR for sustained release of antiretroviral agents, proceeding directly to clinical evaluation without prior animal studies can be risky, since toxicity or unacceptable drug pharmacokinetics may create significant setbacks, resulting in wasted time, effort, and cost. Animal models evaluating microbicide pharmacokinetics and safety would thus be useful at an earlier stage of microbicide IVR development when IVR design and composition are not yet finalized.

Selection of an appropriate animal model to test product safety

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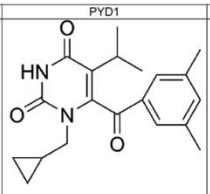
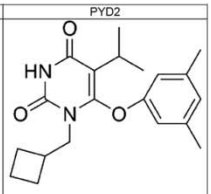
| | PYD1 | PYD2 |
|---|---|---|
| Chemical Structure |  |  |
| MW | 340.42 | 342.43 |
| ClogP | 4.1 | 4.5 |

**FIG 1** Pyrimidinedione analogs PYD1 and PYD2. *C* log *P*, calculated log partition coefficient.

and efficacy is difficult and the subject of debate in the microbicide field because animal models to replicate HIV-1 transmission and pathogenesis vary with the question asked (12, 45). However, nonhuman primate safety and pharmacokinetic studies can assist in the selection and assessment of microbicide candidates (34, 43). The pigtail macaque model is particularly relevant since it closely models human vaginal anatomy, physiology, and bacterial microflora (45, 46). Moreover, female pigtail macaques have a menstrual cycle length and frequency similar to those of women and therefore do not require progesterone administration to synchronize their menstrual cycle, a treatment which also thins the vaginal epithelium and thus increases API and virus permeation (25, 33). Numerous gel-based microbicide candidates have therefore been evaluated for safety and pharmacokinetics in the pigtail macaque model (34), including a tenofovir gel viral challenge study which predicted effectiveness prior to the CAPRISA 004 clinical trial (33). However, only recently has an IVR macaque model been developed (36). Therefore, to aid in the selection of a lead PYD molecule, to determine its concentration in the IVR, and to identify overall safety and vaginal biodistribution, we formulated PYD1 and PYD2 at various concentrations in polyurethane (PU) IVRs and compared the *in vitro* release, chemical stability, and antiviral activity to the *in vivo* 28-day safety and biodistribution in pigtail macaques.

## MATERIALS AND METHODS

**Materials.** Medical-grade Tecoflex EG-85A PU was purchased from Lubrizol (Wickliffe, OH). Dyna-Purge purging compound was purchased from Shuman Plastics (Buffalo, NY). Single lots of micronized IQP-0528 and IQP-0532 (purity, >99.8%; mean particle size, 1.5 $\mu$m) were provided by ImQuest BioSciences (Frederick, MD). 19-Norethindrone and zidovudine (AZT) (purities, >98%) were purchased from Sigma-Aldrich (St. Louis, MO). Solutol HS-15 solubilizer was purchased from BASF (Ludwigshafen, Germany). All solvents and reagents were American Chemical Society grade unless noted.

**Ring fabrication.** PYD-loaded PU rods were fabricated in a two-step process: PU pellets were frozen under liquid nitrogen, ground in a Pulverisette 14 stainless steel variable-speed rotor mill (Fritsch, Germany) to a particle size of roughly 500 $\mu$m, and then roll mixed on a jar mill with micronized PYD1 or PYD2. Upon thorough mixing, the PU-PYD powder mixture was manually fed into a benchtop twin-screw extruder (Thermo Haake, Waltham, MA) set at 140°C and 50-rpm screw speed and extruded continuously through a 4.3-mm-diameter aluminum die. Upon exiting the die, the compressed cylindrical rod was allowed to swell to approximately 5 mm in cross-sectional diameter and wrapped around a tube to form a circular ring shape. Upon extrusion of the entire sample, the machine was purged using 20 g of Dyna-Purge.

A total of four PYD formulations were made; the final PYD loadings in the polymer, as determined by solvent extraction, were 0.5 and 1 wt% PYD1 ($PYD1_{0.5wt\%}$ and $PYD1_{1wt\%}$, respectively) and 4 and 14 wt% PYD2 ($PYD2_{4wt\%}$ and $PYD2_{14wt\%}$, respectively). The upper loading of 14 wt% API was selected, as it is approximately the maximum amount at which PYD2 is soluble in the hydrophobic PU; since the API release rate increases linearly with API loading until maximum solubility is attained in the polymer (given that dissolution into the vaginal fluid is not rate limiting), loading-dependent release was expected over the loadings evaluated (7, 14). Placebo IVRs were also made as controls. IVRs were fabricated by cutting the extruded rods to 6.6 cm in length using a PU butt-welding kit (Fenner Drives, Manheim, PA) to melt and subsequently join the ends together to form an IVR with an outer diameter of 25 mm. The 5- by 25-mm IVR dimensions were selected on the basis of previous work concluding that a 5- by 25-mm IVR is well tolerated and retained in the pigtail macaque vaginal tract (36).

***In vitro* release.** To simulate the *in vivo* drug elution from the ring into the surrounding vaginal fluid, IVRs in triplicate were placed in either sink or nonsink *in vitro* release medium solutions which were replaced every 24 h. The sink release medium consisted of the 2 wt% nonionic surfactant Solutol in pH 4.2 25 mM sodium acetate buffer, whereas the nonsink release medium contained 0.05 wt% surfactant. A 0.05 wt% surfactant was selected as the nonsink release medium since it was above the critical micelle concentration of approximately 0.02 wt% and therefore had increased PYD solubility compared to water but still resulted in PYD saturation (2, 42). The volume of release medium was adjusted over the course of the study so that the concentration for either PYD in the 2 wt% surfactant release medium was roughly 1/10 to 1/15 the maximum solubility. On days 1, 3, 7, 10, 15, 20, 25, and 30, an aliquot of release medium from each sample was transferred to a high-pressure liquid chromatography (HPLC) vial for drug content analysis.

**Antiviral activity.** The antiviral activities of PYD1 and PYD2, both unformulated and formulated in the 0.05 wt% surfactant release medium from the 30-day IVR *in vitro* release assay, were evaluated by the cytopathic effect (CPE) inhibition and the cell-cell HIV transmission (syncytium-forming unit [SFU]/RT) assays. The CPE inhibition assay was performed in CEM-SS cells with $HIV\text{-}1_{IIIB}$. Briefly, 6 serial dilutions of test or control compound in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin (10% complete medium) was added to appropriate wells of a 96-well round-bottom microtiter plate. CD4-expressing CEM-SS cells at a con-

**TABLE 1** Antiviral activity of PYD1 and PYD2, unformulated and after *in vitro* release

| | $EC_{50}$ (nM)[a] | | | |
|---|---|---|---|---|
| | Unformulated | | Day 30 nonsink condition *in vitro* | |
| Compound | CPE inhibition assay with CEM-SS/$HIV\text{-}1_{IIIB}$ | SFU assay with CEM-SS/CEM-SS-$HIV\text{-}1_{RF}$ | CPE inhibition assay with CEM-SS/$HIV\text{-}1_{IIIB}$ | SFU assay with CEM-SS/CEM-SS-$HIV\text{-}1_{RF}$ |
| PYD1 | 4 | 5 | 60 | 7 |
| PYD2 | 10 | 7 | 40 | 7 |

[a] The mean values presented are from a single representative antiviral assay with control compounds evaluated in parallel and derived from three replicate wells.

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centration of $2.5 \times 10^3$ cells per well and HIV-$1_{IIIB}$ at an appropriate predetermined titer yielding 90% or greater cell killing in virus control wells were sequentially added to the microtiter plate. The cultures were incubated in 5% $CO_2$ at 37°C for 6 days. Following incubation, the microtiter plates were stained with 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) dye to evaluate the efficacy and toxicity of the test compound(s). XTT dye was metabolized to a colored formazan product by active mitochondria in viable cells. The HIV-1 RTI AZT was evaluated in parallel as a positive control for HIV inhibition. Cell, virus, precipitation/color, and medium controls were evaluated in parallel on each plate. Each test concentration was evaluated in triplicate and then averaged to determine the 50% effective concentration ($EC_{50}$).

Assessment of the inhibition of cell-to-cell transmission of HIV was performed using a syncytial inhibition (SFU/RT) assay (41). CEM-SS cells chronically infected with HIV-$1_{RF}$ (CEM-$SS_{RF}$ cells) were cocultivated in serial logarithmic dilutions (range, $10^5$ to $10^1$ cells) with uninfected $CD4^+$ CEM-SS cells at $5 \times 10^5$ cells per well along with test compound in flat-bottom 96-well microtiter plates at 37°C in 5% $CO_2$. Following cocultivation for 24 h, the number of syncytia in each well was counted by microscopic observation. Cell-to-cell transmission was also evaluated by quantification of the burst of virus production, which occurs at between 24 and 48 h after the cocultivation of the infected and uninfected cells. Virus production in the cell-free supernatant for determination of RT activity was quantified using a standard radioactive incorporation polymerization assay (3). Toxicity of the test compounds was determined using the XTT dye assay as described above. Dextran sulfate was used as a positive control for the assay. Each test concentration was evaluated in triplicate and then averaged to determine the $EC_{50}$.

**PYD chemical stability and quantification of IVR drug content.** To determine the chemical stability of the PYDs in the IVR formulations, extruded rods were cut into approximately 50-mg pieces and randomly selected, weighed, and placed into separate aluminum pouches. The pouches were then sealed using a heated impulse sealer (recycle setting = 3, sealing setting = 7, 155°C) and placed in a stability chamber set at 40°C with 75% relative humidity (Caron, Marietta, OH). At 0, 30, and 90 days, the pouches were removed and stored at −80°C until completion of the 90-day time point. PYD1 and PYD2 were then extracted from IVR segments at each drug loading concentration using a previously developed PU dissolution method (21). Briefly, the segments were dissolved in a 5 ml dimethyl acetamide-dichloromethane mixture along with the internal standard progestogen 19-norethindrone. One milliliter was transferred to a centrifuge tube, and methanol was added to precipitate the polymer. The sample was then centrifuged and the supernatant was quantified via HPLC. PYD extraction efficiency, as determined using spike blank samples, was 97.3% ± 0.4% ($n = 5$).

**Drug content analysis for *in vitro* studies.** The following methods were used to quantify and detect PYD1 and PYD2 *in vitro*. Samples were injected on an Agilent 1200 series HPLC with an Agilent Zorbax octyldecyl silane (ODS) column (particle size, 5 μm; 4.6 by 250 mm). For PYD1, a 12-min isocratic method was used with 35/65 (vol/vol) double-deionized water-acetonitrile at a flow rate of 1.5 ml/minute. Typical retention times for PYD1 and norethindrone were 6.4 and 3.0 min, respectively. For PYD2, a 15-min gradient water-acetonitrile method was used at a flow rate of 1 ml/minute. Typical retention times for PYD2 and norethindrone were 13.0 and 6.3 min, respectively. PYD1 and PYD2 were detected at 267 nm, whereas norethindrone was detected at 245 nm.

**Nonhuman primate studies.** Two 28-day IVR safety and vaginal biodistribution studies using pigtail macaque monkeys were performed following procedures approved by the CDC Institutional Animal Care and Use Committee according to the *Guide for the Care and Use of Laboratory Animals* as previously described (36). A total of six macaques divided into three groups with two animals per group were used for each study. PYD1 and PYD2 were evaluated since they both possess promising microbicide IVR attributes. A wide range of drug loadings was evaluated, as there are no microbicide IVR macaque studies reported in literature and therefore we were unsure what loading might achieve acceptable safety and pharmacokinetics from this type of delivery system. Furthermore, the PYDs have not been extensively studied *in vivo*, and therefore, their vaginal and systemic toxicological profile was uncertain. For the PYD1 study, the three groups were placebo IVR, $PYD1_{0.5wt\%}$ IVR, and $PYD1_{1wt\%}$ IVR. For the PYD2 study, the three groups were placebo IVR, $PYD2_{4wt\%}$ IVR, and $PYD2_{14wt}$ IVR. IVRs were inserted in the posterior vagina (ectocervix) on day 0 and left in place for 28 days. All vaginal fluid and tissue samples were collected at both the vaginal introitus (distal to the ring) and the ectocervix (proximal to the ring). Upon completion of the 28-day time point, IVRs were removed and analyzed for remaining drug content as described above.

**Safety.** Prior to IVR insertion, baseline safety, including evaluation of colposcopy samples, plasma and vaginal cytokine levels, and vaginal microflora levels, were established in all animals to allow inter- and intra-animal comparisons. IVRs were then inserted and the safety parameters were monitored for any changes. Vaginal microflora was characterized for all macaques on days 0, 7, and 21, as well as 10 days after IVR removal (day 38) as previously described (35). The aerobic and anaerobic organisms *Lactobacillus* species, viridans group streptococci, and *Gardnerella vaginalis* were identified to the genus, species, or group of bacteria level using phenotypic tests, Gram stain and colony morphology, and aerotolerance, as described in the *Manual of Clinical Microbiology* (30). *Lactobacillus* species and viridans group streptococci were tested for hydrogen peroxide production in a qualitative assay on tetramethylbenzidine agar plates (37).

Blood plasma and vaginal swab specimens were collected from each macaque to analyze the induction of any mucosal or systemic cytokines. All macaque-specific antibodies were used for plasma cytokine analysis, including gamma interferon (IFN-γ), interleukin-2 (IL-2), tumor necrosis factor alpha (TNF-α), IL-6, macrophage inflammatory proteins 1α and 1β (MIP-1α and MIP-1β), RANTES, eotaxin, IL-15, IL-12p40, granulocyte colony-stimulating factor (G-CSF), IL-8, and interleukin-1 receptor antagonist (IL-1Ra). Likewise, all vaginal cytokines available for analysis included IL-1β, IL-6, IL-8, G-CSF, RANTES, IL-1Ra, TNF-α, MIP-1α, and MIP-1β. Both plasma and vaginal cytokines were analyzed using fluorescent multiplexed bead-based assays (Invitrogen, Carlsbad, CA, and Bio-Rad, Hercules, CA) as previously described (36). All cytokine assays utilized individual cytokine standard curves created by serial dilution and the in-house internal positive control of phorbol myristate acetate-ionomycin-stimulated pigtail macaque peripheral blood mononuclear cell supernatant. Introitus and ectocervix vaginal swab specimens were collected from each animal at days −11, 0, 3, 7, 14, 21, 28, and 38. The swabs were weighed prior to and after vaginal fluid collection to determine vaginal fluid recovery. The swabs were then immersed in 200 μl of a 1:100 dilution of a protease inhibitor cocktail (1 ml; P8340; Sigma) in phosphate-buffered saline (PBS) for 30 min at 4°C. A Costar Spin-x centrifuge filter unit (catalog no. CLS8160; Sigma) was used to separate the extracted medium from the swab. Samples were centrifuged at 12,000 rpm for 20 min at 4°C. In calculating the final concentration of the cytokines, a dilution factor was calculated on the basis of a dilution factor of $(x + y)/x$, where $x$ equals the volume of the material collected (assuming a vaginal fluid density of 1.0 g/ml) and $y$ is the volume of the extraction buffer added. This dilution factor was used to calculate the final concentration of the cytokines in the mucosal secretions. Systemic cytokine quantification was carried out in the same manner using blood plasma collected at the same time points as vaginal swabs.

The surfaces of the IVRs postimplantation were examined for cellular and noncellular adhesion and drug surface crystallization. A 1% Alcian blue mucin stain was prepared in water, and 200 μl was placed on top of the ring segment for 10 s and subsequently rinsed with water and examined. A 4′,6-diamidino-2-phenylindole (DAPI) cell nuclear stain was prepared at 1 μM, 200 μl was added on top of ring segments, and the segments were viewed under a fluorescence microscope. Lastly, crystalli-

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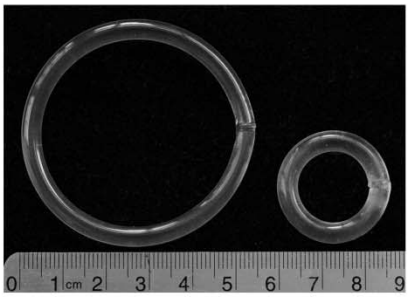


FIG 2 Human IVR (left) and macaque IVR (right).

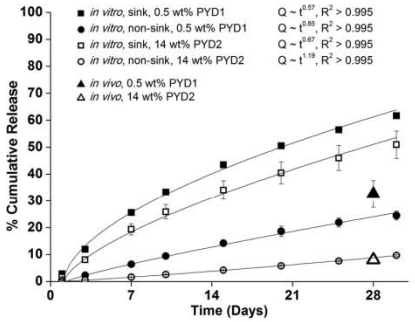


FIG 3 Percent cumulative release of PYD1 and PYD2 *in vitro* over 30 days (sink and nonsink conditions) compared to 28-day *in vivo* data. Mean ± SD, $n = 3$ for all points.

zation of either PYD on the IVR surface was observed using a polarized light microscope.

**PYD measurement in plasma, vaginal fluid, and vaginal tissue.** Plasma and vaginal fluid (days −11, 0, 3, 7, 14, 21, 28, and 38) and vaginal tissue (days −11, 7, and 38 for PYD1; days −11, 7, 21, and 38 for PYD2) were taken from the animals for drug content analysis. Weck-Cel wicks (Medtronic Xomed, Inc.) were used to collect vaginal fluid at both the introitus and ectocervix. Wicks were inserted and allowed to absorb fluid for approximately 5 min prior to removal. Biopsy samples were taken at the introitus and ectocervix using a Miltex Townsend model 30-1445 minibite cervical biopsy punch forceps with a bite of 4.2 by 2.3 by 1 mm.

PYD in biological samples was extracted and analyzed by modifying a previously developed liquid chromatography-tandem mass spectrometry method (23). Briefly, each sample was extracted with acetonitrile, followed by concentration and resuspension in mobile phase A, which was composed of 0.04% formic acid, 0.04% ammonium hydroxide, and 0.057% acetic acid in water. The final processed sample was then injected onto a 150- by 2-mm Ascentis phenyl reverse-phase column (Supelco, Bellefonte, PA) connected to a Shimadzu Prominence HPLC system (Durham, NC) and eluted with a water-acetonitrile mobile-phase gradient. A Model 3200 QTrap mass spectrometer (Applied Biosystems, Foster City, CA) in multiple-reaction-monitoring mode was used to monitor the transitions for the compounds of interest. For PYD1, *m/z* 341 to 287 and 341 to 133 were monitored, while *m/z* 343 to 275 and 343 to 122 were monitored for PYD2. The lower limit of quantification (LLOQ) was defined as the minimum level at which quantitative results could be obtained and was calculated to be 10 times the standard deviation of injections at the lowest concentration which were statistically different from blank injections using a 99% confidence interval. The LLOQ of both PYDs for this assay was determined to be 5 ng/ml. The amount of vaginal fluid absorbed onto the wick was measured by weighing the wicks before and after fluid absorption. All biopsy samples were also weighed after collection. The vaginal tissue LLOQ was determined to be 5 ng/sample. The vaginal fluid LLOQ was 170 ng, for an average collected vaginal fluid mass of 5.5 mg. To convert weight/weight concentrations of PYD (ng of PYD/mg of vaginal fluid or tissue) to molarity ($\mu$M), vaginal fluid and tissue densities of 1.0 g/ml were assumed.

**Statistical analysis.** Statistical difference between unpaired microflora groups was determined using the Mann-Whitney U test. Chemical degradation of the PYDs as a function of time was determined using one-way analysis of variance (ANOVA). For both tests, a $P$ value of $<0.05$ was considered significant.

## RESULTS

***In vitro* release.** PYD-PU hot-melt extrusion produced amorphous, molecularly dissolved PYD in PU whose cylindrical rod ends were subsequently butt welded to create macaque IVRs (Fig. 2). The *in vitro* release profile was determined for both the lowest loading ($PYD1_{0.5wt\%}$) and highest loading ($PYD2_{14wt\%}$), where the cumulative percentage of PYD released ($Q$) under sink conditions was significantly higher than that under nonsink conditions (Fig. 3). The cumulative amounts released from $PYD1_{0.5wt\%}$ and $PYD2_{14wt\%}$ at 30 days were 3 and 71 mg, respectively, under sink conditions and 1 and 14 mg, respectively, under nonsink conditions. The cumulative release profiles were quite different: the sink condition exhibited matrix-controlled kinetics of $Q \propto \text{time}^{1/2}$, whereas the nonsink condition exhibited partition-controlled kinetics of $Q \propto$ time.

**Antiviral activity and chemical stability.** To confirm that the two PYDs maintained their nanomolar-level activity against HIV-1 after being released from the IVRs, we compared the antiviral activity of PYD1 and PYD2 before formulation and after *in vitro* release from the IVRs. The activity of both unformulated PYDs in the CPE and SFU assays ranged from 4 to 10 nM (Table 1). The *in vitro* release samples exhibited diminished (4 to 15 times) nanomolar-level activity in the CPE assay but maintained similar nanomolar-level activity in the cell-to-cell transmission assay. Likewise, the chemical stability of PYD1 and PYD2 in the IVR formulation was evaluated under the accelerated stability condition of 40°C for 90 days (Table 2). There was no significant chemical degradation of either compound over the 90-day period (one-way ANOVA, $P > 0.05$), suggesting that these formulations are chemically stable for an extended period of time. Ninety-day HPLC chromatograms of the extracted PYD showed no new peaks to suggest chemical degradation, thus providing further support that no degradation of these com-

TABLE 2 Chemical stability of formulated PYD1 and PYD2 stressed at 40°C

| Formulation | % recovery after the following no. of days at 40°C[a]: | | |
|---|---|---|---|
| | 0 | 30 | 90 |
| PYD1 | 100.0 ± 1.8 | 100.0 ± 3.4 | 99.4 ± 0.7 |
| PYD2 | 100.0 ± 0.9 | 100.5 ± 1.0 | 99.6 ± 1.6 |
| Spike blank | 97.3 ± 0.4 | | |

[a] Data are means ± standard deviations ($n = 5$). Percent recovery was determined by HPLC and normalized to that at day 0.

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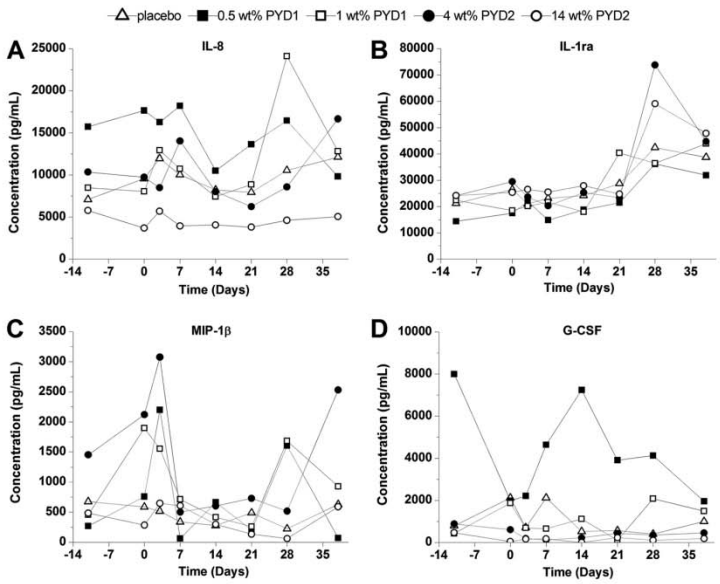


FIG 4 Mean vaginal cytokine levels as a function of time of IL-8 (A), IL-1ra (B), MIP-1$\beta$ (C), and G-CSF (D) for placebo IVRs, PYD1 IVRs, and PYD2 IVRs. IVRs were inserted at day 0 and removed at day 28. The mean data point was the average of single measurements in each animal ($n = 4$ and 2 animals for placebo and PYD IVRs, respectively).

pounds occurred under the stability conditions tested (see the figure in the supplemental material).

**Safety of PYD IVRs in pigtail macaques.** The *in vivo* safety of the PYD formulations was evaluated over the study duration by analyzing colposcopy specimen, plasma, and vaginal cytokine levels and microflora levels. Colposcopies revealed no obvious tissue inflammation or abrasions that would indicate IVR irritation (data not shown). Cytokine levels fluctuated greatly between time points and animals both in plasma (see Table S1 in the supplemental material) and vaginally (displaying four cytokines whose levels were consistently above the detection limit to allow comparisons between the formulations) (Fig. 4; see Table S2 in the supplemental material). However, there were no discernible trends in cytokine levels when comparing the level for each animal to its corresponding day −11 or day 0 level (intra-animal) or between groups (placebo versus drug loaded). Likewise, there was no significant difference (Mann-Whitney test, $P > 0.05$) in vaginal microflora levels between placebo and IVR groups (Fig. 5). Altogether, these results suggest that the placebo and PYD-loaded IVRs did not perturb the vaginal tissue and native microflora.

To further assess device performance and biocompatibility, the surfaces of the removed IVRs were examined for cellular and macromolecular adhesion as well as PYD surface crystallization. The IVRs changed little in appearance, maintaining a transparent look, except for the slight presence of blood on IVRs removed from menstruating macaques (data not shown). Additionally, the $PYD2_{14wt\%}$ IVR showed API surface crystallization, as observed by polarized light microscopy (data not shown). DAPI cell nucleus stains revealed considerable cellular adhesion on all IVRs, and all IVRs stained positive for mucin using an Alcian blue stain (data not shown).

**PYD vaginal fluid levels.** PYD vaginal fluid levels were quantified over the 28-day period to aid in evaluation of release kinetics and biodistribution. Wicks collected and analyzed for drug content prior to IVR placement (days −11 and 0) or 10 days after IVR removal (day 38) revealed no detectable drug for any of the four

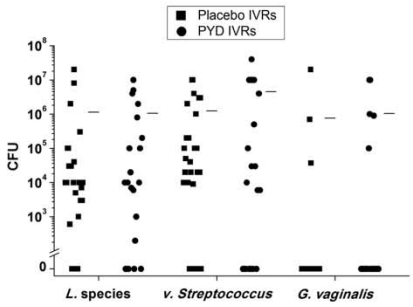


FIG 5 Vaginal microflora levels (numbers of CFU) of *Lactobacillus* species, viridans group streptococci, and *G. vaginalis* from animals with placebo and PYD IVRs (bars, mean value for all four time points; $n = 27$ and 21 for placebo and PYD IVRs, respectively).

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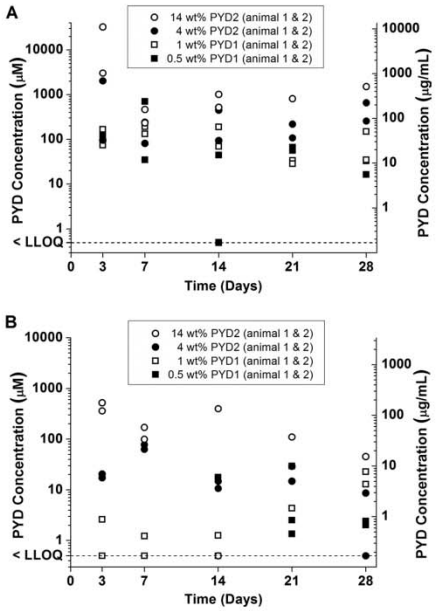


FIG 6 PYD levels in vaginal fluid at the ectocervix (proximal) (A) and introitus (distal) (B) for PYD2 IVRs and PYD1 IVRs. $n = 2$ animals per group, except on days 21 and 28 of 14 wt% PYD2 loading ($n = 1$). PYD1 and PYD2 levels were below the LLOQ prior to IVR insertion and after IVR removal (days −11 and 38; data not shown).

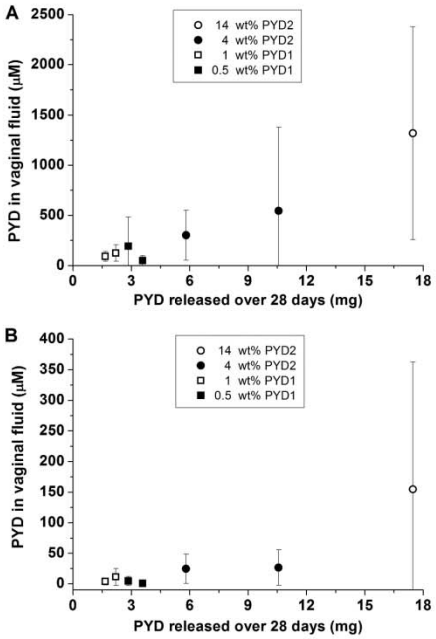


FIG 7 *In vivo* correlation between PYD levels in vaginal fluid and mg PYD released from the individual IVRs after the 28-day pharmacokinetic study at both the ectocervix (A) and introitus (B) for PYD2 IVRs and PYD1 IVRs. Vaginal fluid levels were averaged over the 28-day implantation period (mean ± SD, $n = 5$ time points). The amount (mg) of PYD1 or PYD2 released *in vivo* from the IVR was determined by drug solvent extraction from the IVR following the 28-day vaginal implantation period.

drug-loaded formulations. With the exception of one sample, PYD vaginal fluid levels at the ectocervix exceeded micromolar concentrations across all PYD IVR loadings, animals, and time points during ring insertion, although the higher-drug-loaded IVRs generally maintained higher respective PYD vaginal fluid levels (Fig. 6A). The highest-loaded IVRs (14 wt%) also exhibited a significant burst (day 3 PYD levels several log units higher than day 7 levels) yet still maintained near millimolar PYD vaginal fluid levels at 28 days. At the introitus, PYD vaginal fluid levels were roughly an order of magnitude lower than those in the ectocervix but demonstrated the similar trend of greater PYD concentrations with higher IVR drug loadings (Fig. 6B). Although most vaginal fluid samples contained micromolar PYD levels, at least one introitus vaginal fluid sample for each loading had levels below the LLOQ, highlighting the *in vivo* variability. Overall, however, increased PYD IVR loading correlated with increased vaginal fluid concentrations at both the introitus and ectocervix, with Pearson correlation coefficients ($R$ values) of 0.57 and 0.65, respectively. A positive correlation also existed between the cumulative amount of PYD released at 28 days *in vivo*, as determined by API solvent extraction from IVRs after study completion at 28 days, and time-averaged PYD concentrations in vaginal fluid (Fig. 7), with Pearson correlation coefficients at the introitus and ectocervix of 0.53 and 0.64, respectively. The cumulative percent PYD released *in vivo* was more similar to that under nonsink than sink *in vitro* release conditions (Fig. 3).

**Vaginal tissue drug levels.** Vaginal tissue biopsied and analyzed for PYD concentration prior to IVR placement (day −11) and 10 days after IVR removal (day 38) revealed no detectable API (data not shown). All ectocervical tissues exposed to PYD IVRs (collected at day 7 for PYD1 and days 7 and 21 for PYD2) contained micromolar PYD levels, except for the lowest-loading $PYD1_{0.5wt\%}$ group, where the tissue concentration was below the LLOQ (Table 3). However, at the introitus, only the highest-loading $PYD2_{14\ wt\%}$ IVR group gave detectable drug levels, with 2 of 3 biopsy samples having micromolar drug levels.

## DISCUSSION

The PYD-PU powder blend hot-melt extrusion method was an improvement over previous multistep fabrication techniques employing a solvent (14, 21). The *in vitro* PYD release from IVRs under nonsink conditions compared well with the *in vivo* release, and the antiviral activity and chemical stability of the PYDs in the formulation were maintained. We observed an increase in PYD vaginal fluid concentrations with increasing PYD IVR loadings (and amount of PYD released from the IVR). The vaginal fluid and tissue drug levels attained in the highest-loaded group were greater than 1,000 times the *in vitro* $EC_{50}$, suggesting that the

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**TABLE 3** Ectocervical and introitus PYD vaginal tissue levels for each individual biopsy specimen

| Location | Formulation | Concn[a] | | | |
|---|---|---|---|---|---|
| | | Day 7 | | Day 21 | |
| | | Animal 1 | Animal 2 | Animal 1 | Animal 2 |
| Ectocervix | 0.5 wt% PYD1 | 6.4 (2.2) | <3.5 (1.2) | | |
| | 1 wt% PYD1 | 16.6 (5.6) | 1.3 (0.4) | | |
| | 4 wt% PYD2 | 27.4 (9.3) | 20.9 (7.1) | 10.5 (3.6) | 12.3 (4.2) |
| | 14 wt% PYD2 | 8.7 (3.0) | 9.1 (3.1) | Sample lost | 7.3 (2.5) |
| Introitus | 0.5 wt% PYD1 | <4.2 (1.4) | <1.5 (0.5) | | |
| | 1 wt% PYD1 | <4.2 (1.4) | <2.9 (1.0) | | |
| | 4 wt% PYD2 | <3.2 (1.1) | <3.9 (1.3) | <8.6 (2.9) | <5.4 (1.8) |
| | 14 wt% PYD2 | 14.0 (4.8) | <4.5 (1.5) | Sample lost | 4.2 (1.4) |

[a] Data are in $\mu$M ($\mu$g/g). For PYD1, biopsy specimens were collected only on day 7, whereas for PYD2, biopsy specimens were collected on both day 7 and day 21. In tissue samples where drug was not detected, the individual sample LLOQ is given (indicated by <), as it differs for each biopsy specimen on the basis of its tissue mass. PYD1 and PYD2 levels were less than LLOQ prior to IVR insertion (day −11) and after IVR removal (day 38) and are not shown.

$PYD2_{14\ wt\%}$ IVR may sufficiently load PYD in vaginal tissue along the length of the vaginal tract at concentrations sufficient to prevent HIV-1 infection.

***In vitro* studies.** Typically, *in vitro* drug release testing utilizes sink conditions, wherein the API concentration in the release medium never exceeds one-third the equilibrium saturation concentration (42). Conversely, nonsink conditions exist when the concentration of API in the release medium nears its maximum solubility, resulting in attenuated drug release due to a smaller concentration gradient as well as API partitioning between the polymer and medium (6, 17). Although utilization of sink conditions does in fact classify the device type—the most common being time dependent (matrix devices) or time independent (reservoir devices)—it seldom reflects *in vivo* pharmacokinetics. This and other studies utilizing novel delivery platforms have shown a disparity between *in vitro* release under sink conditions and *in vivo* release, particularly for poorly water soluble compounds, which constitute the majority of antiretrovirals being developed as microbicides (20, 42). With hydrophobic compounds such as the PYDs, achieving sink conditions requires large volumes of aqueous release medium (up to hundreds of milliliters per day) and/or a high concentration of surfactants or organic solvents to increase the API's solubility in the medium.

We performed *in vitro* release under sink and nonsink conditions to determine an *in vitro*/*in vivo* release correlation that may be beneficial for future PYD IVR development. The cumulative percentages of PYD1 and PYD2 released in nonsink release medium was quite similar to those observed *in vivo*, whereas sink conditions overestimated drug release. *In vitro*, the 14 wt% PYD2 IVR released 23 and 11 times more API than the 0.5 wt% PYD1 IVR under sink and nonsink conditions, respectively. The lower differential release between the 14 wt% PYD2 and 0.5 wt% PYD1 IVRs under nonsink conditions was likely due to rapid PYD saturation of the release medium with the high loading, therefore hindering further drug release from the IVR. Given the low volume and variable production of vaginal fluid, it is not surprising that sink conditions did not predict *in vivo* pharmacokinetics for either hydrophobic PYD at the loadings evaluated (28, 32). Therefore, the nonsink release condition established an *in vivo*/*in vitro* release correlation that may be useful for future hydrophobic API microbicide formulation development.

All formulations pose the potential problem of API chemical instability and subsequent loss of biological activity upon storage (19). A successful microbicide formulation must be able to withstand elevated temperatures, which may occur during storage, without causing chemical degradation of the API. We stressed the PYD IVRs at 40°C for 90 days and observed no PYD1 or PYD2 chemical degradation, as evaluated by HPLC. Similarly, the PYD1 and PYD2 released from the IVRs *in vitro* showed nanomolar-level antiviral activity, as evaluated in CPE inhibition and SFU assays. Although the CPE inhibition assay showed diminished (4 to 15 times) activity compared to that of the unformulated PYD, this relatively subtle change could be due to inherent error in the assay and/or presence of surfactant in the release medium. Therefore, the PU carrier and processing conditions were able to maintain the chemical stability of both drugs upon fabrication and storage, and the released compounds maintained nanomolar-level antiviral activity.

***In vivo* safety and PYD biodistribution.** Safety is an essential component of a microbicide's efficacy since irritation, inflammation, or disruption of the vaginal epithelium can increase the susceptible immune cell population in the cervicovaginal mucosa (22). Similarly, suppression of lactic acid-secreting microflora such as *Lactobacillus* spp. and viridans group streptococci may be deleterious to maintenance of an acidic pH, which can neutralize pathogens such as HIV-1 (8). We monitored epithelial integrity, microflora, and plasma and vaginal cytokines throughout the 28-day *in vivo* studies. Although the inter- and intra-animal cytokine and microflora variability was quite high, we observed no clear indication that the PYDs were detrimental locally or systemically. Furthermore, cytokine levels were similar to those described in previously published work comparing naïve and placebo IVR exposure (36).

Thorough examination of the IVR surface postimplantation revealed dispersed adherent cells and Alcian blue staining of cervicovaginal mucus. Biological matter has been observed on the surface of IVRs in other *in vivo* IVR studies and has not noticeably increased vaginal inflammation (13, 27, 40). The impact of biological matter adhesion on drug release has not been reported in literature but could potentially affect drug elution and the corresponding pharmacokinetics. However, since the diffusion coefficient of small-molecule APIs through mucus is comparable to that of IVR polymers (approximately $10^{-7}$ $cm^2/s$ [10, 17, 26]), it is unlikely that a thin layer of biological material on the ring surface significantly impedes drug transport. This assumption holds true only for uncharged molecules such as the PYDs, as mucus is known to bind charged molecules and thereby hinder transport (9, 10). Crystallized PYD was also observed on the surface of the 14 wt% PYD2 IVR after removal, suggesting partition-controlled drug release where dissolution into vaginal fluid is the rate-limiting step as a result of the PYDs' high polymer solubility and low aqueous solubility (6). This phenomenon may be advantageous, as the matrix IVR could provide relatively constant drug release throughout the dosage duration, as suggested with the $PYD2_{14wt\%}$ IVR vaginal fluid levels.

Also in regard to API transport, the impact of biopsy specimen collection and frequency of collection on drug release and pharmacokinetics from sustained-delivery devices has not been stud-

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ied. Potentially, large biopsy specimen and/or frequent biopsy specimen collection could create significant tissue trauma, inducing a local wound healing or inflammatory response and possibly altering drug release and local biodistribution. To mitigate this risk, we used a miniature-sized biopsy punch forceps (average tissue mass, 5 mg) and limited biopsy frequency to 14 days to minimize any local tissue trauma and associated alteration of PYD pharmacokinetics.

Since PYDs primarily act to inhibit RT inside susceptible immune cells, they must be sufficiently concentrated in vaginal tissue, where initial infection of these cells is thought to occur (18). The efficacy of an RTI formulation therefore likely hinges on the API's ability to diffuse into the surrounding vaginal fluid and subsequently partition into the local vaginal tissue. We collected vaginal fluid and tissue samples at two anatomical sites (introitus and ectocervix) to evaluate PYD distribution. Most ectocervical vaginal fluid and tissue samples attained micromolar PYD concentrations for all of the PYD IVR loadings, yet at the introitus, only the highest loading (14 wt%) demonstrated PYD levels above the LLOQ. Encouragingly, PYD vaginal tissue concentrations for this 14 wt% loading were quite similar to those measured in the dapivirine IVR clinical study (3.5 $\mu$g PYD per g of tissue), whereas PYD vaginal fluid levels were higher than those of dapivirine (7.1 $\mu$g PYD per ml of fluid) (38). Considering that the PYDs and dapivirine have nanomolar-level $EC_{50}$ activity against HIV-1 RT, the micromolar PYD concentrations attained in the vaginal tract—over 1,000 times the $EC_{50}$—suggest that this formulation may be effective and warrants further evaluation.

In both vaginal fluid and tissue, PYD levels at the ectocervix were greater than those at the introitus, a trend also observed in the dapivirine IVR clinical trial (38). Despite this gradient, the near steady-state PYD levels attained at the introitus after 3 days of IVR implantation argue for significant drug transport by vaginal fluid convection, an advantageous phenomenon that IVR efficacy likely depends upon for adequate biodistribution (11, 39). Collecting vaginal fluid and tissue at earlier time points, such as hours after IVR insertion, would be beneficial for determining when the PYDs are sufficiently concentrated along the length of the vaginal tract to prevent viral infection. Likewise, attaining the vaginal washout kinetics of the PYDs after IVR removal (no PYD was detected 10 days after IVR removal in our study) would provide insight as to how long an IVR can be absent from the vagina before protection is lost.

In sum, the nonsink *in vitro* release medium condition accurately predicted PYD *in vivo* release and may prove useful for many of the hydrophobic APIs formulated in microbicide IVRs. The potent nanomolar-level activity of the PYDs was maintained after *in vitro* release, and the PYDs were chemically stable in the PU IVR formulation. None of the four IVR formulations evaluated in the pigtail macaque model caused any obvious vaginal irritation, inflammation, or toxicity. The highest-loading IVR formulation appeared to be the most promising, as it was the only formulation that achieved drug levels in excess of 1,000 times the *in vitro* $EC_{50}$ along the entire length of the vaginal tract 3 days after insertion and maintained these levels throughout the 28-day study. Furthermore, since only approximately 10% of loaded drug was released by day 28, it may be possible to increase the dose duration and thus reduce the daily cost of the device in resource-poor areas. Due to the inherent data variability when utilizing a low number of animals in a pilot safety and pharmacokinetic study, statistical analyses and depth of conclusions were limited. A follow-on study with the lead formulation using a larger number of animals and improved bioanalytical methods with lower detection limits is planned to confirm safety and pharmacokinetics. Despite these limitations, the reported study was beneficial, as it demonstrated successful PYD formulation in an IVR and promising *in vivo* safety and pharmacokinetic evaluation in pigtail macaques to justify further development of the PYD IVR as a sustained-dosage prophylactic to prevent HIV infection.

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The use of trade names is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services, the Public Health Service, or the Centers for Disease Control and Prevention.

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CHAPTER 3

# SEGMENTED POLYURETHANE INTRAVAGINAL RINGS FOR THE SUSTAINED COMBINED DELIVERY OF ANTIRETROVIRAL AGENTS DAPIVIRINE AND TENOFOVIR

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# Segmented polyurethane intravaginal rings for the sustained combined delivery of antiretroviral agents dapivirine and tenofovir

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ABSTRACT

Dual segment polyurethane intravaginal rings (IVRs) were fabricated to enable sustained release of antiretroviral agents dapivirine and tenofovir to prevent the male to female sexual transmission of the human immunodeficiency virus. Due to the contrasting hydrophilicity of the two drugs, dapivirine and tenofovir were separately formulated into polymers with matching hydrophilicity via solvent casting and hot melt extrusion. The resultant drug loaded rods were then joined together to form dual segment IVRs. Compression testing of the IVRs revealed that they are mechanically comparable to the widely accepted NuvaRing® IVR. Physical characterization of the individual IVR segments using wide angle X-ray scattering and differential scanning calorimetry determined that dapivirine and tenofovir are amorphous and crystalline within their polymeric segments, respectively. In vitro release of tenofovir from the dual segment IVR was sustained over 30 days while dapivirine exhibited linear release over the time period. A 90 day accelerated stability study confirmed that dapivirine and tenofovir are stable in the IVR formulation. Altogether, these results suggest that multisegment polyurethane IVRs are an attractive formulation for the sustained vaginal delivery of drugs with contrasting hydrophilicity such as dapivirine and tenofovir.
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Women comprise roughly 60% of all human immunodeficiency virus (HIV) infections in sub Saharan Africa, highlighting the need for a simple, cost-effective, and reliable prophylactic method to prevent the male to female sexual transmission of HIV in a region disproportionately afflicted by the disease (UNAIDS, 2008). To satisfy this need, topically applied vaginal products called microbicides are currently being designed to prevent HIV infection in resource-poor countries (Ndesendo et al., 2008). Of the common microbicide formulations pursued, intravaginal rings (IVRs) have an advantage over high frequency dosing formulations, such as gels, in that they likely improve acceptability and adherence (Woolfson et al., 2000). In microbicide acceptability studies, women exhibited greater preference for and adherence to vaginal rings over gels (Hardy et al., 2007). A recent clinical trial evaluating the efficacy of a carrageenan based microbicide gel echoed this reality, concluding that only 42.1% of women who participated in the trial adhered to the dosage regimen, thereby shortening the trial and attenuating any meaningful outcomes (Skoler-Karpoff et al., 2008). IVRs, on the other hand, have improved adherence likely due to longer dose duration, noncoital dependence, and the possibility of covert use. In turn, this likely contributed to the success of the contraceptive IVR NuvaRing® (EVA-R), which boasted 96% user satisfaction in large scale international clinical trials (Novak et al., 2003; Roumen, 2002; Dieben et al., 2002; Sarkar, 2005).

Microbicide formulations often incorporate a reverse transcriptase inhibitor (RTI) which binds and inactivates reverse transcriptase, a conserved viral enzyme responsible for viral DNA synthesis leading to integration into the host genome. Reverse transcriptase poses an attractive target since its inactivation interrupts the HIV viral replication cycle and thereby halts infection. Two RTIs that have been extensively evaluated for microbicide use include the nonnucleoside RTI (NNRTI) dapivirine (Fletcher et al., 2009; Nuttall et al., 2008; Gupta et al., 2008; Woolfson et al., 2006; Di Fabio et al., 2003; Romano et al., 2009; Saxena et al., 2009) and the nucleotide analogue RTI (NtRTI) tenofovir (Terrazas-Aranda et al., 2008; Rosen et al., 2008; Cranage et al., 2008; Mayer et al., 2006; Saxena et al., 2009). Although both drugs target reverse transcriptase, they inactivate the enzyme via different mechanisms. Dapivirine binds to a conserved region on reverse transcriptase and subsequently prevents flexural movement required for transcription. Tenofovir, on the other hand, is a nucleotide analogue molecule that lacks the 3′-hydroxyl on the deoxyribose. As a result, tenofovir incorporation into the growing

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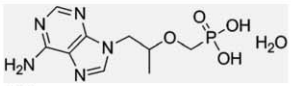
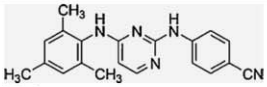
**Table 1**
Antiretroviral agents tenofovir and dapivirine.

| | Tenofovir monohydrate | Dapivirine |
|---|---|---|
| Chemical structure |  |  |
| MW | 305.2 | 329.4 |
| ClogP | −1.56 | 6.3 |

viral DNA chain halts transcription since the next nucleotide cannot form a 5′–3′-phosphodiester bond with tenofovir.

Due to the promising nanomolar activity of these two molecules (Van Herrewege et al., 2004; Das et al., 2004; Srinivas and Fridland, 1998), monomicrobicide formulations of dapivirine and tenofovir are in phase 1/2 and 3 clinical trials, respectively (Microbicideupdate, 2009) and have demonstrated efficacy in viral challenged animal models (Cranage et al., 2008; Di Fabio et al., 2003). Furthermore, a recent clinical study using dapivirine-loaded silicone IVRs showed significant dapivirine levels throughout the human vaginal tract over 7 days, validating microbicide IVRs as a vehicle for sustained prophylactic HIV therapy (Romano et al., 2009).

Drug-related local tissue toxicity is a concern for microbicide formulations since an effective microbicide may be exposed to vaginal tissue for years. Cytotoxicity of the vaginal tissue could in fact promote HIV infection by damaging the protective epithelial barrier and thereby allow free viral passage. Fortunately, safety studies using dapivirine-loaded IVRs have indicated the product is safe in women (Romano et al., 2009; Nel et al., 2009). Likewise, a vaginal tenofovir gel has shown no significant toxicity in women (Mayer et al., 2006). Animal studies with various microbicide formulations have also shown overall safety (Di Fabio et al., 2003; Fletcher et al., 2009). Lastly, unformulated combinations of dapivirine and tenofovir, both separately and combined, have shown no cellular or tissue toxicity at typical microbicide formulation concentrations (Herrera et al., 2009). Altogether, previous microbicide safety studies indicate that dapivirine and tenofovir are nontoxic to the vaginal mucosa when delivered at typical microbicide concentrations.

Despite the preliminary success of either drug in microbicide formulations, the microbicide field has expressed concern over the use of monotherapeutic microbicides that may select for drug resistant viral variants (Grant et al., 2008; Wilson et al., 2008; Coplan et al., 2004). Consequently, there is now heightened interest in the development of microbicides incorporating multiple therapeutics that have different viral targets, thereby reducing the probability of HIV infection and subsequent production of drug resistant strains (Trapp et al., 2006; Klasse et al., 2008; Veazey et al., 2005; Turpin, 2002; Herrera et al., 2009; Klasse et al., 2006). This combination therapeutic approach has been established as the most effective way to reduce HIV infection and replication in vitro and in vivo (Tremblay, 2004). Moreover, HIV challenge studies using cellular and tissue models has confirmed that the combination of dapivirine and tenofovir is more active than either drug alone, both in resistant and non-resistant strains (Herrera et al., 2009; Schader et al., 2007). Herrera et al. (2009) studied the effect of unformulated dapivirine and tenofovir on the NNRTI-resistant HIV-1 A17 isolate and NRTI-resistant isolate HIV-1 71361-1 in TZM-bl cells, PMBCs, and colorectal explants. Individually, the NNRTI dapivirine and NtRTI tenofovir were deficient in their ability to inhibit HIV-1 A17 and HIV-1 71361-1 replication, respectively. However, when both compounds were simultaneously used against the resistant strains, HIV replication was completely inhibited in both the cellular and tissue models. Therefore, in this study we propose the simultaneous controlled delivery of the NNRTI dapivirine and the NtRTI tenofovir from a polyurethane (PU) IVR to minimize the emergence of drug resistant viral mutants.

Currently, most microbicide IVRs are manufactured via injection molding, resulting in a reservoir or monolithic IVR that has the same composition throughout its length. This formulation design is quite successful for delivering multiple compounds with similar hydrophilicity such as hormones for contraception and hormone replacement therapy (Woolfson et al., 1999). However, it is improbable that dapivirine and tenofovir, which have opposing ClogPs (Table 1), could be effectively delivered from a single polymeric segment in a sustained manner. As a result, we explored the unique capability of fabricating a ring via extrusion with hydrophobic and hydrophilic segments corresponding to the relatively hydrophobic and hydrophilic nature of dapivirine and tenofovir, respectively.

By using this novel two segment PU ring approach, we optimized the IVR to simultaneously deliver dapivirine and tenofovir. Upon fabrication of a dual segment dapivirine/tenofovir PU IVR, the physical state of both drugs in the dosage form was analyzed using differential scanning calorimetry and wide angle X-ray scattering. We also tested ring joint integrity and compared the IVR mechanical properties to the widely used EVA-R IVR to ensure easy ring insertion, retention, and biocompatibility. Lastly, we investigated the release and stability of both drugs from the dual segment PU IVR. Using this segmented PU IVR approach, drugs throughout the hydrophilicity spectrum could likely be optimized for sustained delivery to reduce infection and the emergence of drug resistant strains.

## 1. Materials and methods

### 1.1. Materials

Acetonitrile (HPLC grade), trifluoroacetic acid (purity > 97%), tetrahydrofuran (HPLC grade), and phosphoric acid (ACS grade) were obtained from Fisher Scientific (Fairlawn, NJ). Potassium phosphate monobasic (ACS grade), potassium phosphate dibasic (ACS grade), tetrabutyl ammonium bisulfate (purity > 98%), 5% hydrogen peroxide (ACS grade), 19-norethindrone (purity > 98%), were purchased from Sigma–Aldrich. Methanol (HPLC grade) and glacial acetic acid (ACS grade) were obtained from EMD (Germany). Isopropanol (ACS grade) and dichloromethane (ACS grade) were received from Mallinckrodt (Phillipsburg, NJ). Dimethylacetamide (purity > 99%) was supplied by Alfa Aesar (Ward Hill, MA). Dapivirine and tenofovir were provided by the International Partnership for Microbicides (Silver Spring, MA). Medical grade water swellable Tecophilic® HP-60D-20 and nonwater swellable Tecoflex® EG-85A polyurethane, hereby referred to as WS-PU and NWS-PU, respectively, were obtained from Lubrizol (Wickliffe, OH). Dyna-purge® polymer was purchased from Shuman plastics (Buf-

falo, NY). High density polyethylene was obtained from American Plastics (Arlington, TX). Loctite® adhesive was purchased from Henkel (Rocky Hill, CT). NuvaRing® (subsequently referred to as EVA-R) was obtained from a local pharmacy.

### 1.2. Thermal analysis

To verify drug stability under extrusion temperatures as well as the physical state of both drugs in the IVR, differential scanning calorimetry (DSC) was performed. Between 3 and 5 mg of sample was weighed and sealed in an aluminum 40 μL pan. To allow water evaporation, the tenofovir pan was punctured with a needle. Scans were run from 25 to 300 °C for dapivirine and 25 to 350 °C for tenofovir at a heating rate of 10 °C/min under nitrogen gas with a flow rate of 40 mL/min (Model DSC821e, Mettler Toledo, Columbus, OH). System calibration was verified using indium. Tenofovir was also analyzed via thermal gravimetric analysis (model Q500, TA instruments, New Castle, DE). Tenofovir was weighed in an open aluminum pan (13.5 mg) and 20.5 °C isothermal heat was applied for 10 min followed by heating to 200 °C at a heating rate of 5 °C/min. Meanwhile, argon gas was flown over the sample and balance at 90 and 10 mL/min, respectively.

### 1.3. Vaginal ring fabrication

Solvent casting was used to load dapivirine and tenofovir in their respective polyurethane matrix. Briefly, 15 g of polymer (either NWS-PU or WS) was added to 500 mL tetrahydrofuran (THF) in 1 L roundbottom flasks. The flasks were then attached to a reflux condenser and the polymer was dissolved by stirring the solution at 60 °C for 4 h using a heating mantle controlled by a Powerstat variable transformer (Superior Electric, Bristol, CT). After complete dissolution of polymer, the contents of each flask were allowed to cool to room temperature. Dapivirine and tenofovir in THF and double deionized water, respectively, were added to their respective roundbottom flasks containing the dissolved polymer and allowed to mix for 0.5 h. Once thoroughly mixed, each flask was poured into a silanized 190 mm × 100 mm crystallizing dish and the THF was evaporated in a fume hood, resulting in polymer films. The films were then placed on high vacuum and considered free of THF when the film mass remained constant over a 24 h period. Lastly, the resulting dried matrix was cut into approximately 1 $cm^2$ pieces suitable for hot melt extrusion.

Extrusion temperatures for NWS-PU and WS polymer were chosen based upon manufacturer recommendations. Solvent casted film samples were extruded using a Minilab twin screw extruder (Thermo Haake, Waltham, MA). For each sample, extrusion was performed by setting the extruder temperature to 180 °C for WS and 175 °C for NWS-PU. When the set temperature was reached, the sample was loaded into the feed hole of the extruder. The motor screw speed was set to 50 rpm and the sample was extruded continuously by being pushed through a 4 mm aluminum die, resulting in a cooled cylindrical rod approximately 4.5 mm in cross-sectional diameter. Upon extrusion of the entire sample, the machine was purged using 5 g of Dyna-purge®.

Rings were fabricated by cutting the NWS-PU and WS extruded rods to 8.3 cm in length. A Fenner Drives polyurethane butt welding kit (Manheim, PA) was used to melt and subsequently join the NWS-PU and WS rods together. The resulting NWS-PU/WS rods were allowed to cool for 5 min before the proximal and distal ends were joined in the same fashion, resulting in two segment rings. After further cooling for 1 h, polymer stress relaxation was performed by placing the rings in a 100 °C oven for 10 min. The rings were then slightly stretched over the mouth of a truncated cone and allowed to cool. Upon completion of this step the rings were circular in geometry. Flashing at the joint was then removed with flashing scissors.

### 1.4. Mechanical testing

Rings were tested for compression/retraction force using an Instron model 3342 mechanical testing system with Bluehill Lite software (Norwood, MA). A grooved aluminum rectangular piece served to minimize ring movement during the test while a custom acrylic probe provided axial pressure which measured axial load. The custom probe consisted of a 5 mm diameter rod with a flat base at its distal end while its proximal end was secured in the jaw clamps mounted on the moving arm of the Instron (see supplementary Fig.). The probe descended at a rate of 1 mm/s to compress the ring 50% of its initial diameter. Once the compression was complete, the probe was retracted at the same rate of 1 mm/s to the starting point. The force and position data was recorded over the entire compression/retraction cycle. To evaluate ring joint integrity, tensile testing was performed on the Instron by stretching the ring at a rate of 1 mm/s up to a tensile force of 100 N.

### 1.5. X-ray scattering

The physical state of dapivirine and tenofovir in their respective polymers were further analyzed using wide angle X-ray scattering (WAXS). Scattering data was measured using an Anton Paar SAXSess line collimation instrument (Ashland, VA) with a sealed tube source and 1D elliptical focusing graded multilayer mirror optics ($d$ spacing = 4 ± 0.07 nm) with a Max Flux Osmic block collimator (flatness < 14 μm/cm). Rods of 4.5 mm cross-sectional diameter 40 mg/g dapivirine in NWS-PU and 50 mg/g tenofovir in WS were cut to 1 mm long length and the scattering intensities from samples were recorded for 2–5 min at 22 °C using a 10 mm slit beam geometry. Data were recorded using 2D position sensitive image plates (42.3 μm × 42.3 μm pixel size; sample to image plate distance, 264.5 mm; sample to detector distance, 309.0 mm). Image plates were read using a Perkin Elmer Cyclone phosphor storage system driven with OptiQuant software. Anton Parr SAXSquant2D software was used to average the 2D images over a 10 mm integration width to produce the slit smeared 1D scattering profiles as $I(q)$ versus $q$ for $q \approx 0.007$–$0.6\ Å^{-1}$ ($q = (4\pi/\lambda)\sin\theta$, $\lambda = 1.5418$ Å (Cu Kα), $\theta$ = half the scattering angle).

### 1.6. Release study

Drug loaded extruded rods were cut into approximately 15 mm long pieces. To prevent drug diffusion out of the rod ends, the rods were capped with 1/16 inch thick high density polyethylene sheets and adhered with a two component Loctite® plastic glue consisting of an activator and an adhesive. Previous work in our lab has shown there to be no difference in the release rate from end capped rods and complete IVRs as measured as amount of drug per area of device (Gupta et al., 2008). The capped rods were weighed and their dimensions were recorded. The 30 day release study was performed as previously reported (Gupta et al., 2008) using 25/75 (v/v) isopropanol/25 mM acetate buffer at pH 4.2 as the release media. Drug quantification was performed on an Agilent 1200 Series HPLC equipped with ChemStation32 software. The area of the analyte was computed for each sample using the ChemStation software. For each sample, the concentration of analyte was determined from a standard curve relating peak area to analyte concentration.

Dapivirine release was quantified as previously reported (Gupta et al., 2008) while tenofovir release was quantified via a modified HPLC method (Furst and Hallstrom, 1992). Tenofovir samples were run on a 5 μm 4.6 mm × 250 mm Agilent Zorbax ODS column at

a flow rate of 0.8 mL/min. Mobile phase A consisted of a 115 mM phosphate buffer at pH 5.5 with 1 g/L tetrabutylammonium bisulfate. Mobile phase B consisted of 30/70 (v/v) acetonitrile/buffer A. A 22 min gradient method was run (see supplementary) with a typical retention time of 10.0 min. The injection volume for all samples was 20 μL. Tenofovir was detected at λ = 214 nm.

### 1.7. Accelerated stability study

The extruded rods were cut into 50 ± 10 mg pieces and randomly selected, weighed, and placed into semi permeable or aluminum pouches. The pouches were then sealed using a heated impulse sealer, recycle setting = 3, sealing setting = 7, 155 °C (AIE Inc, Whittier, CA). The pouches were placed in a 50 °C convection oven (Thermo Haake, Waltham, MA) or a humidity stability chamber (Caron, Marietta, OH) set at 40 °C/75% relative humidity. At 0, 15, 30, 60, and 90 days, the pouches were removed and stored at −80 °C until the 90 day time point, at which time drug extraction and analysis was performed.

### 1.8. Drug extraction

Due to the contrasting hydrophilicity of dapivirine and tenofovir, a separate drug extraction method from the polymer was necessary for each drug. The dapivirine extraction utilized an internal standard (19-norethindrone).

Dapivirine-loaded NWS-PU segments were weighed and added to 5 mL volumetric flasks and dissolved in 2.5 mL of dimethylacetamide (DMA) at 250 rpm overnight. Next, 1 mL of 3.3 mM norethindrone stock in DMA was added by mass to the volumetric flask and filled to volume with dichloromethane (DCM). The flask was vortexed vigorously and 1 mL of solution was aliquoted to a 15 mL centrifuge tube. The polymer was precipitated by addition of 4 mL methanol to the centrifuge tube followed by vortexing and further addition of 5 mL of methanol. To ensure complete polymer precipitation, the centrifuge tube was spun at 1000 rpm for 5 min. Approximately 1 mL of supernatant was removed and analyzed via HPLC for dapivirine and norethindrone concentration. All samples were run on a Kromasil 10 μm, 4.6 mm × 250 mm C18 column at 25 °C with a flow rate of 1.0 mL/min. Mobile phase A consisted of 99.9% DDI water/0.1% trifluoroacetic acid (TFA), and mobile phase B consisted of 99.9% acetonitrile/0.1% TFA. A 45 min gradient method was run (see supplementary). Sample injection volume was 40 μL. Dapivirine was detected at λ = 245 nm, with a typical retention time of 18.0 min.

Tenofovir loaded WS-PU segments were weighed and added to 5 mL volumetric flasks. Approximately 3.5 mL of DMA was added to each volumetric flask. The volumetric flask was then capped and placed on the orbital shaker at 250 rpm overnight to dissolve the polymer followed by addition of DMA to fill up to volume. After vortexing, 100 μL was removed and added to a 2 mL centrifuge tube. Next, 900 μL of 50 mM ammonium acetate buffer was added and vortexed to precipitate the polymer. The supernatant was drawn up with a 1 mL syringe and pushed through a 0.2 μm nylon filter into a HPLC vial for tenofovir concentration analysis. All samples were run on a 5 μm, 150 mm × 4.6 mm Phenomenex Luna C18(2) column at 25 °C with a flow rate of 1.5 mL/min. Mobile phase A consisted of 50 mM phosphate buffer at pH 6.0 filtered through a 0.45 μm nylon filter. Mobile phase B consisted of 70% Mobile phase A/30% acetonitrile. A 40 min, four gradient method was run (see supplementary). Sample injection volume was 20 μL. Tenofovir was detected at λ = 260 nm, with a typical retention time of 12 min.

For both drugs, a control was made to determine method efficiency and quantified whenever drug extraction occurred. Drug was dissolved in DMA/DCM in a volumetric flask at a similar concentration to the expected sample (for the dapivirine control, norethindrone was also added). A portion of the solution was then removed and added to a smaller volumetric flask. Placebo polymer pellets were then added to the flask at expected sample concentration and the polymer was dissolved overnight by shaking. The solution was then filled to volume, and both solutions (with and without polymer) were aliquoted and stored. The solution containing polymer was placed through the same extraction process as actual samples. The non-polymer solution was injected directly on the HPLC and compared to the extracted control to determine the extraction efficiency of the method.

## 2. Results and discussion

### 2.1. Ring fabrication

Prior to formulation of tenofovir and dapivirine into the polymer matrices, a DSC scan of both drugs was performed to identify possible polymorphs as well as confirm their stability at extrusion temperatures. Typically, PU extrusion occurs between 100 and 200 °C for up to 30 s. Therefore, candidate drugs for the extrusion process must be able to withstand these conditions without degrading. We have previously reported a dapivirine melting endotherm at 220 °C with a small endothermic release of water at 100 °C (Gupta et al., 2008), both of which are identified in spectrum 3 of Fig. 1B. Dapivirine did not exhibit any exothermic degrada-

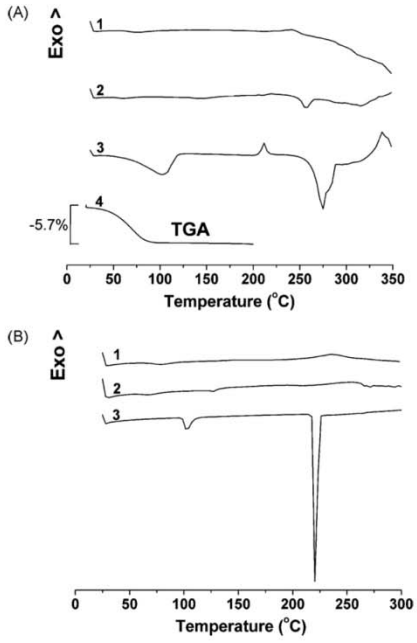



**Fig. 1.** DSC scan of (A) tenofovir and (B) dapivirine in their respective polymer matrices. Spectrum (1) corresponds to pure polymer, (2) corresponds to 10% (w/w) formulation, and (3) is the pure drug. A TGA scan was performed on tenofovir (4) to confirm that the broad DSC endothermic peak up to 100 °C was due to the release of the monohydrate from tenofovir (5.7% mass loss between 20 and 100 °C).

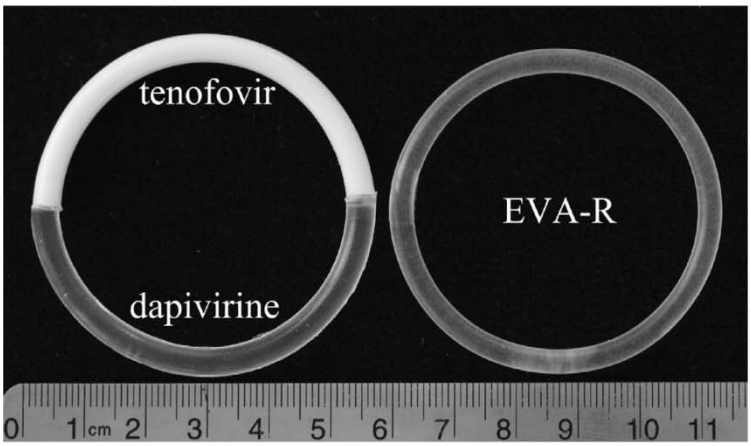



**Fig. 2.** Left: segmented ring consisting of tenofovir in WS-PU (Tecophilic® HP-60D-20) (top section) and dapivirine in NWS-PU (Tecoflex® 85A) (bottom section). Right: EVA-R (Nuvaring®).

tion peaks up to 300 °C. The melting point of tenofovir is known to be 279 °C (Merck Index, 2006). However, in spectrum 3 of Fig. 1A, tenofovir exhibited a broad endothermic peak from 25 to 100 °C. TGA combined with DSC has been used to study if additional DSC endotherms are due to water evaporation or the presence of a polymorph (Baronsky et al., 2009). Since we used the monohydrate form of tenofovir, we used TGA to determine whether the broad DSC peak was due to the release of bound water molecules from tenofovir monohydrate. As shown in spectrum 4, the 5.7% mass loss between 20 and 100 °C corresponds to 97.1% water release from tenofovir, assuming every tenofovir molecule was originally bound to a single water molecule. Following water loss, recrystallization of tenofovir occurred around 210 °C. Subsequently, melting occurred around 275 °C, consistent with literature values (Merck Index, 2006), followed by a broad exothermic peak indicative of degradation. Altogether, these results indicate that dapivirine and tenofovir were free of polymorphs and stable at extrusion temperatures.

Upon confirmation of dapivirine and tenofovir thermal stability, both drugs were solvent cast with their respective PU matrix. The resultant films were separately extruded to create dapivirine in Tecoflex® EG-85A (NWS-PU) and tenofovir in Tecophilic® HP-60D-20 (WS-PU) rods with 4.5 mm cross-sectional diameter. Afterward, equal length segments of each extruded rod were cut and heat welded together to form segmented rings as shown in Fig. 2. The segmented IVRs were fabricated to have similar geometry to the well accepted EVA-R ring.

### 2.2. Mechanical testing

The mechanical properties of an IVR, although often neglected, are likely crucial to its efficacy and acceptability in vivo. An IVR that is too stiff may be difficult to insert and could cause tissue damage and inflammation (Bounds et al., 1993; Weisberg et al., 2000), whereas a ring that is too soft may lack retention and thus slip or be expelled from the vagina (Koetsawang et al., 1990). The EVA-R ring thus serves as a good model for our design due to its reported easy insertion, retention, and biocompatibility in the vaginal tract (Barnhart et al., 2005; Novak et al., 2003). As a result, we manufactured rings of similar dimensions to EVA-R (Fig. 2) and compared their mechanical properties to EVA-R.

In our test, we compressed the rings to 50% of their initial outer diameter since this deformation is observed with EVA-R in vivo (Barnhart et al., 2005). Throughout the compression/retraction process force was recorded. Since the grade of WS-PU we used achieves 20% (w/w) equilibrium swelling in water, we examined the mechanical properties before and after equilibrium swelling (Fig. 3). At maximum ring compression, the PU ring exerted a force very similar to EVA-R both before and after equilibrium swelling. However, the PU ring demonstrated slightly lower hysteresis upon hydration as indicated by the diminished difference between the compression and retraction curves. The decreased hysteresis upon hydration is probably due to the plasticizing effect of water which reduces the glass transition temperature of the WS-PU polymer, thereby allowing the polymer chains to align and become more elastic (Siepmann and Peppas, 2001). The initial slower recoil will likely be advantageous since rapid recoil could make ring inser-

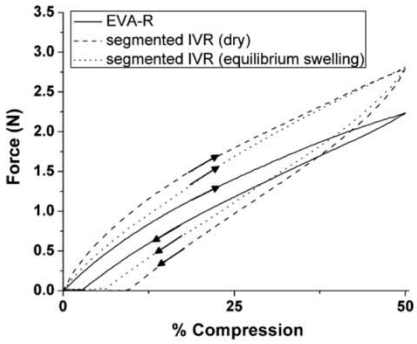



**Fig. 3.** Force versus percent ring compression for segmented and EVA-R IVRs. Equilibrium swelling, as determined by mass change, was achieved at 3 days in water. Each ring was brought to 50% compression and subsequently allowed to recover to its original diameter as indicated by the arrows.

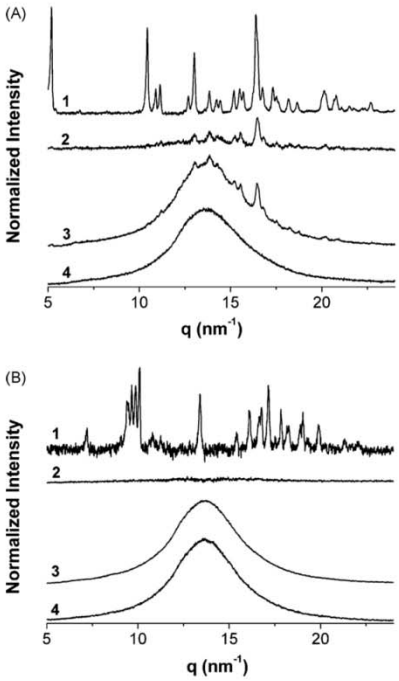



**Fig. 4.** WAXS spectra of (A) tenofovir and (B) dapivirine in their respective polymer matrices. Spectrum (1) corresponds to pure drug, (4) corresponds to pure polymer, (3) is the formulation, and (2) is the subtracted spectra of the polymer alone from the formulation spectra.

tion difficult. Once hydrated, however, the faster recoil should help ensure that the PU ring is retained in the vaginal tract since a ring that does not mechanically recover quickly could slip out of place and therefore be expelled or cause discomfort. We also compared the stress strain curves of the segmented IVRs in our compression retraction apparatus with the weld joints in the vertical and horizontal orientations. We observed little difference in the force exerted as a function of displacements for these orientations.

We also performed a simple ring tensile test before and after equilibrium swelling to ensure that our ring joint maintained mechanical integrity. Rings were stretched up to 100 N of tensile force at a rate of 1 mm/s. All PU rings tested withstood joint failure and upon test completion were able to recover their initial geometry. Altogether, these results indicate that our ring has similar mechanical properties to EVA-R and therefore should be biocompatible and easy to use.

### 2.3. IVR physical characterization

DSC and WAXS experiments were performed to characterize the physical state of dapivirine and tenofovir in their polymer matrices since the physical state of an active pharmaceutical ingredient often affects its release and pharmacokinetics (Huynh-Ba, 2009). Faheem et al. (2009) have reported crystalline dapivirine melting endotherms at 10% (w/w) concentration in silicone due to the drug's low solubility and thus dispersion in the polymer whereas we have shown absence of a dapivirine peak up to 20% (w/w) when dapivirine is dissolved in NWS-PU polymer (Gupta et al., 2008). Therefore, dispersed (but not solubilized) drug in the polymer matrix should be detectable by DSC at concentrations of 10% (w/w). As a result, we selected 10% (w/w) of both dapivirine and tenofovir in their respective polymer matrices to see whether the drugs were dissolved or dispersed. As shown in the tenofovir formulation spectrum (1) of Fig. 1, an endothermic peak around 255 °C corresponds well with the endothermic peak of the drug alone (3), implying that tenofovir is crystalline and dispersed in WS-PU (Crowley et al., 2007). However, lack of an endothermic peak in the corresponding dapivirine spectrum indicates dapivirine is dissolved in the NWS-PU matrix as previously concluded (Gupta et al., 2008). WAXS was also used to determine the physical state of dapivirine and tenofovir in the IVR since this technique is commonly used to characterize amorphous/crystalline drug domains in bulk pharmaceutical formulations (Huynh-Ba, 2009). In the resulting normalized spectrum (Fig. 4), both dapivirine and tenofovir exhibited sharp, crystalline peaks in spectra (1) whereas the broad humps in the polymer alone spectra (4) indicate there is no large scale, crystalline order on the length scale probed. By subtracting the spectra of the polymer alone from the spectra of drug-in-polymer, it is clear from spectra (2) that tenofovir maintains many crystalline, dispersed domains within the WS-PU polymer whereas dapivirine appears to be amorphous within the NWS-PU polymer. These conclusions are consistent with the DSC data as well as visual observations (Fig. 2).

In total, DSC and WAXS spectra strongly suggest that dapivirine is amorphous and dissolved and tenofovir is crystalline and dispersed in the IVR. This is of direct importance since compounds in the crystalline form often display higher stability yet lower aqueous solubility and bioavailability than when in the amorphous form (Newman and Byrn, 2003). Therefore, physical characterization is critical as it may have an impact on the performance and success of the device. As discussed further in the next section, this notion became apparent with respect to the differing release kinetics of tenofovir and dapivirine.

### 2.4. In vitro release

We have previously reported linear, loading dependent in vitro release of dapivirine from NWS-PU over 30 days (Gupta et al., 2008). Due to this success, we attempted co-delivery of both dapivirine and tenofovir from the NWS-PU matrix. Tenofovir release was barely detectable and independent of the initial tenofovir loading (data not shown). Likely, the hydrophilic tenofovir was virtually insoluble and thus dispersed in the hydrophobic NWS-PU matrix, consequently preventing tenofovir diffusion through the PU matrix. The low release rate of tenofovir from the hydrophobic PU matrix was probably exacerbated due to lack of polymer hydration, thus inhibiting water interaction with tenofovir which would enable dissolution. Subsequently, we attempted to release both dapivirine and tenofovir from the hydrophilic WS-PU matrix. Although tenofovir release was significantly improved (Fig. 5), the dapivirine release profile was nonlinear as opposed to the linear release profile previously shown from NWS-PU (Fig. 6).

As a result, we decided to pursue a segmented ring that had optimized drug release from separate polymer compositions. Tenofovir release from WS-PU polymer was analyzed to determine its release profile and dependency on initial loading. The 30 day release of tenofovir from WS-PU displayed sustained release which was dependent on tenofovir loading. However, the high tenofovir loading released more drug at later time points than the lower loadings ($p < 0.05$ at day 20 and beyond using a Student's $t$-test). The lower loadings most likely experienced early depletion of tenofovir from the polymer matrix resulting in an insufficient concentration

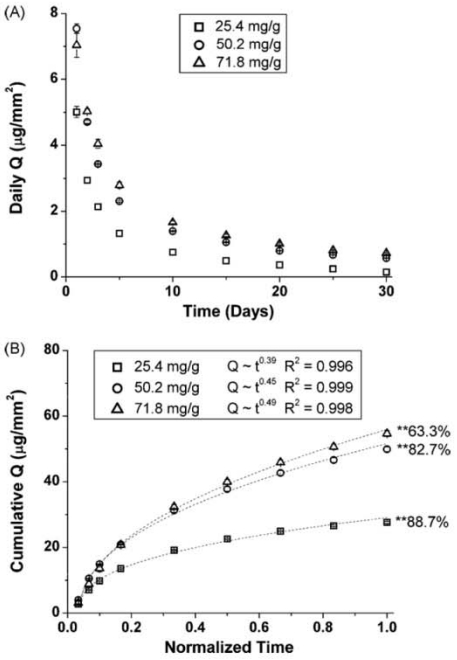



**Fig. 5.** Daily release (A) and cumulative release (B) of tenofovir as a function of time for 25.4, 50.2, and 71.8 mg/g loading concentrations in WS-PU. Mean ± SEM; $n = 4$. **wt% cumulative release of tenofovir over 30 days.

at later time points (88.7% of tenofovir was depleted from the lowest drug loading at day 30 compared to 82.7% and 63.3% for the middle and high loadings, respectively). To further compare the different release profiles we performed a power law curve fit as this is commonly used to categorize drug release from polymeric systems. As shown in Fig. 5B, the time exponent increased with drug loading. The exponent values for the low and middle loadings suggest Fickian diffusion whereas the high loading exponent corresponds

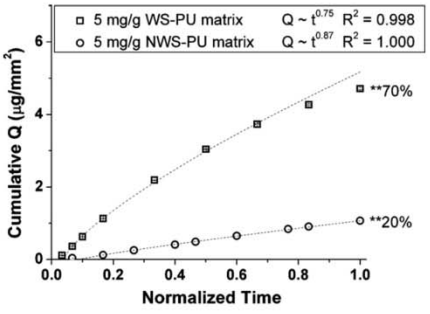



**Fig. 6.** Cumulative flux of dapivirine as a function of time for 5 mg/g loading concentrations in WS and NWSPU (Gupta et al., 2008). Mean ± SD; $n = 3$. **wt% cumulative release of dapivirine over 30 days.

to anomalous transport (release kinetics displaying both time-independent and Fickian diffusion characteristics) (Siepmann and Peppas, 2001). In sum, these results suggest that by further increasing the loading of tenofovir the release profile would become more linear, particularly at later time points.

The mechanism behind improved tenofovir release from the hydrophilic WS-PU matrix is likely analogous to that of the well studied hydrophilic polymer hydroxypropyl methylcellulose (Siepmann and Peppas, 2001). Initially, water penetration into the hydrophilic WS matrix changed the polymer and drug concentration, acted as a plasticizer, and increased the IVR dimensions. Once in contact with water inside the IVR, crystalline tenofovir previously unable to diffuse could now dissolve and diffuse out of the device due to concentration gradients. Furthermore, increased water content in the IVR amplified the tenofovir diffusion coefficient by increasing the concentration gradient. At later time the IVR core, now with diminished drug content, became more porous and less restrictive for tenofovir diffusion out of the polymer. Consequently, if WS-PU undergoes the proposed polymeric changes, it would explain the significantly higher tenofovir flux out of the WS-PU matrix than the hydrophobic NWS-PU matrix which has minimal water penetration.

Dapivirine, like tenofovir, was also released from WS-PU in a nonlinear fashion in contrast to its linear release from NWS-PU (Fig. 6). Since dapivirine was dissolved in both NWS-PU and WS-PU, the difference in dapivirine release kinetics from the two polymers is likely due to water penetration into WS-PU. For example, dapivirine was likely transported by water inside the WS-PU IVR via osmotic pressure and a steep concentration gradient unlike passive diffusion through the polymer as in the case of NWS-PU. Furthermore, swelling of the polymeric matrix likely increased the polymer mesh size, thereby increasing dapivirine diffusion. Since the cumulative dapivirine release at 30 days from WS-PU was 70% compared to just 20% from NWS-PU, depletion of dapivirine from the IVR at later time points, as in the case of tenofovir, may be an explanation for the deviation in linearity. A power law fit of dapivirine release was also performed (Fig. 6) and the time exponent correlated to anomalous dapivirine transport from the WS-PU but time-independent transport from NWS-PU (Siepmann and Peppas, 2001). We have previously mentioned possible explanations for linear release of dapivirine from NWS-PU such as differential partitioning of drug into the microcrystalline domains of the PU hard segment, thereby acting as a dapivirine reservoir which can release drug through transport channels in the abundant PU amorphous soft segments (Gupta et al., 2008). The linear release of dapivirine from NWS-PU is attractive since it should help ensure consistent dapivirine levels in the vaginal tract over the entire dosage period. As a result, we decided to pursue a segmented ring design where dapivirine is linearly released from NWS-PU.

We confirmed that the antiretroviral compounds released from the IVRs were not degraded by LC and LC/MS (see supplementary). Both dapivirine and tenofovir effectively inhibited viral replication in human cervical explants at roughly 1 nM and 16 μM concentrations, respectively (Fletcher et al., 2009; Gupta et al., 2002). In this study, we have observed dapivirine and tenofovir in vitro concentrations greater than 20 fold higher. Moreover, a recent study combining both molecules in vitro against RTI-resistant and non-resistant viral strains demonstrated a significant reduction in the $IC_{50}$ of both molecules when used simultaneously (Herrera et al., 2009). Since this IVR design is able to store and release a large amount of non-degraded dapivirine and tenofovir our data suggests that these combination IVRs warrant further study as devices to prevent HIV infection in the vaginal tract. However, the efficacy of a prophylactic IVR depends on additional factors beyond the antiviral activity of the antiretroviral agent(s); Issues such as phamacokinet-

ics, depth of tissue penetration, biodistribution, and user adherence together play important roles in the efficacy of microbicide devices (Hladik and Hope, 2009; Romano et al., 2009; Buckheit et al., 2009).

Extrapolating the cumulative flux from Figs. 5 and 6 to the segmented PU ring geometry shown in Fig. 2, we achieved roughly 64 mg tenofovir and 2.5 mg dapivirine cumulative release at 30 days for initial loadings of 71.8 and 5 mg/g, respectively. It is uncertain what quantity of dapivirine and tenofovir should be released from the IVR in vitro since a disparity often exists between in vitro release and in vivo vaginal release and subsequent biodistribution. In terms of $IC_{50}$ values, discrepancy between in vitro, in vivo biodistribution in animals, and human clinical trial data makes dosage approximation even more difficult (Klasse et al., 2006) and requires human dose ranging studies. For these reasons, controlled release is critical to the formulation of a microbicide IVR. Given that dapivirine and tenofovir show initial loading dependent release in our formulation in vitro, the loading can likely be adjusted to release an amount of drug that is safe and deemed adequate to prevent infection in humans. Furthermore, our segmented IVR design allows for adjustment of the relative length of the NWS-PU/WS segments. As a result, we can further modulate the dosage by changing the relative length of the two PU segments. Lastly, the extent of swelling in the WS polymer can be modified to control water interaction with water soluble tenofovir, thereby controlling the release rate from the polymer matrix to ensure a more sustained release profile. In total, these results suggest that the release of dapivirine and tenofovir is suitable from a dual segmented NWS-PU and WSPU IVR. Further optimization of the dosage form loading and geometry can be performed as necessary.

### 2.5. Accelerated stability

To determine the long-term stability of dapivirine and tenofovir in the IVR, a 90 day stability study was performed under accelerated conditions. We studied drug degradation using high temperature and humidity conditions since this would be indicative of long-term drug degradation under ambient conditions and may suggest possible degradation mechanisms. Samples were placed at either 40 °C/75% relative humidity or 50 °C for up to 3 months (Fig. 7). Upon conclusion of the last time point, drug was extracted from all polymer samples and quantified for percent recovery via HPLC. The results indicate no significant degradation of tenofovir in WS-PU or dapivirine in NWS-PU polymer at 90 days when comparing to day 0 using a Student's $t$-test ($p < 0.05$). Furthermore, tenofovir/dapivirine stability was not impacted by temperature or humidity as determined by 2-way ANOVA. In addition, there were no new peaks in the sample chromatograms that would indicate possible degradation. Altogether, these results indicate that dapivirine and tenofovir are stable in their respective PU segments and are suitable for long-term delivery from an IVR.

Our proposed antiretroviral delivery from a segmented PU IVR has many advantages over other IVR microbicide formulations currently being pursued. Saxena et al. (2009) have successfully formulated and released multiple antiretroviral agents from a hydrogel IVR. However, their ring fabrication relies on in situ free radical polymerization whereby residual toxic compounds in the IVR could result in complications if used long-term in vivo. Malcolm et al. (2005) have used a straightforward and proven manufacturing approach to deliver hydrophobic compounds from silicone IVRs with sustained and loading dependent release. Unfortunately, delivery of hydrophilic molecules such as tenofovir from matrix or reservoir silicone IVRs have proven problematic.

Medical grade polyurethanes, on the other hand, represent a class of polymer that can be chemically modified to satisfy the unique design specifications of vaginal delivery. Changing the composition and ratio of the hard/soft PU segments (which together form the PU structure) as well as addition of hydrophilic groups will consequently impact properties such as melting temperature, mechanical rigidity, drug stability, and release kinetics. Since most medical grade PUs can be continuously extruded or injection molded, PU fabrication on an industrial scale is simple and can be performed without the use of toxic compounds. Furthermore, extrusion and fabrication of multiple PU segments into an IVR allows for formulation optimization of drugs with different hydrophilicity, as reported herein.

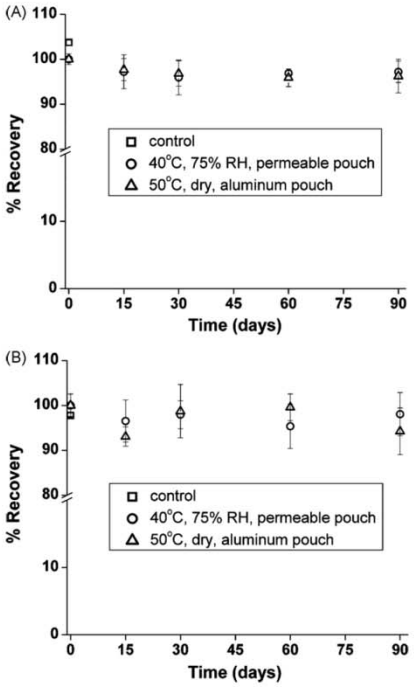



**Fig. 7.** Percent recovery of tenofovir from WSPU (A) and dapivirine from NWS-PU PU (B) as a function of time (normalized to day 0). Two environmental conditions (40 °C/75%RH, 50 °C dry) as well as packaging (permeable, aluminum) were tested. Mean ± SD, $n = 5$. All samples were analyzed on the same day along with an extraction efficiency control.

## 3. Conclusion

The results of this study demonstrate that dapivirine and tenofovir, two antiretroviral agents with contrasting hydrophilicity, can be stably formulated into a segmented polyurethane intravaginal ring for 30 day sustained release. By adjusting properties such as hard/soft segment ratio, composition, degree of hydrophilicity, and length each segment can be tailored to control the release of a specific compound. Most likely this multisegmented polyurethane approach can be used to deliver a diverse set of antiviral therapeutics from a single IVR to reduce the probability of HIV infection and the emergence of drug resistant strains. Furthermore, the simple and economical fabrication process makes them attractive for use in resource-poor regions where HIV infection is most rampant.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2009.11.007.

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CHAPTER 4

# A NINETY DAY TENOFOVIR RESERVOIR INTRAVAGINAL RING FOR MUCOSAL HIV PROPHYLAXIS

## 4.1 Abstract

A vaginal gel containing the antiretroviral tenofovir (TFV) recently demonstrated 39% protection against HIV infection in women. We designed and evaluated a novel reservoir TFV intravaginal ring (IVR) to potentially improve product effectiveness by providing a more controlled and sustained vaginal dose to maintain cervicovaginal concentrations. Polyurethane tubing of varying hydrophilicity was filled with a high density 65/33/2 weight percent TFV/glycerin/water semisolid paste and then end-sealed to create IVRs. *In vitro*, TFV release increased with polyurethane hydrophilicity, with 35 weight percent water swelling polyurethane IVRs achieving approximately 10 mg/day release for 90 days with similar mechanical stiffness to commercially available NuvaRing®. This design was evaluated in two 90 day *in vivo* sheep studies for TFV pharmacokinetics and safety. Overall, TFV vaginal tissue, vaginal fluid, and plasma levels were relatively time-independent over the 90 day duration at approximately $10^4$ ng/g, $10^6$ ng/g, and $10^1$ ng/mL, respectively, near or exceeding peak concentrations in a TFV 1% gel control group. TFV vaginal fluid concentrations were approximately 1000-fold greater than levels shown to provide significant protection in women with the TFV

1% gel. There were no toxicological findings following placebo or TFV IVR treatment for 28 or 90 days, although modest increases in inflammatory infiltrates in the vaginal epithelia were considered to be TFV- and/or ring-related and warrant additional safety and efficacy assessment. In summary, this reservoir IVR provided controlled yet significant daily release of TFV to maintain elevated sheep vaginal concentrations for 90 days and merit further evaluation as a HIV prophylactic.

## 4.2 Introduction

Recent progress in antiretroviral HIV prevention research has advanced the field from concept toward medical practice (46). The CAPRISA 004 study demonstrated that a vaginal gel containing the reverse transcriptase inhibitor tenofovir (TFV) was partially effective in preventing HIV transmission in women (1), with significant protection observed in women who maintained preventative TFV concentrations of at least 1000 ng/mL in vaginal fluid (23). However, the overall effectiveness (39%) likely was reduced by poor user adherence to the inconvenient "before-and-after-sex" dosing regimen. The correlation of adherence and TFV vaginal fluid concentrations to protection was a key finding (23, 24, 45), indicating the need for vaginal drug delivery systems that attain and maintain elevated user adherence and vaginal drug concentrations. More recently, the VOICE trial tested the same TFV 1% gel formulation as CAPRISA 004 but with a once daily dosage regimen, and failed to show any effectiveness in women. Here as well, low adherence may have contributed to the gel's inability to prevent HIV transmission (54). As a result, we (6, 21) and others (4, 35, 36, 44, 49) aim to develop

TFV drug delivery systems to provide sustained protective vaginal tissue concentrations and potentially increase user adherence.

The micromolar anti-HIV activity of TFV motivated selection of the high dose in the CAPRISA 004 trial (up to two 40 mg doses within 24 hours). Inter- and intra-user TFV vaginal fluid and tissue concentrations were likely dependent on several poorly understood factors and processes, such as adherence, time between gel applications, vaginal product clearance, menstrual cycle phase, vaginal fluid volume and composition, and frequency of intercourse (i.e., more sex acts over a given time would result in higher vaginal TFV administration since the dosing regimen was coitally dependent). Moreover, episodic dosage forms like gels are intrinsically short acting; the TFV 1% gel formulation attains peak vaginal tissue concentrations in women two hours post-vaginal application and diminishes rapidly thereafter (45). Finally, the anatomical site and kinetics of HIV transmission itself are poorly understood (19). Therefore, a drug delivery system that maintains elevated yet controlled and consistent TFV cervicovaginal tract drug concentrations over a duration longer than HIV transmission and throughout multiple episodic HIV exposures has the potential to increase efficacy over the dynamic drug levels provided by vaginal gels.

The silicone intravaginal ring (IVR), invented in 1970 (8, 34), was designed to elute hormones for a 30 day duration and provide sustained drug levels in the range of 10 to 100 μg/day. Since then there has been little innovation in IVR technology. In fact, current IVR technology is inadequate to meet the high topical dose requirements of TFV. Groups have claimed successful TFV formulation and delivery from silicone- and ethylene vinyl acetate-based IVRs (4, 35, 36, 44, 49), yet the *in vitro* and *in vivo* daily

delivery rates reported were micrograms, rather than milligrams, as is required for HIV prophylaxis with TFV. The TFV release from silicone and ethylene vinyl acetate polymers is therapeutically insignificant primarily due to TFV's hydrophilicity and resultant low solubility in these elastomeric polymers commonly used for IVRs.

Although IVRs have a higher per-unit cost compared to gels, for a global health application they offer the advantage of distributing the per-unit cost over many days, weeks, or months (15). Therefore, from an economic perspective a long-lasting and low manufacturing-cost IVR could be more affordable than a frequently applied gel since hundreds of gel units would be needed per year compared to several rings. However, developing a long-duration IVR to deliver milligrams-per-day of TFV for at least 90 days requires a design capable of delivering approximately 1 gram of TFV from a 3 - 6 gram IVR. With such a high weight fraction of drug incorporated in the IVR, maintaining both time-independent TFV release and ring mechanical stiffness for a 90 day duration presents a significant design challenge. We recently reported TFV delivery from hydrophilic polyether urethane (HPU) matrix IVRs formulated with up to 20 weight percent (wt%) TFV (6, 21). Although TFV release rates were greatly improved compared to hydrophobic polymer IVRs, matrix IVRs inherently demonstrate decreased drug release rates with time. Matrix IVRs with a high fraction of undissolved drug similarly display time-dependent decreases in mechanical stiffness as the drug is released. Therefore, we developed and evaluated *in vitro* and *in vivo* the first reservoir IVR using a water-absorbable polyurethane as a rate controlling membrane capable of delivering 10 to 30 mg of TFV daily for up to 90 days. Herein, we report the ring's *in vitro/in vivo* TFV release, mechanics, and vaginal safety and TFV pharmacokinetics in a sheep model.

## 4.3 Materials and Methods

### 4.3.1 Materials

Hydrophilic aliphatic polyether urethane (HPU) Tecophilic® HP-60D-20, HP-60D-35, and HP-60D-60 grades were purchased from Lubrizol Advanced Materials (Wickliffe, OH), with equilibrium aqueous mass absorption of approximately 20, 35, and 60 wt% and shore hardnesses of 43D, 42D, and 41D, respectively. A custom-synthesized 35 wt% swelling HPU with 78A shore hardness was provided by DSM Biomedical (Berkeley, CA). Tenofovir monohydrate was supplied by Gilead Sciences (Foster City, CA). TFV 1% gel was supplied by Patheon Pharmaceuticals (Cincinnati, OH). USP grade glycerol and water were purchased from Spectrum Chemical (New Brunswick, NJ). Starch 1500® USP grade partially pregelatinized maize starch was provided at no cost by Colorcon (Harleysville, PA). Unless noted, all solvents and reagents were ACS grade.

### 4.3.2 Ring fabrication

HPU resins were dried overnight in a compressed air micro dryer (Dri-Air, East Windsor, CT) to less than 0.05 wt% water content as determined using Karl Fisher titration (Mettler-Toledo, Columbus, OH). The dried pellets were fed into a 3/4-inch single screw hot-melt extruder attached to an advanced torque rheometer drive (C.W. Brabender, South Hackensack, NJ) with a tubing crosshead (Guill Tool, West Warwick, RI). The extruder heating zones (1 - 3) were set at 150, 160, 170°C and the tubing crosshead tip and die temperatures were 150°C and 130°C, respectively. Upon leaving the crosshead, the extrudate was drawn down using a CPC2-12 combination puller/cutter (Conair, Cranberry Township, PA) to create a final tubing product with 0.7 mm wall

thickness and 5.5 mm cross-sectional diameter. The extruded tubes were cut to 171 mm in length, weighed, and the tubing lumens were filled with either 100% TFV powder or a 65/33/2 wt% TFV/glycerol/water mixture, resulting in 1.6 g of TFV loaded into each IVR lumen. The TFV/glycerol/water semisolid was mixed using a Hobart mixer with "B" beater attachment (Troy, OH) and back-filled into the tubing lumen using a high pressure hydraulic filling system (Dymax, Torrington, CT). TFV powder was manually filled into the tubing lumen. The tubing ends were sealed using an induction welder equipped with a stainless steel reverse bonding die, and the resultant plugged ends were welded together using a stainless steel split die induction welder (PlasticWeld Systems, Inc., Newfane, NY) to create an IVR. A custom machined 12 cavity aluminum mold was used to shape anneal the IVRs in a circular conformation and minimize tubing lumen kinking. IVRs were placed in the mold which was first heated via water circulation to 65°C for 15 minutes followed by 5 minutes cooling at 10°C.

4.3.3 *In vitro* TFV release and IVR mechanical testing

*In vitro* release and ring mechanical compression testing were performed as previously described (6, 21). Briefly, IVRs were immersed in 50 mL of 25 mM sodium acetate buffer (pH 4.2) at 37°C and 80 rpm. Release media was periodically collected for TFV concentration measurement by high-performance liquid chromatography (HPLC) and replaced every 24 hours to maintain sink conditions. Simultaneously, IVRs were periodically subjected to mechanical compression testing. Briefly, a custom machined probe attached to an Instron 3342 uniaxial mechanical testing system was used to compress the IVR 25% of its initial 55 mm diameter at a rate of 1 mm/sec and record the

result required force. The force value corresponding to 10% ring compression was compared across all samples.

4.3.4 Drug content analysis for *in vitro* studies

TFV *in vitro* release sample concentrations were determined by HPLC using a method previously described (6). Briefly, 2 μL of sample was injected on an Agilent 1200 series HPLC with diode array detector and Phenomenex Luna C18 5 μm, 150 x 4.6 mm column. A 15 minute gradient method consisted of 100% mobile phase A (potassium phosphate buffer, pH 6.0) switching to 100% mobile phase B (acetonitrile/ potassium phosphate buffer, pH 6.0) upon run completion. The flow rate was 1.5 mL/minute with a typical TFV retention time of 7 min with TFV detection at 260 nm. Cumulative percent release was calculated by numerically integrating between collection time points using the trapezoidal rule and subsequently dividing by the original amount of TFV in the IVR.

4.3.5 Sheep pharmacokinetic study (study 1)

The 78A shore hardness HPU IVR (35 wt% swelling) and the clinically tested TFV 1% gel were comparatively evaluated for pharmacokinetics in 1 - 2 year old Dorset Crossbred sheep. The study was conducted at MPI Research (Mattawan, MI), which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The study protocol was reviewed and approved according to the standard procedures of the performing laboratory's Institutional Animal Care and Use Committee. The sheep were housed indoors, and fluorescent lighting was provided for approximately 12 hours per day. On occasion, the

dark cycle was interrupted due to study-related activities. Reproductive cycles were not synchronized, and the study was performed September through December. Eight animals in treatment Group 1 received a TFV IVR that was inserted in the posterior vagina on day 0 and retained in the vaginal tract for 90 days. The exterior of the vagina was cleaned with chlorhexadine solution and the IVR was inserted as aseptically as practical using a gloved hand. Following insertion, a speculum was used to confirm correct placement of the ring and the animals were examined daily for evidence that the ring was still in place. If the vaginal ring was expelled or otherwise needed to be replaced, a new ring was inserted and the old ring was recovered for residual drug content analysis (if found). Seven animals in treatment Group 2 received once daily vaginal administration of 4 mL of the TFV 1% gel for 28 days (40 mg/day). Each gel dose was administered using a pre-filled, single-dose polypropylene applicator (HTI Plastics, Lincoln, NE) similar to that used in the clinical gel administration regimen. Vaginal tissue, vaginal fluid, and blood plasma periodically were collected for drug content analysis throughout the dosage duration and up to three days following the last gel dose or IVR removal, as depicted in Figure 4.1. Blood samples (approximately 1 mL) were collected from all animals via the jugular vein, placed in tubes containing $K_3$EDTA anticoagulant, and centrifuged under refrigeration to isolate the blood plasma. At all specified time points, two Weck-Cel® (distributed by Beaver-Visitec International, Inc., Waltham, MA) swabs and biopsies were collected per animal for TFV concentration determination in vaginal fluid and tissue, respectively, one proximal to the ring (posterior vagina, 5-7 cm from the introitus) and one distal to the ring (2-4 cm from the introitus). Weck-Cel swabs were pre-weighed, inserted and allowed to absorb fluid for approximately 1 minute and subsequently were

removed and re-weighed to determine fluid mass uptake. A Kevorkian-Younge biopsy forceps (Miltex, York, PA) and speculum were used to acquire approximately 50 mg vaginal tissue biopsies. Local and general anesthetics and analgesics were given on the day of biopsy collection and a chlorhexidine solution was used to clean the exterior vagina. Vaginal tissue biopsy location (left or right side) was alternated with timepoint to minimize tissue trauma and encourage healing. If bleeding was observed after biopsy collection, direct pressure with gauze or gauze with thrombin was applied to encourage clotting. Upon completion of the 90 day time point, IVRs were removed and analyzed for remaining drug content as described below. At study termination on days 93 (Group 1, 72 hours post-IVR removal) and 31 (Group 2, 72 hours post-last gel dose), surviving animals were euthanized by an intravenous overdose of sodium pentobarbital solution followed by exsanguination via severing the femoral or axillary vessels, and vaginal tissues were collected for final drug content determination. All samples (blood plasma, Weck-Cel swabs, and tissues) were snap frozen and stored at -70°C until bioanalysis was performed, as described below.

4.3.6 TFV measurement in plasma, vaginal fluid, and vaginal tissue

TFV was extracted from vaginal tissue, vaginal fluid, and plasma and quantified by LC/MS/MS similar to methods described elsewhere (7, 36). Briefly, for TFV plasma quantification, 100 µL of standard, quality control sample or study sample was mixed with 50 µL of a working internal standard TFV-$d_6$ solution at 50 ng/mL. A 500 µL aliquot of 0.5% formic acid in water was added to all plate wells, and the samples were transferred to a pre-conditioned Oasis MCX solid-phase extraction plate. The plate was

then washed with 800 µL of 0.5% formic acid in water and 800 µL of methanol, followed by sample elution with 600 µL of 5:95 ammonium hydroxide:methanol. The eluent was evaporated to dryness, and the residue was reconstituted with 200 µL of 0.1% ammonium hydroxide in water. The samples were vortexed and an aliquot of each sample was injected onto an LC-MS/MS system for analysis. Similarly, TFV was measured in tissue by mixing 200 µL of standard, quality control sample or study sample with 50 µL of a working internal standard solution containing TFV-$d_6$ at 50 ng/mL. The samples were vortexed and centrifuged, and an aliquot of each sample was injected onto an LC-MS/MS system for analysis. Lastly, TFV levels in swabs were processed by mixing 50 µL of standard, quality control sample or study sample with 50 µL of a working internal standard TFV-$d_6$ solution (200 ng/mL) in a tube containing a clean spear or study sample. A 950 µL aliquot of 50:50 methanol:water was added to the standards and quality control samples, whereas a 1000 µL aliquot of 50:50 methanol:water was added to the study samples. The samples were vortexed and centrifuged, and an aliquot of the supernatant was injected onto an LC-MS/MS system for analysis.

The chromatography varied with sample type: plasma samples were analyzed using a Phenomenex Synergi Polar-RP column, 75 mm x 2 mm (4 µm particle size) with a gradient flow of 0.1% acetic acid in water, 0.1% acetic acid in acetonitrile, and 0.2% ammonium hydroxide in water at a flow rate of 300 to 500 mL/minute. Swabs were analyzed using the same column but with an isocratic flow of water/acetonitrile/acetic acid/ammonium hydroxide (930:70:5:1) (v/v/v/v) at a flow rate of 200 µL/minute. Tissue samples were analyzed using a BioBasic AX column, 50 x 3.0 mm (5 µm particle size) with a gradient flow of acetonitrile:10 mM ammonium acetate in water at pH 6 (30:70)

and acetonitrile:1 mM ammonium acetate in water pH 10.5 (30:70) at a flow rate of 400 to 1000 mL/minute.

The analyte and internal standards were detected using a Sciex API 5000 triple quadrupole LC-MS/MS system equipped with an ESI (TurboIonSpray®) ionization source operated in the positive and negative ion mode. Multiple-reaction-monitoring mode transitions of the respective ions were used to monitor TFV and TFV-$d_6$ and may have been slightly modified to optimize system performance. For TFV, m/z 288 to 176 was monitored with a retention time of 1.15 – 3.67 min. For TFV-$d_6$, m/z 294 to 182 was monitored with a retention time of 1.15 – 3.65 min. The lower limits of quantification (LOQ) for TFV in plasma, Weck-Cel, and vaginal tissue were 1.00 ng/mL, 5.00 ng/spear, and 20.0 ng/g, respectively.

4.3.7 TFV pharmacokinetic analysis

Individual pharmacokinetic parameters were determined by non-compartmental methods using WinNonlin® Phoenix software (Pharsight Corporation, Sunnyvale, CA). Area under the curve (AUC) estimates were determined using the linear trapezoidal rule with the linear interpolation calculation method. Values below the respective LOQs were treated as "0" for the analysis. Nominal sample collection time was used for the analysis. Pharmacokinetic parameters were defined as follows: $T_{max}$: Time of maximum observed concentration; $C_{max}$: Maximum observed concentration occurring at $T_{max}$; and $AUC_{0\text{-}90\ days}$: Area under the curve from the time of dosing to 90 days (IVR group only).

4.3.8 Quantification of IVR residual TFV content

TFV was extracted out of the recovered IVRs to determine the total amount of TFV released. Each IVR was cut into approximately 1 cm segments that were placed together in a 50 mL volumetric flask, and 100 mM pH 7.4 phosphate buffer was added to dissolve the entirety of the residual TFV. After removing the polymer segments, an aliquot of the resulting solution was diluted volumetrically and analyzed for TFV content by HPLC as previously described (21). Original semisolid material used for IVR manufacture was kept and extracted in the same manner to calculate percent recovery based on IVR fill mass.

4.3.9 Sheep toxicology study (study 2)

The local tolerance and systemic toxicity of the 78A shore hardness HPU TFV IVR (35 wt% swelling) was assessed in female Welsh Mule sheep (age: 49 months; weight at start of study was 59.5 - 83.5 kg) over a 1 month (28 day) or 3 month (90 day) period, in comparison with a placebo IVR or sham control (i.e., underwent restraint and sham insertion, but no ring was inserted). The placebo IVR consisted of 60/38/2 wt% starch/glycerol/water in the tubing lumen but otherwise the IVR was manufactured using identical HPU tubing, processes, and equipment as the TFV IVR. The study was conducted at Huntingdon Life Sciences (Huntingdon Life Sciences, United Kingdom), which is fully accredited by AAALAC International. The study design, outlined in Table 4.1, was based on the current International Conference on Harmonisation (ICH) Harmonised Tripartite Guidance on Non-Clinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals (20), and the

study protocol was reviewed and approved according to the standard procedures of the performing laboratory's Ethical Review Process Committee. Physical examinations were conducted on all animals pre-treatment. The animals were observed at least twice daily throughout the study for any clinical abnormalities or signs of reaction to treatment. More frequent observations were made on the day of dosing. Body weights were recorded weekly and food consumption was recorded on a daily basis. Hematology, blood chemistry, and urinalysis were conducted on all animals pre-treatment and pre-termination (day 28 or day 90). Upon termination of the study, all animals were sacrificed and gross necropsy was conducted. Most major body organs were isolated and weighed. Tissues were then fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at approximately 4 - 5 μm thickness and stained with haematoxylin and eosin (H&E) for histological examination by light microscopy.

In addition to the standard analyses described above, H&E stained slides of the posterior vaginal tissue (i.e., proximal to IVR location) were used for vaginal irritation scoring in accordance with the rabbit vaginal irritation method of Eckstein *et al.* (9). Approximately 10 areas were analyzed using a Nikon 600 microscope and individual scores from 0 - 4 were assigned depending on the extent of congestion, edema, leukocyte infiltration and epithelial damage. The individual scores were combined to yield a total group score (0 - 16) that in rabbits correlates with human irritation potential.
Similar to the sheep pharmacokinetic study, TFV IVRs were retrieved from the animals prior to termination at 28 and 90 days and analyzed for remaining drug content as described above. In addition, blood plasma, vaginal fluid (Weck-Cel swabs), and vaginal

tissue was collected from the TFV IVR treated animals prior to termination and analyzed for TFV content using methods described above.

4.3.10 Statistical analysis

Data were analyzed using one-way and two-way ANOVA and unpaired two-tailed Student's t-test. A p-value less than 0.05 was considered statistically significant.

## 4.4 Results

4.4.1 *In vitro* characterization and optimization

An elastomeric HPU tubular IVR design was utilized as it maximized the weight ratio of drug to total device, could be filled with solid or liquefied drug, and provided sufficient time-independent mechanical stiffness and drug release. Previous research beyond the scope of this publication determined that HPU IVRs of 5.5 mm outer cross-sectional diameter, 0.7 mm wall thickness, and 55 mm outer diameter (Figure 4.2) would provide ring mechanical properties, lumen volume, and drug release kinetics suitable for the proposed TFV IVR (see Chapter 5). The *in vitro* TFV release rate was evaluated using various core compositions and equilibrium percent swelling HPU IVRs. IVRs containing only TFV powder in the lumen showed low yet steadily increasing TFV release rates with time which did not reach steady-state by 28 days (Figure 4.3A). When the highly water miscible and HPU-permeable compound glycerol was added into the tubing lumen, the lumen was rapidly hydrated and attained the equilibrium TFV release rate after only a 1 day transient state. The TFV release rate increased with polymer equilibrium swelling for 20, 35, and 60 wt% swelling Tecophilic HPUs. The 20 wt%

swelling IVRs did not achieve the desired 10 mg/day minimum release rate and were stopped after 28 days, whereas the 35 and 60 wt% swelling IVRs achieved approximately 17 and 25 mg/day steady-state release rates, respectively. However, TFV was depleted from the 60 wt% swelling IVRs before the target 90 day duration. The TFV percent cumulative release profile was linear until greater than 90% of the initial drug load was depleted from the IVRs (Figure 4.3B). The softer shore hardness 35 wt% swelling HPU IVR released 10 mg/day of TFV for 90 days (Figure 4.4A) and demonstrated mechanical stiffness similar to NuvaRing (Figure 4.4B). Furthermore, the HPU IVR stiffness did not change considerably with time when comparing the dry state, hydrated state, and after a majority of the TFV was released (i.e., 0, 1, and 90 days). Ten percent ring compression values were compared as this is approximately the *in vivo* compression of NuvaRing, as determined by Magnetic Resonance Imaging (3).

### 4.4.2 TFV pharmacokinetics in sheep (study 1)

The HPU TFV IVRs were evaluated in a sheep model to compare the TFV pharmacokinetics to the clinically tested TFV 1% gel. Throughout the 90 day duration one animal expelled multiple rings, one animal expelled one ring, and two animals required a replacement ring due to a ring manufacturing defect and a procedural error during pinch biopsy collection which damaged the ring. However, pharmacokinetic data from these sheep did not differ from sheep that retained a single IVR for the full 90 day duration and thus no data were excluded. One sheep in the TFV gel group expired on day 15, with complications from anesthesia being determined as the cause of death (non-treatment related). The calculated time-averaged *in vivo* TFV release rate from devices

that were retained for 90 days *in vivo* was 17.0 ± 1.1 mg/day (mean ± standard deviation, N = 5) as determined by residual TFV extraction, approximately 70% higher than the 10 mg/day *in vitro* release rate.

TFV vaginal fluid concentrations from the IVR group attained their steady-state levels of approximately $10^6$ ng/g by day 1, which were maintained for the remainder of the 90 day duration (Figure 4.5A). Mean TFV concentrations from IVR insertion through 90 days were 3.5-fold higher proximal to the ring than distal. Comparatively, steady-state mean TFV vaginal fluid concentrations from the IVR group (approximately $10^6$ ng/g) were similar to mean TFV vaginal fluid concentrations at 8 hours post-gel dose (Figure 4.5B). Mean TFV concentrations from the gel group were 1.4-fold higher proximal than distal. Up to 3 days beyond IVR removal (Figure 4.6) and after the last gel dose, vaginal fluid concentrations for both groups were approximately $10^4$ ng/g.

Eight hours post-IVR insertion, TFV vaginal tissue concentrations were approximately $10^2$ ng/g (Figure 4.7A). TFV tissue concentrations at day 14 (the next biopsy time point) through day 90 were approximately $10^4$ ng/g, with concentrations trending slightly higher with time. For the full duration of IVR residence in the vaginal tract (0 to 90 days), mean TFV tissue concentrations were similar proximal and distal to the ring. Mean TFV tissue concentrations were $10^4$ ng/g 8 hours after the $15^{th}$ gel dose, similar to day 14 TFV concentrations with the IVR group (Figure 4.7B). Eight hours after the $15^{th}$ dose and 24 hours after the $28^{th}$ gel dose, mean TFV levels decreased to approximately $10^3$ and $10^2$ ng/g, respectively. TFV tissue concentrations 3 days after IVR removal and 3 days after the last gel administration were significantly lower, with several biopsies from both groups below the LOQ. Across all gel group time points, mean TFV

tissue concentrations were 3.1-fold higher proximal than distal. TFV-diphosphate concentration determination in vaginal tissue was also attempted but was undetectable or highly variable in both IVR and gel groups and thus was excluded from further analysis (data not shown).

Mean TFV plasma concentrations at day 7 through day 90 from the IVR group were steady at approximately 15 ng/mL, with the exception of day 14 where mean concentrations were  28 ng/mL (Figure 4.8A). In contrast, the gel group achieved similar TFV concentrations at 2 hours but rapidly decayed thereafter (Figure 4.8B). Also in the gel group, mean post-dose TFV plasma concentrations decreased with increased dose (mean TFV concentrations from the $1^{st}$ dose > $15^{th}$ dose > $28^{th}$ dose). TFV was undetectable in plasma at 24 hours post-IVR removal or gel dose. TFV pharmacokinetic parameters for vaginal fluid, vaginal tissue, and plasma (Table 4.2) were consistent with the data presented above.

### 4.4.3 Toxicological evaluation

A second sheep study was performed to evaluate the systemic toxicity and local irritation potential of TFV IVRs compared to placebo IVRs and a sham control. IVRs were well tolerated and retained, with only one IVR expulsion noted on the day of scheduled removal (day 90).  No treatment-related toxicological findings were observed regarding body weight, food consumption, hematology, blood chemistry, urinalysis, organ weight, and gross pathology. As determined by histopathology, the degree or incidence of leukocytic infiltration in the vagina at 1 and 3 months increased with ring presence although no epithelial disruption was observed (TFV or placebo, Figure 4.9).

When quantifying the irritation potential in sheep, slight increases in the mean vaginal irritation scores were observed for both placebo and TFV IVRs compared to sham controls (Figure 4.10). Specifically, mean vaginal irritation scores for sham, placebo IVRs, and TFV IVRs at 1 month were 1.8, 2.4 and 3.2, respectively, and at 3 months were 2.2, 3.2 and 3.8, respectively. Differences between the TFV IVR and sham control were found to be statistically different at both time points ($p=0.03$ and 0.01 for 1 and 3 months, respectively).

Upon animal necropsy at 28 and 90 days, IVRs were recovered for drug content analysis and plasma, vaginal fluid, and vaginal tissue were analyzed for TFV concentration. The time-averaged TFV release rate of IVRs retrieved from sheep after 28 and 90 days of treatment was $14.0 \pm 2.8$ and $16.9 \pm 1.6$ mg/day, respectively (mean ± SD, N = 5). TFV vaginal tissue, vaginal fluid, and plasma concentrations at 28 and 90 days were generally similar to the pharmacokinetic study (Table 4.3).

## 4.5 Discussion

We designed and tested a new reservoir intravaginal ring for long-duration TFV vaginal delivery. IVR fabrication incorporated materials and manufacturing methods commonly utilized by the pharmaceutical and medical device industries, making the IVR a scalable and cost-effective product for resource-poor regions where the HIV pandemic is most prevalent (51). The 35 wt% swelling HPU released at least 10 mg/day of TFV *in vitro* for 90 days with time-independent IVR mechanical properties. In sheep receiving IVRs, TFV concentrations in plasma, vaginal fluid, and vaginal tissue were near time-independent for 90 days at levels similar to peak concentrations in the TFV 1% gel group.

Lastly, in the sheep safety study, no significant toxicological effects were observed although modest increases in inflammatory infiltrates in the vaginal epithelia were considered to be TFV- and/or ring-related.

4.5.1 *In vitro* studies

The steady-state *in vitro* TFV release rate increased with HPU equilibrium swelling, thus allowing simple modulation of the release rate by selecting an HPU with appropriate equilibrium swelling. Since TFV is virtually insoluble in the non-swelling polymer phase (6), increased water presence allowed for the water-soluble TFV to dissolve and diffuse through the aqueous phase of the hydrated polymer. The lower durometer 35 wt% swelling HPU released approximately 10 mg/day TFV for 90 days with time-independent IVR mechanical stiffness similar to NuvaRing, which was utilized as a benchmark for mechanical stiffness due to its high user acceptance and extensive safety record in women (43).

Glycerol incorporation in the IVR core formulation yielded several benefits over 100% TFV powder including easier lumen filling and increased TFV density to maximize TFV loading. Although TFV comprised approximately 36% of the ring's total mass, the ring mechanical stiffness remained unchanged throughout the 90 day duration even after the majority of the TFV was released. This unique performance attribute was due to the semisolid core whose presence or absence did not significantly impact the overall ring elasticity, in contrast to the solid core TFV matrix IVR that was initially quite stiff but softened upon hydration and as TFV was released (6).

Glycerol also acted as an osmotic agent to rapidly draw water into the TFV-loaded core and nearly eliminated the time to steady-state TFV release. Following initial HPU hydration, we hypothesized that the low amount of water at the inner surface of the HPU lumen mixed with the high concentration of glycerol present, resulting in a large inward osmotic driving force since glycerol and water are infinitely miscible. Following lumen hydration, TFV quickly saturated the aqueous solution to establish a fixed TFV concentration gradient across the tubing wall and allow TFV release by membrane-controlled steady-state diffusion. In the absence of a highly water-miscible molecule in the tubing lumen, TFV steady-state release was not achieved after several weeks since TFV alone is a relatively poor osmotic agent (~1% w/v solubility in the acidic aqueous media utilized) (6). The excess of undissolved and highly mobile TFV in the tubing lumen allowed for true time-independent TFV release until virtually the entire load was depleted, thus minimizing drug waste. Conventional solid core polymeric reservoir IVRs, such as NuvaRing, create environmental and cost concerns since up to 85% of the initial drug load remains in the IVR at the end their planned durations (60).

Another advantage of the HPU reservoir IVR design is its tunability, where HPU equilibrium swelling, cross-sectional diameter, wall thickness, and shore hardness (elastic modulus) may be independently varied to achieve the desired TFV loading, TFV release rate, and ring stiffness. Commonly utilized silicone IVRs typically demonstrate a lower elastic modulus than thermoplastic elastomers and therefore require a wider cross-sectional diameter and/or the addition of an inert filler excipient to increase ring stiffness (12). As increasing IVR dimensions may negatively impact user acceptability (48), we

minimized the HPU IVR cross-sectional diameter while still ensuring that the target TFV release rate and duration was achieved.

When accounting for the overall societal costs associated with HIV infection including healthcare and inability to work, TFV 1% gel is expected to be quite cost effective by South African standards at a projected cost of approximately $5/month (58, 61). The 90-day reservoir TFV IVR, with an estimated per-unit manufacturing cost of less than $1 (personal communication and estimate from ProMed Pharma LLC, Plymouth, MN), should therefore cost significantly less per month than the gel. Despite its unconventional design, the reported IVR combines existing pharmaceutical and medical device materials, techniques, and equipment to minimize associated manufacturing costs and ensure affordability in resource-poor countries. From a supply chain and distribution perspective, a year's supply of gel would require significant transportation efforts both for the supplier and the end user as compared to 4 rings. In addition to lower transportation costs and end-user convenience, the IVR would also minimize waste management issues associated with the number of gel applicators and packaging required for frequent use.

4.5.2 *In vivo* pharmacokinetics

In the CAPRISA 004 clinical trial evaluating the TFV 1% gel, women with vaginal fluid concentrations greater than $10^3$ ng/mL were significantly more protected against HIV infection than women with concentrations less than $10^3$ ng/mL (23). In the sheep pharmacokinetic study described in this report, the TFV IVR attained near steady-state vaginal fluid concentrations at day 1 ($10^6$ ng/g or ng/mL), which were

approximately 1000 times higher than the clinically protective concentration, and this level was maintained for the remainder of the 90 day duration. Vaginal dosing of the TFV 1% gel both in a controlled clinical pharmacokinetic study previously reported by Schwartz *et al.* (45) and in our sheep gel group demonstrated peak $10^6$ ng/g TFV vaginal fluid concentrations several hours post-dose which were equivalent to the observed steady-state TFV IVR concentrations.

Although TFV tissue concentrations were not measured in the CAPRISA 004 trial, the clinical pharmacokinetic study by Schwartz *et al.* reported approximately $10^5$ ng/g peak vaginal tissue concentrations at 2 hours post-dose which plateaued at around $10^4$ ng/g thereafter (45). Our sheep gel group attained approximately $10^3$ - $10^4$ ng/g tissue levels 8 hours post-dose, but were lower at 24 hours post-dose. TFV tissue concentrations at 8 hours post-IVR insertion were comparatively lower than 8 hours post-gel dose. However, mean TFV tissue concentrations of approximately $10^4$ - $10^5$ ng/g were observed at the next sampling time point (day 14) through the remaining dosing duration (day 90) which were similar to $C_{max}$ gel tissue concentrations. Since tissue biopsy time points between 8 hours and 14 days were not collected, it is not known whether similarly high tissue concentrations were achieved earlier than 14 days with the TFV IVR. However, since vaginal fluid and plasma concentrations were near steady-state by days 1 and 7, respectively, it seems probable that near steady-state tissue levels were reached within the first several days as vaginal tissue acts as the intermediary compartment for drug between vaginal fluid and the systemic circulation (62). The elevated sheep tissue TFV concentrations reported herein from the TFV HPU IVR were approximately 1000 times

higher than sheep tissue TFV concentrations from an alternative TFV IVR design (35), reflecting the nearly 1000-fold greater TFV *in vitro* release rate from the HPU IVR.

The mean TFV concentrations in vaginal fluid, vaginal tissue, and plasma in the IVR group were similar to or greater than peak concentrations attained in the gel group. This is an interesting result considering that the time-averaged daily TFV release from the IVR, as determined by residual device extraction, was less than one-half of the daily TFV gel dose. The percent of dosed TFV which is absorbed following TFV 1% vaginal gel administration has not been reported, but it is likely that a significant fraction is not absorbed because the gel leaks out of the vaginal tract (2, 33). The gel more rapidly achieved high TFV vaginal and systemic concentrations and therefore may be advantageous for coitally dependent, intermittent use where it is applied just prior to sex (similar to CAPRISA 004 dosing). Conversely, the IVR may be advantageous when women desire a long-lasting, coitally independent, and discreet dosage form.

It is generally assumed that antiretrovirals should be well distributed throughout the vaginal tract to maximize HIV prevention. TFV vaginal fluid and tissue concentrations measured proximal and distal to the IVRs' placement were nearly identical over the 90 day period and trended similar to the TFV 1% gel. This finding corroborates previous TFV IVR pharmacokinetic studies in sheep where TFV levels proximal and distal were indistinguishable (36). Conversely, vaginal biodistribution of hydrophobic antiretrovirals delivered from IVRs has been evaluated in women and macaques and both studies have shown a higher drug concentration proximal to the ring than distal (22, 40). Therefore, if uniform vaginal biodistribution proves necessary,

hydrophilic antiretrovirals such as TFV may be better suited for IVR delivery than hydrophobic antiretrovirals.

Reservoir drug delivery devices typically offer near time-independent release of molecules given sink conditions are satisfied (25, 42). Time-independent TFV release rates were attained by day 2 *in vitro*, and similarly TFV vaginal fluid concentrations stabilized by day 2 *in vivo*, suggesting that the device was releasing drug in a zero-order fashion as expected. Following a 3 - 7 day lag time, TFV plasma concentrations in the IVR group were steady for the remaining 90 days with the exception of day 14. It is known that hydrophobic compounds can be more rapidly transported across the epithelium for systemic absorption than hydrophilic compounds (62), yet there has been limited reports of hydrophilic API plasma pharmacokinetics from IVRs, in part due to IVR formulation limitations of such APIs as previously discussed. Also, the reported TFV IVR did not provide an initial burst of drug as do conventional matrix and reservoir IVRs. Nonetheless, a similar plasma concentration-time profile has been observed with the contraceptive NuvaRing whereby plasma concentrations of the hydrophobic small molecules ethinyl estradiol and etonogestrel peaked approximately 1 week after IVR insertion in women (50).

A significantly higher release rate was observed *in vivo* than *in vitro,* which is atypical for controlled release devices. Of note is that TFV is an acidic compound, with increased aqueous solubility at higher pH. The drug release rate from reservoir IVRs is proportional to the dissolved concentration of drug in the tubing lumen (55). Therefore, the sheep neutral vaginal pH may increase the dissolved TFV concentration in the IVR lumen and thus demonstrate higher TFV flux than in the *in vitro* pH 4.2 acetate buffer

release media. The pH of the healthy human vagina is acidic due to buffering by lactic acid-secreting microflora (62). Therefore, future studies in women will be needed to determine whether the increased *in vivo* flux in sheep is also observed in women.

The 35 wt% swelling HPU TFV IVR demonstrated similar TFV *in vivo* release rates and TFV vaginal concentrations in both sheep studies, albeit the second study did not include as many time points, as its primary objective was toxicological evaluation. Nonetheless, the studies at separate institutions utilizing two different sheep breeds confirmed the ability of the HPU TFV IVR to reproducibly provide elevated and sustained TFV vaginal concentrations for up to 90 days.

4.5.3 *In vivo* toxicology

An efficacious microbicide product must not compromise vaginal barrier function. Thinning or disruption of the protective epithelium and/or recruitment of susceptible immune cells via an inflammatory response could increase HIV infection, as was observed with early HIV prophylaxis trials using surfactants and anionic polymers (26, 52, 53). Microbicide IVRs are designed to be present in the vagina for a duration of weeks to months, and beyond brief excursions would likely be reinserted or replaced throughout a woman's sexually active life. The potential long-term vaginal effects of an IVR and its corresponding influence on HIV prophylaxis are therefore a concern. Bounds *et al*. studied an early version of Femring® that had remarkable mechanical stiffness, and it was concluded that the ring contributed to the creation of ulcerative lesions in the vagina (5). Subsequently, several large clinical safety studies have evaluated an array of medicated and non-medicated IVRs composed of silicone and ethylene vinyl acetate

polymers comprising various dimensions and ring stiffness (14, 27, 41, 43, 47, 59). None of these studies found a significant ring contribution in creating epithelial lesions and/or altering vaginal microflora when tested for up to 1 year. Furthermore, several marketed IVRs have now recorded thousands of women-years of use with favorable safety records (16, 43). Polyurethane IVRs have not been clinically evaluated to date, although evaluation in pigtail macaque monkeys shows no significant alteration in native microflora or mucosal and proinflammatory cytokines when compared to naïve animals (22) or animals receiving silicone IVRs (personal communication with J. Smith, Centers for Disease Control and Prevention, Atlanta, GA).

Care was taken at the onset of product development to utilize materials that possess strong evidence of safety in humans. Medical-grade polyurethanes have demonstrated long-term biocompatibility in many biomedical and drug delivery applications (28). Of the potential water-miscible excipients, glycerol was utilized, as it is generally recognized as safe by the Food and Drug Administration and is one of the most commonly used excipients in vaginal formulations (17) including in the clinically efficacious TFV 1% gel (1). The overall amount of glycerol in the TFV IVR (825 mg) is approximately equivalent to that incorporated in a single dose of the TFV 1% gel (800 mg), which demonstrated no significant adverse events in women (1, 32). Greater than 95% of the glycerol was released from the ring in the first 3 days (data not shown). Over a 90 day duration, the TFV IVR would deliver significantly less glycerol vaginally than the gel which could be dosed up to twice daily.

Following 28 or 90 days of treatment with a single IVR, no signs of systemic toxicity were observed. Minimal cellular debris or bacterial adhesion was found on the

IVR surface after 28 days in sheep as observed by electron microscopy (data not shown). Slight but notable changes in the microscopic pathology of the cervicovaginal epithelium were found to be associated with the presence of the placebo and TFV IVRs. These histopathological findings also correlated with a mild increase in the vaginal irritation potential of the IVRs. Though sheep have been used for the systemic safety evaluation of microbicide IVRs (37) and to evaluate the effects of vaginal gels on the epithelium (56), they are yet to be considered a validated model for the testing of vaginal microbicide products and therefore the significance of the results reported here may still be unclear. Nonetheless, this is the first time, to the best of the authors' knowledge, in which the vaginal irritation scoring methodology developed by Eckstein *et al.* (9) has been applied to sheep. As the methodology is relatively simple to perform, continued evaluation of vaginal irritation scoring of clinically tested microbicide products may help to define its usefulness as a nonclinical vaginal irritation model.

The modest increase in leukocytic infiltration in the vaginal epithelium of sheep receiving IVRs (placebo and to a greater extent TFV IVRs) warrants further evaluation to determine any potential effects on long-term safety and efficacy. The optimal TFV daily dose to deliver from a ring is unknown as dose ranging efficacy studies with any vaginal prophylactic TFV dosage form have not been performed. The reported TFV IVR demonstrated approximately 10 mg/day *in vitro* release rates which correspondingly maintained vaginal TFV concentrations similar to or exceeding peak concentrations with the TFV 1% gel. Future nonhuman primate and human toxicity and efficacy studies will aid in determining an optimal TFV daily dose that provides maximum efficacy. The TFV release rate from this flexible IVR platform may be easily modulated to attain this desired

dose by varying the HPU equilibrium percent swelling and/or tubing cross-sectional dimensions (as reported herein and in Chapter 5).

Sheep possess several additional advantages and limitations for HlV prophylactic IVR safety and pharmacokinetic evaluation beyond that mentioned above (for a more detailed discussion of the female sheep reproductive anatomy and physiology, the reader is referred elsewhere (10, 11, 13)). In contrast to the more commonly utilized rhesus and pigtail macaque nonhuman primates (18, 22, 30, 38), most sheep breeds demonstrate body mass and vaginal anatomical dimensions similar to women, allowing for human-sized IVR testing and avoidance of scaling issues when extrapolating ring mechanics, drug dosing, biodistribution, and pharmacokinetics (35, 36). Although sheep are not susceptible to infection by related immunodeficiency viruses, they are more affordable and easier to acquire than nonhuman primates. Similar to humans, the sheep vaginal epithelium is stratified squamous and thus is pertinent for toxicological irritation studies such as that described herein. The vaginal microflora species and vaginal fluid pH of sheep and women is generally different, although large variation in both variables exists in both populations (29, 31, 57).

Sheep are seasonal breeders with a shorter estrous cycle (approximately 17 days) than women, which may potentially alter drug pharmacokinetics via changes in vaginal epithelium thickness and permeability (33, 39). The estrous cycle was not monitored or controlled in this study, although over the IVR and gel test duration the animals would have undergone approximately 5 and 1 complete estrous cycles, respectively. Generally, vaginal fluid, vaginal tissue, and plasma concentrations in the IVR group (individual and mean) increased or were steady with time, and did not appear to follow any cyclical

trend. Conversely, concentrations in the gel group generally decreased with time. As a result, the hormonal cycle appeared to not significantly influence local or systemic TFV pharmacokinetics although further studies may consider monitoring this potential effect in greater detail.

In summary, we believe that the HPU reservoir IVR provided sustained sheep TFV vaginal fluid, vaginal tissue, and plasma concentrations for 90 days which were similar to peak concentrations in women using the TFV 1% gel. The TFV IVR showed no attributable toxicological effects although a modest increase in inflammatory infiltration of the vaginal epithelium was observed with the placebo IVR and to a greater extent with the TFV IVR when comparing to the sham control. While human studies are needed to confirm the safety, pharmacokinetics, and potential efficacy of this new HPU reservoir IVR design, the IVR achieved what are likely prophylactic concentrations for the 90 day study duration. This IVR design addressed conventional IVR limitations in delivering significant quantities of drug for sustained duration, and further demonstrated flexibility in modulating drug release kinetics and ring mechanical properties. Altogether, these results support further exploration of the HPU reservoir IVR as a sustained TFV delivery system.

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Table 4.1. Sheep safety study design.

| Group | Treatment | Dose | Number of Animals | |
|---|---|---|---|---|
| | | | 28 days | 90 days |
| 1 | Control | Sham dose only | 5 | 5 |
| 2 | Placebo IVR | 1 IVR/animal | 5 | 5 |
| 3 | TFV IVR | 1 IVR/animal | 5 | 5 |

Table 4.2. Mean (standard deviation) pharmacokinetic parameters for the TFV IVR and TFV gel.

| **Parameter** | **Group 1 (IVR)** | | **Group 2 (Gel)** | |
|---|---|---|---|---|
| **Vaginal Fluid** | | | | |
| | **Proximal** | **Distal** | **Proximal** | **Distal** |
| $T_{max}$ (hr)* | 504 | 72 | 336 | 8 |
| $C_{max}$ (ng/g) | 2.4E+7 (3.9) | 5.1E+6 (2.5) | 1.1E+6 (0.8) | 9.0E+5 (6.2) |
| $AUC_{0\text{-}90\ days}$ (ng·hr/g) | 1.3E+10 (2.2) | 2.4E+9 (2.2) | NA | NA |
| **Vaginal Tissue** | | | | |
| | **Proximal** | **Distal** | **Proximal** | **Distal** |
| $T_{max}$ (hr)* | 1.1E+3 | 2.2E+3 | 8 | 8 |
| $C_{max}$ (ng/g) | 4.7E+4 (2.6) | 8.8E+4 (9.7) | 2.7E+4 (2.8) | 8.1E+3 (11.2) |
| $AUC_{0\text{-}90\ days}$ (ng·hr/g) | 5.1E+7 (2.0) | 4.7E+7 (3.6) | NA | NA |
| **Plasma** | | | | |
| $T_{max}$ (hr)* | 336 | | 2 | |
| $C_{max}$ (ng/mL) | 31.3 (8.3) | | 46.8 (20.8) | |
| $AUC_{0\text{-}90\ days}$ (ng·hr/g) | 3.2E+4 (0.6) | | NA | |

*Median $T_{max}$ reported. For the gel group, refers to time post-1$^{st}$ dose (336 hours = pre-15$^{th}$ gel dose).

Table 4.3. Study 2 TFV vaginal fluid, vaginal tissue, and plasma concentrations from the TFV IVR.

| Collection Time and Location | Animal Number | | | | | Mean | SD |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | | |
| **Vaginal fluid (ng/g)** | | | | | | | |
| Proximal, Day 28 | 5.8E+6 | 5.6E+6 | 9.6E+6 | 7.9E+6 | 4.8E+6 | 6.8E+6 | 2.0E+6 |
| Distal, Day 28 | 1.8E+6 | 1.3E+7 | 1.4E+6 | 4.4E+6 | 1.9E+6 | 4.6E+6 | 5.0E+6 |
| Proximal, Day 90 | 3.0E+6 | 1.7E+6 | 3.6E+5 | 1.6E+6 | 1.4E+6 | 1.6E+6 | 9.5E+5 |
| Distal, Day 90 | 3.3E+6 | 1.5E+6 | 2.1E+5 | 1.5E+6 | 1.3E+6 | 1.6E+6 | 1.1E+6 |
| **Vaginal tissue (ng/g)** | | | | | | | |
| Proximal, Day 28 | 8.1E+4 | 4.7E+3 | 6.1E+3 | 7.6E+3 | 4.0E+3 | 2.1E+4 | 3.4E+4 |
| Distal, Day 28 | 6.7E+4 | 2.5E+4 | 9.2E+3 | 1.7E+4 | 4.5E+3 | 2.4E+4 | 2.5E+4 |
| Proximal, Day 90 | 7.8E+3 | 2.0E+3 | 1.4E+3 | 1.3E+3 | 1.7E+3 | 2.9E+3 | 2.8E+3 |
| Distal, Day 90 | 3.6E+3 | 1.4E+3 | 6.7E+2 | 5.5E+3 | 1.6E+3 | 2.5E+3 | 2.0E+3 |
| **Plasma (ng/mL)** | | | | | | | |
| Day 28 | 23.8 | 18.0 | 29.4 | 24.0 | 31.7 | 25.4 | 5.4 |
| Day 90 | 11.0 | 2.0 | * | 8.1 | 15.6 | 7.3 | 6.4 |

* BLQ<1.0 ng/mL

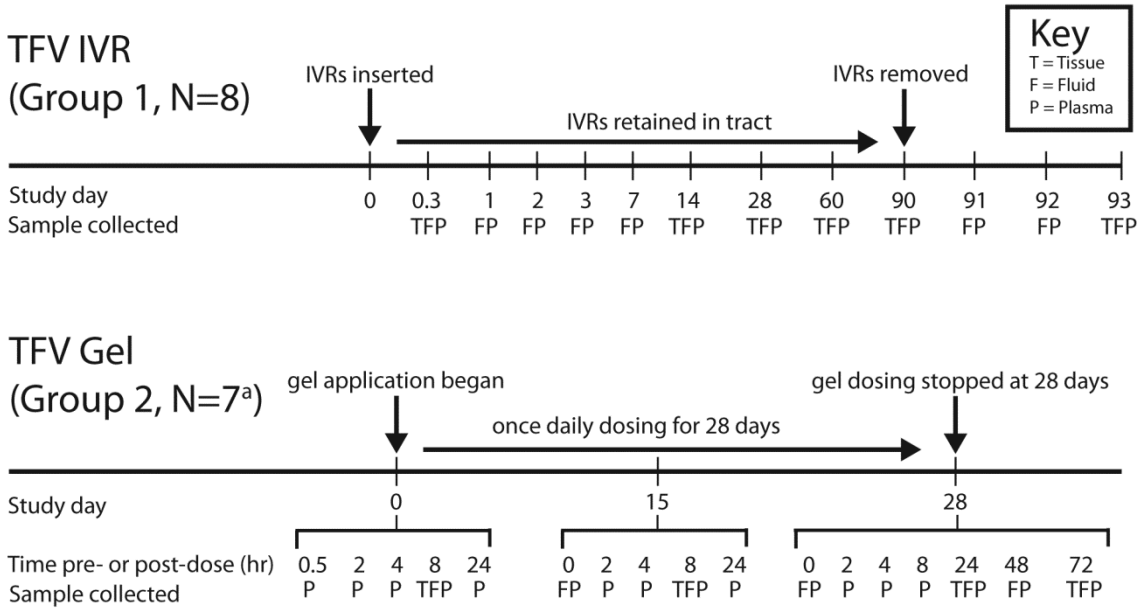


Figure 4.1. Diagram of the sheep pharmacokinetic study design/dosing protocol for TFV IVR Group 1 (top) and TFV Gel Group 2 (bottom). [a]N = 6 for Group 2, day 28 time points.

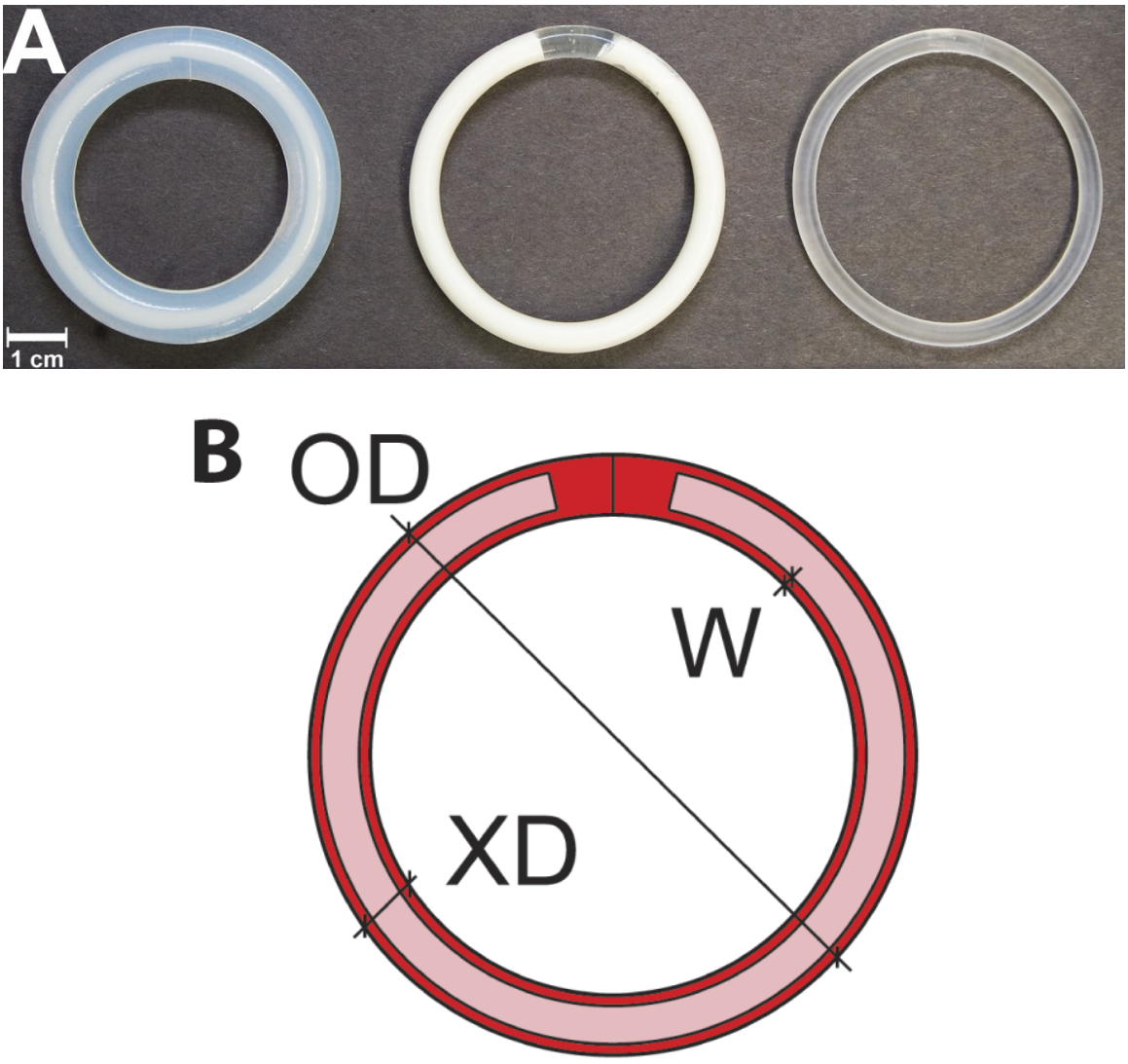


Figure 4.2. (A) Comparison of HPU IVR with commercially available IVRs: Estring (left), TFV IVR with novel HPU reservoir design (center), and NuvaRing (right). (B) Diagram of HPU IVR with 55 mm outer diameter (OD), 0.7 mm wall thickness (W), and 5.5 mm outer cross-sectional diameter (XD).

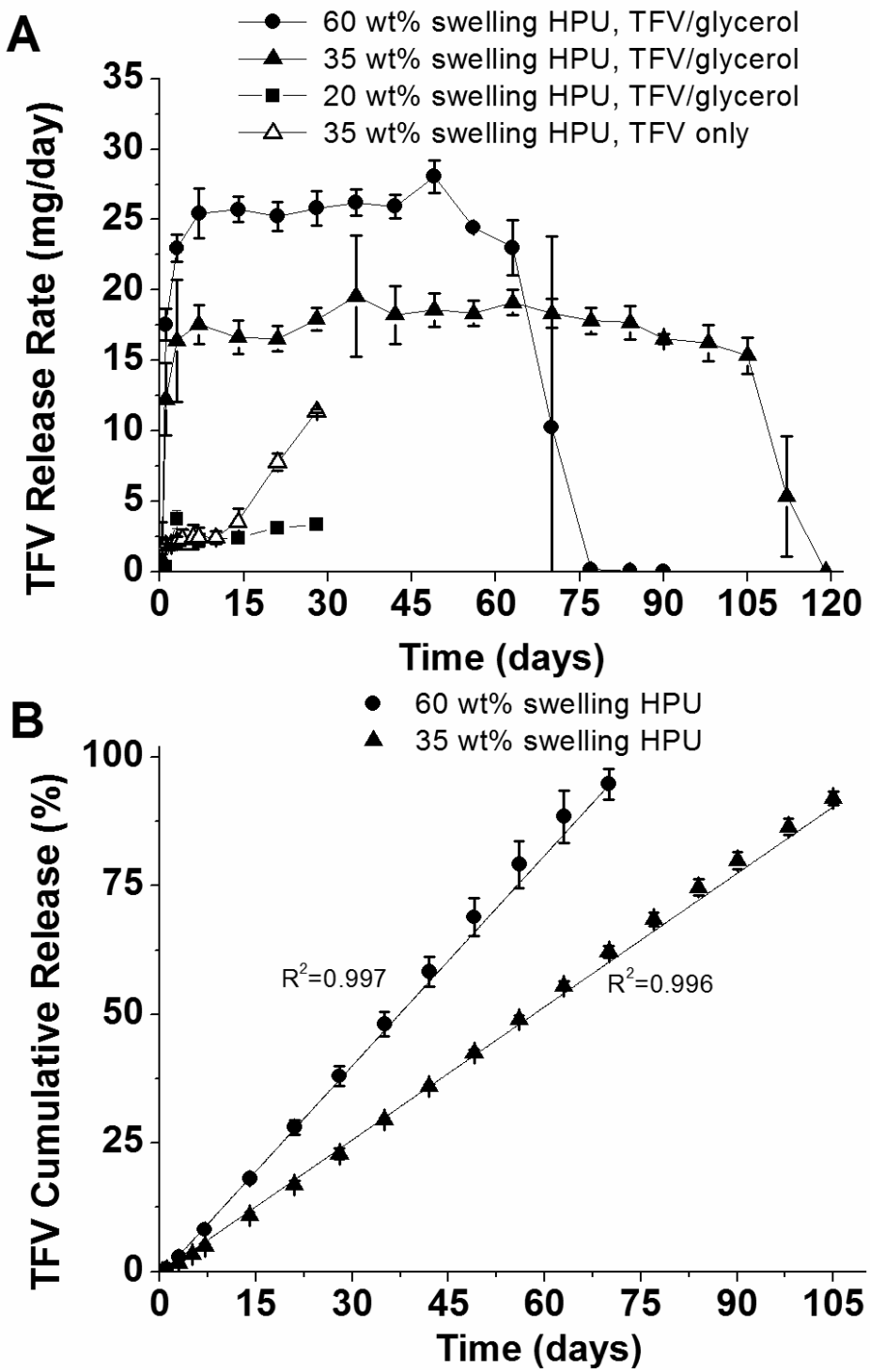


Figure 4.3. (A) The *in vitro* TFV daily release rate as a function of time from prototype IVRs composed from different swelling HPU tubing whose lumen contained 65/33/2 wt% TFV/glycerol/water (solid symbols) or TFV only (open triangles). (B) Percent cumulative release as a function of time for 60 and 35 wt% swelling HPUs with glycerol in the tubing lumen. Data are mean ± SD, N = 3.

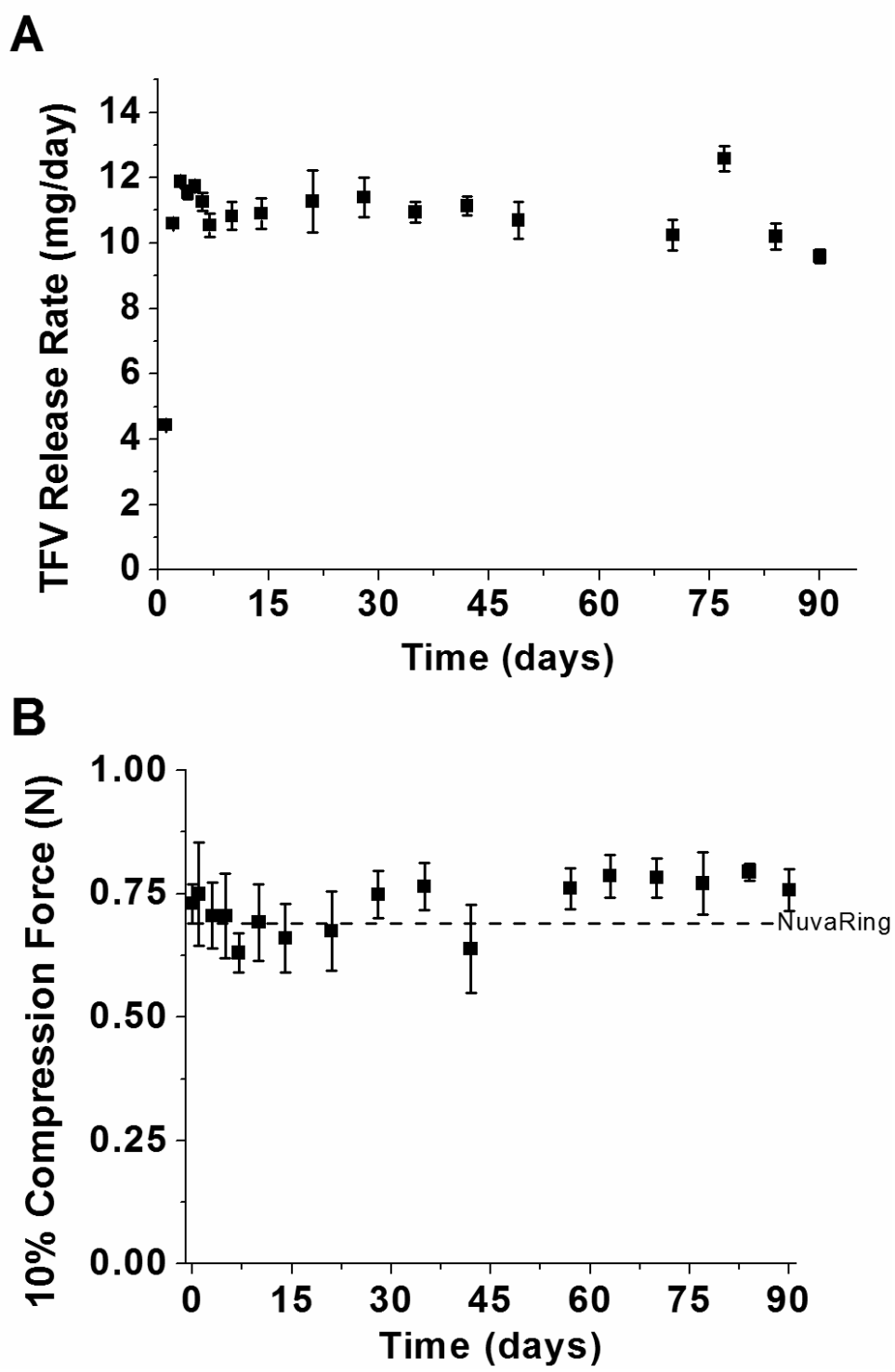


Figure 4.4. *In vitro* TFV release rate (A) and the force required for 10% compression of the ring diameter compared to NuvaRing (B) for the softer shore hardness 35 wt% swelling HPU IVRs composed of 65/33/2 wt% TFV/glycerol/water (IVRs were manufactured from the same HPU as tested *in vivo*). Data are mean ± SD, N = 5.

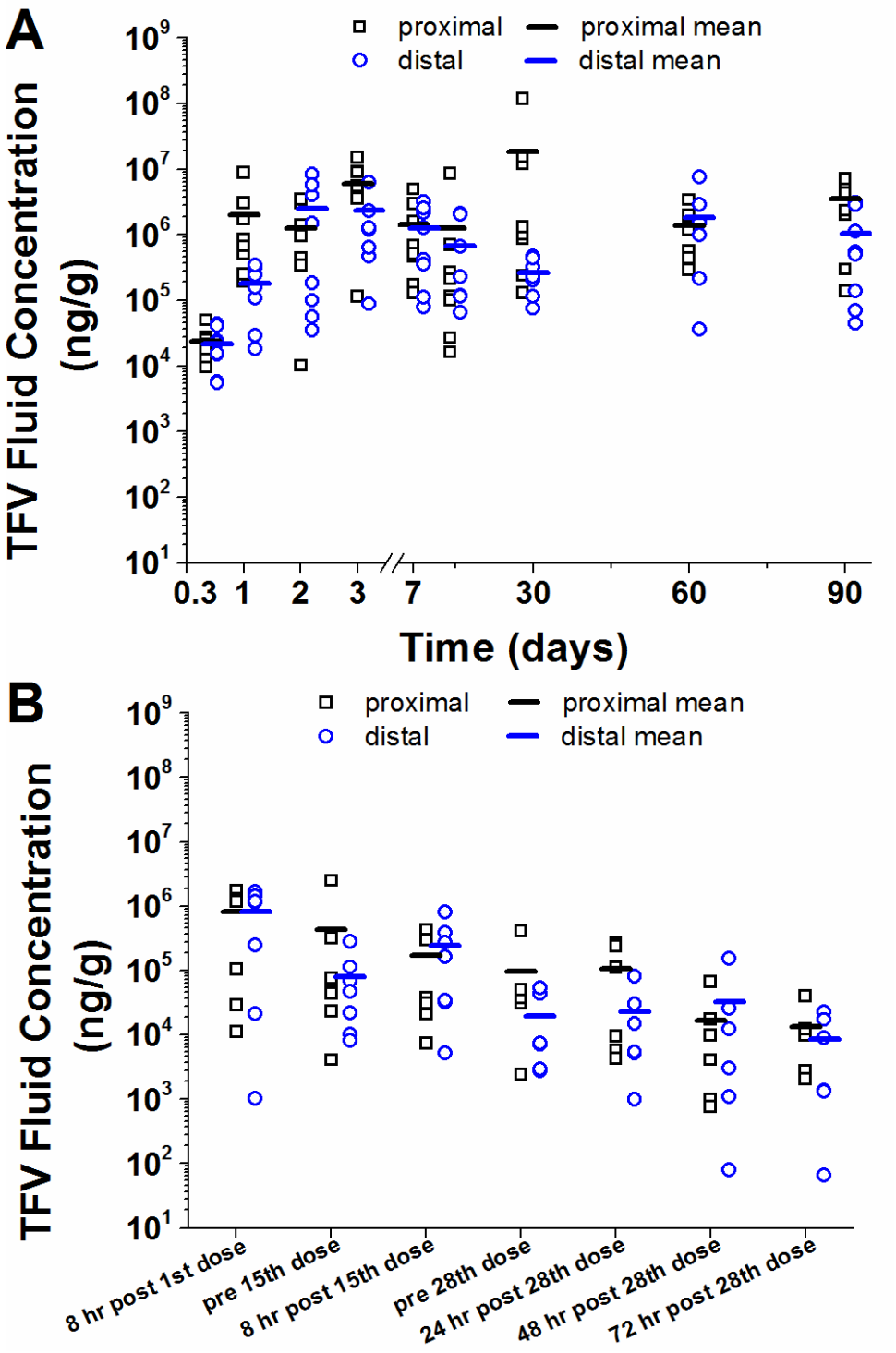


Figure 4.5. Proximal and distal TFV vaginal fluid concentrations for TFV IVR (A) and TFV gel (B).

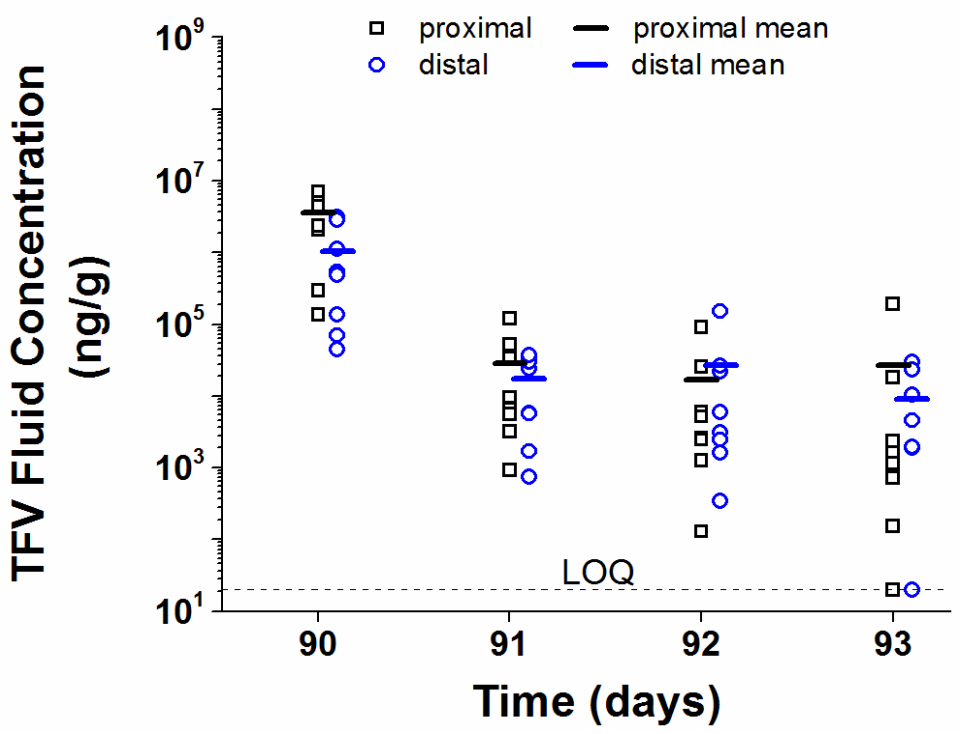


Figure 4.6. Proximal and distal TFV vaginal fluid washout concentrations following IVR removal on day 90.

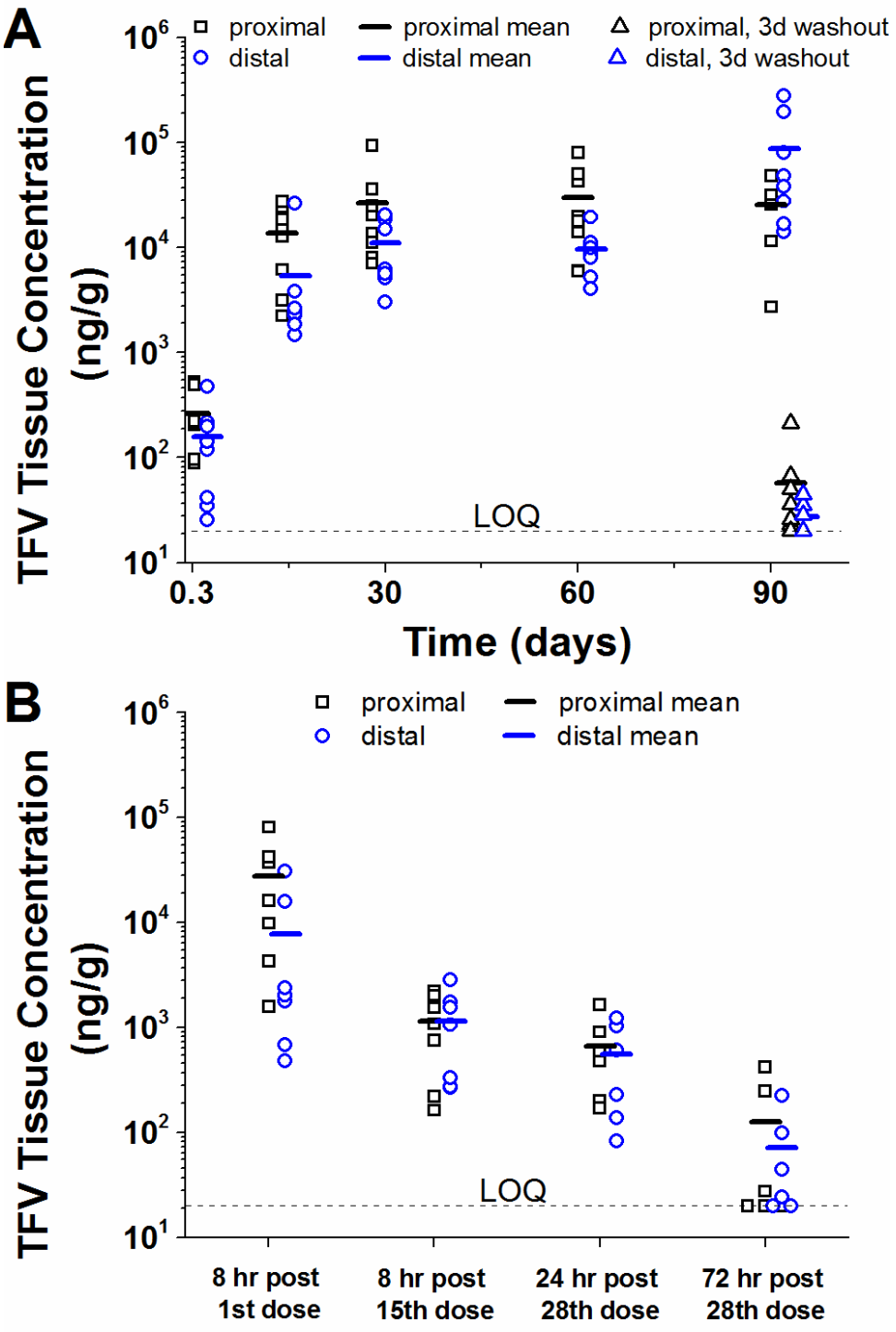


Figure 4.7. Proximal and distal TFV vaginal tissue concentrations for TFV IVR (A) and TFV gel (B).

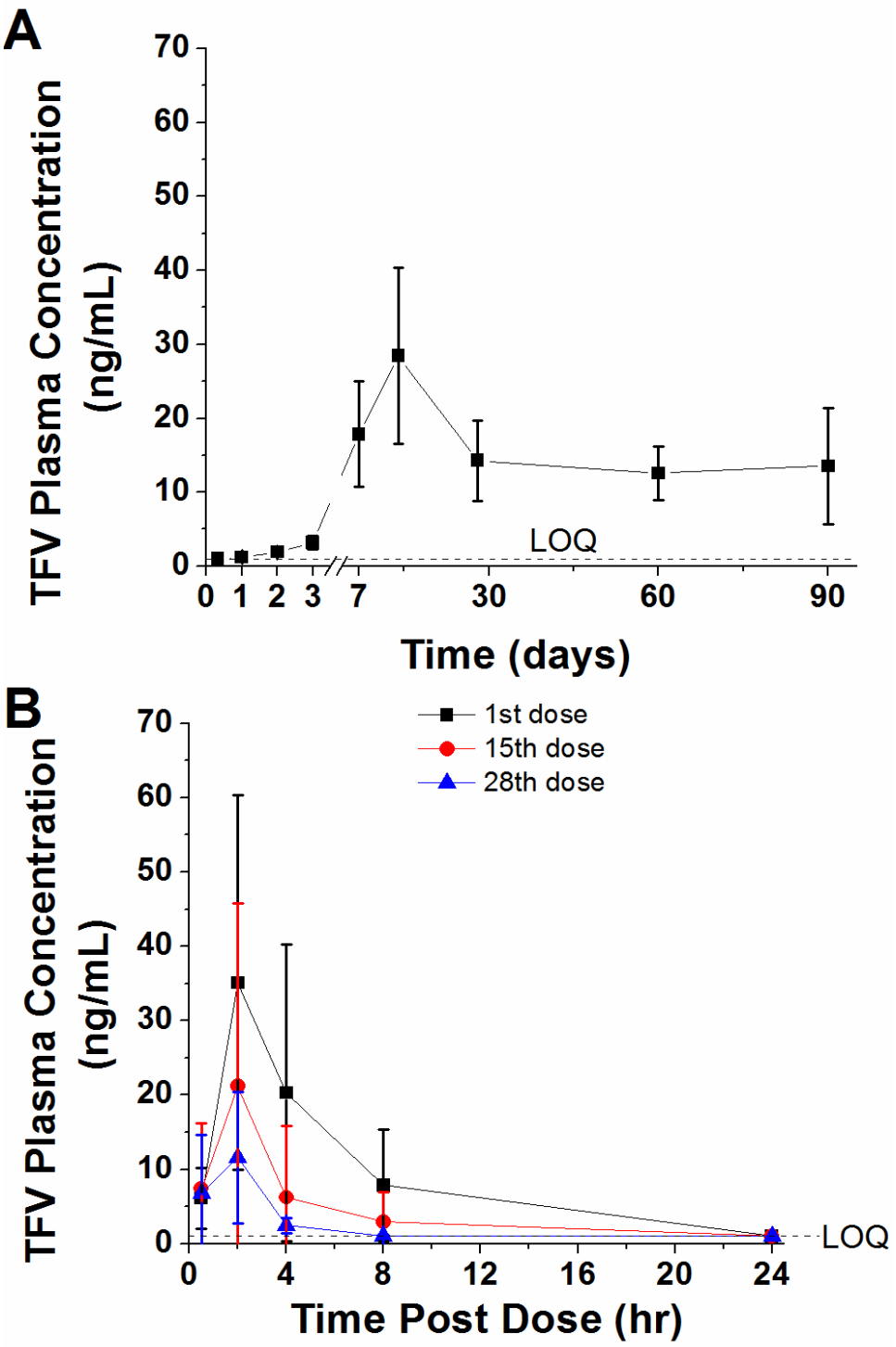


Figure 4.8. TFV plasma concentrations for TFV IVR (A) and TFV gel (B). TFV IVR and gel plasma levels were less than LOQ at 24, 48 and 72 hours post-IVR removal and following the 28$^{th}$ gel dose (only 24 hr post-gel dose is shown). Data represent mean ± SD, N = 8 and N = 7 for IVR and gel, respectively, except N = 6 for the 28$^{th}$ TFV gel dose.

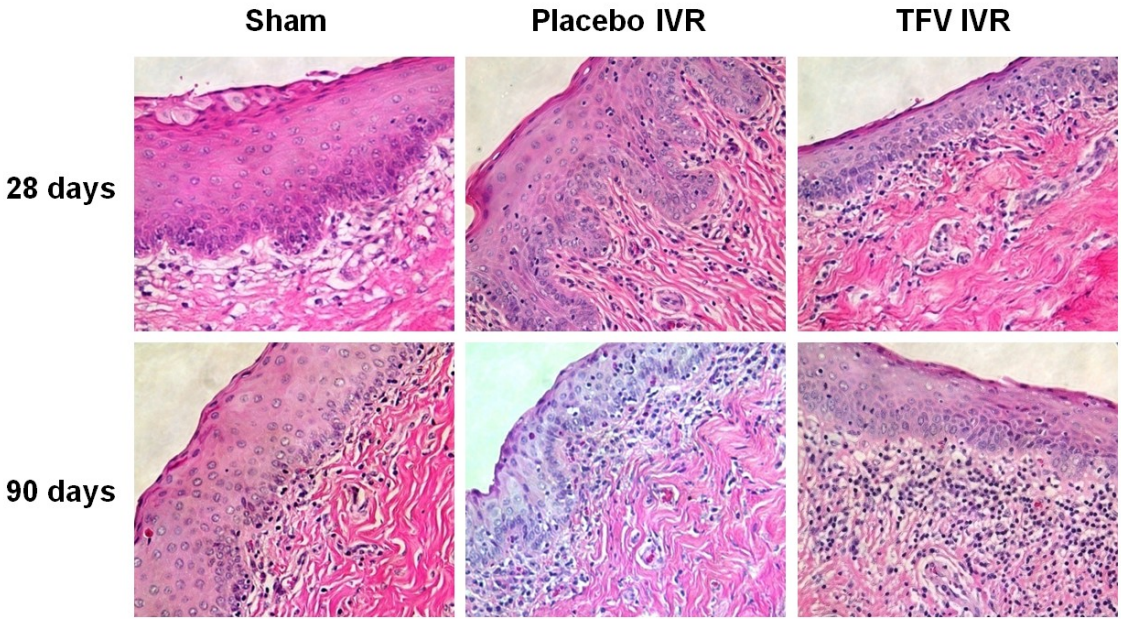


Figure 4.9. Representative micrographs of H&E stained posterior vaginal tissue after 1 or 3 months of treatment with sham control, placebo IVR, or TFV IVR.

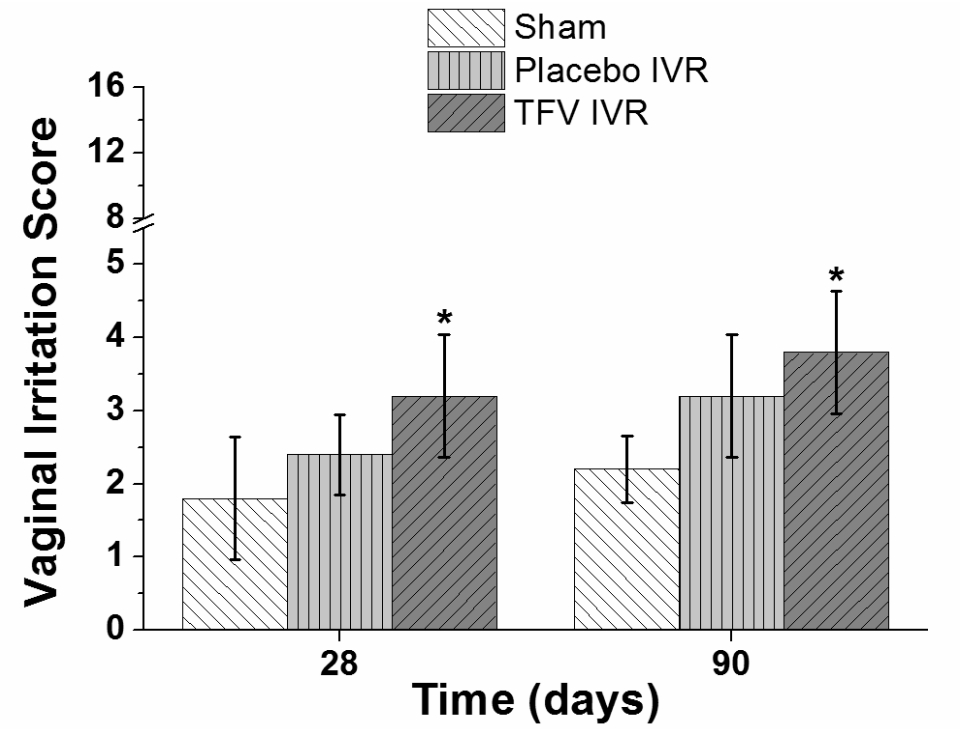


Figure 4.10. Sheep vaginal irritation scores (on a scale of 0 – 16) after 28 or 90 days of treatment with TFV or placebo IVRs or sham control. *Values for TFV IVRs were significantly different compared to sham control ($p<0.05$). Data are mean ± SD, N = 5.

CHAPTER 5

# ENGINEERING OF A NEW INTRAVAGINAL RING TECHNOLOGY FOR HIV PROPHYLAXIS

## 5.1 Abstract

The hydrophilic antiretroviral agent tenofovir (TFV) recently exhibited partial effectiveness against HIV when used prophylactically in a short-lasting vaginal gel formulation. TFV reservoir intravaginal rings (IVRs) were designed and tested to offer a long-lasting and coitally dissociated alternative dosage form. The IVRs were fabricated from hydrophilic polyurethane tubing with varying equilibrium aqueous swelling and wall thickness, and the tubing lumens were filled with a high-density semisolid paste primarily comprising TFV and glycerol. Release of TFV *in vitro* followed geometry- and swelling-dependent zero-order kinetics for up to 90 days. The steady-state TFV release rate and amount of water partially bound to the hydrated polymer displayed similar exponential decay kinetics when the IVRs were stored at 40°C prior to *in vitro* release testing, but eventually stabilized. The lead ring design and composition was well tolerated in sheep and the time-averaged mean *in vivo* TFV release rate was approximately 75% greater than the 13 mg/day observed *in vitro*. Overall, we designed and characterized a physically and chemically stable IVR platform to provide tunable, time-independent TFV release and ring mechanical stiffness for up to 90 days which may

potentially prevent HIV sexual transmission.

## 5.2 Introduction

The human immunodeficiency virus (HIV) infects over a thousand women in sub-Saharan Africa every day, motivating the development of vaginal prophylactic technologies to protect women against the sexual transmission of HIV (37). Despite poor user adherence, a short-lasting gel delivering 40 mg of the antiretroviral agent tenofovir (TFV) was 39% effective at preventing HIV infection in women when vaginally applied before and after coitus (1). HIV prophylaxis was positively correlated with TFV vaginal concentrations and user adherence (13), suggesting that TFV dosage forms that provide long-term elevated concentrations and increased user adherence will improve the effectiveness of TFV (33, 39). Since dose-ranging studies have not been performed in women to associate the vaginal dose of TFV with the vaginal fluid and tissue concentrations and/or effectiveness against HIV, the optimum TFV gel dose for safety and efficacy is unknown. Similarly, optimum TFV doses are unknown for long-lasting dosage forms such as intravaginal rings (IVRs), which possess a differing dosage regimen and are likely to have different vaginal drug distribution and absorption. As a result, we estimate that an optimum IVR dose will be within the same order of magnitude as that of the clinical TFV 1% gel. Unfortunately, TFV has limited solubility in conventional hydrophobic IVR polymeric platforms, including silicone and ethylene vinyl acetate. As the drug release rate from an IVR is linearly dependent on the molecular concentration dissolved in the polymer (14), it is not surprising that conventional IVRs

deliver micrograms of TFV per day, which is orders of magnitude less than the clinical gel (34).

Hydrophilic polyurethane (HPU) matrix IVRs exhibit promising TFV fluxes that can be configured to release from 1 to 50 mg/day because of the water absorption of HPU and the subsequent aqueous-mediated dissolution and diffusion of TFV through the hydrated polymer (4, 12). Despite the increased TFV flux compared to conventional IVRs, the *in vitro* TFV release rate and the mechanical stiffness of the ring steadily decrease by approximately 10- and 3-fold, respectively, over a 90-day period (4). The apparent square-root of time cumulative drug release kinetics are an inherent characteristic of matrix IVRs because the drug depletion from a cylindrical IVR cross-section begins at the outer edge and moves inward with time, resulting in a longer diffusional distance for the drug to reach the IVR surface (20). Similarly, a loss in ring stiffness occurs as the crystalline dispersed TFV, present at up to 20 wt%, elutes from the IVR and creates a lower modulus polymer matrix. The comparatively high TFV flux at early time and low TFV flux at long time is undesirable because it could result in local toxicity and sub-prophylactic concentrations, respectively. The TFV-matrix IVR exhibited decreasing TFV vaginal concentrations with time in nonhuman primates (unpublished work) that were similar to that observed *in vitro*. Likewise, time-dependent IVR stiffness could have multiple undesirable effects, including difficulty during insertion and removal, ring expulsion from the vaginal tract, and biocompatibility (29). An unconventional semi-solid reservoir HPU IVR may achieve the goal of time-independent mechanical stiffness and milligrams-per-day extended drug release. In particular, IVRs with HPU reservoir tubing offer a wide design space because changing

such variables as the wall thickness, cross-sectional diameter, HPU elastic modulus, HPU percent swelling, and lumen composition could allow the simultaneous optimization of the IVR mechanical properties, lumen volume, and TFV flux. The release rate of a drug (mass/time) from a reservoir-type cylinder has been described in the literature (40):

$$\frac{dM_t}{dt} = \frac{2\pi LDK\Delta C}{\ln (\mathrm{r_o}/\mathrm{r_i})} \qquad \text{Equation (5.1)}$$

where $L$ is the cylinder length, $D$ is the diffusion coefficient in the wall, $K$ is the partition coefficient between the membrane and the core, $\Delta C$ is the concentration gradient across the membrane, and $r_o$ and $r_i$ are the cross-sectional outer and inner radii, respectively. The ring dimensions and the drug diffusion coefficient in the rate-controlling membrane polymer may therefore be modulated to achieve the desired drug release rate. However, the ring design must also consider the IVR stiffness, which may directly influence its ability to be inserted and retained in the vaginal tract without disrupting the vaginal mucosa or being expelled (7, 16, 29, 42). The force required to mechanically compress an elastic tubular ring may be described by (11):

$$F = \frac{\pi^2 E({r_o}^4 - {r_i}^4)}{(\pi^2 - 8)(R - r_o)^3} Y \qquad \text{Equation (5.2)}$$

where $E$ is the elastic modulus, $R$ is the ring outer radius, and $Y$ is the reduction in ring outer diameter. Therefore, altering the ring dimensions and the elastic modulus of the material, which often influences drug diffusivity (40), will likely alter both the drug

release rate and the mechanical stiffness of the ring. Furthermore, changes in the cross-sectional dimensions will also impact tubing kinking, which is not considered in the mechanical equation above. Tubing that is axially flexed will collapse (i.e., kink) when a critical bend radius is achieved, with further flexural deformation resulting in noticeably reduced mechanical stiffness. The critical bend radius of elastic tubes under pure bending is described by the following equation (10):

$$R_{crit} = \frac{3}{\sqrt{2}} \frac{{r_o}^2 \sqrt{1 - v^2}}{w} \qquad \text{Equation (5.3)}$$

where $v$ is Poisson's ratio and $w$ is the tubing wall thickness ($r_o$- $r_i$). The implications of tubing kinking as it pertains to IVR performance will be discussed in greater detail below.

The temperature- and time-dependent microphase separation of the hard and soft segments of polyurethane imparts unique physical properties, including high elasticity yet thermal processability (36). Generally, the hard and soft segments of polyurethane demonstrate miscibility at higher temperatures (i.e., during thermal processing such as hot-melt extrusion), which coincides with the disruption of the crystallinity in the hard segment (26, 36). Post-processing, lower storage temperatures result in the immiscibility of the hard and soft segments, leading to microphase separation and the crystallization of the hard segment. Polyurethanes have received little attention as commercial drug delivery products, partly because conventional oral delivery applications prefer polymers that rapidly dissolve in aqueous media. Furthermore, polyurethanes typically rely on thermoplastic processing techniques, which have seldom been used to manufacture

pharmaceutical products until recently (28). A lack of knowledge about the polyurethane structure-property relationships pertaining to drug delivery is apparent when searching Scifinder® for "polyurethane phase separation" and "polyurethane phase separation drug," which yield 1119 and 3 results, respectively (date of search: 7/10/2012). Although extensive work over the past half-century has advanced the understanding of the microphase separation kinetics of polyurethane (19), no peer-reviewed literature has reported its potential impact on the physical stability of a polyurethane drug product (i.e., drug release kinetics as a function of storage time). We recently reported a 90 day HPU reservoir IVR that exhibited steady-state TFV *in vitro* release rates of 10 mg/day and sustained TFV *in vivo* vaginal concentrations that were similar to or exceeding the peak concentrations of the clinically effective TFV 1% vaginal gel (see Chapter 4). We report herein the observation of a solution to the physical instability of the initial IVR prototype, specifically regarding the decreased steady-state TFV *in vitro* release rates as a function of IVR storage time. Upon achieving a physically stable formulation, extensive engineering and characterization of the finalized IVR design was also performed.

## 5.3 Materials and Methods

### 5.3.1 Materials

Tecophilic® HP-60D-35, HP-60D-60, and HP-93A-100 hydrophilic aliphatic polyether urethane (HPU) grades, hereby referred to as HPU-35, HPU-60, and HPU-100, respectively, were purchased from Lubrizol Advanced Materials (Wickliffe, OH). The manufacturer-specified percent swelling in water at 25°C was 34.3, 51.3, and 94.0 wt% for HPU-35, HPU-60, and HPU-100, respectively. Tenofovir monohydrate was supplied

by CONRAD (Arlington, VA). USP-grade glycerol and water were purchased from Spectrum Chemical (New Brunswick, NJ). All solvents and reagents were ACS grade unless otherwise noted.

5.3.2 IVR fabrication

HPU pellets were dried overnight at 150°F in a compressed air microdryer with a 10 pound hopper (Dri-Air, East Windsor, CT) to a moisture content of less than 0.05 wt%, as verified using a Karl Fisher Titrator (Mettler-Toledo, Columbus, OH). The dried pellets of each grade were used individually. In addition, HPU-60 and HPU-100 were physically mixed at wt/wt ratios of 75/25, 50/50, and 25/75 (hereby referred to as HPU-B1, HPU-B2, and HPU-B3, respectively). The HPU pellets were fed into a 3/4 inch single-screw extruder attached to an advanced torque rheometer drive (C.W. Brabender, South Hackensack, NJ) with a tubing crosshead (Guill Tool, West Warwick, RI). The extruder heating zone temperatures varied with the HPU grade and blend, with barrel temperatures (zones 1-3) ranging from 150-180°C and tubing crosshead tip and die temperatures (zones 4 and 5) ranging from 130-150°C. The extrudate leaving the tubing crosshead was drawn using a CPC2-12 combination puller/cutter (Conair, Cranberry Township, PA) to create final tubing products with a 5.5 mm cross-sectional diameter. Various crosshead tips were inserted to create 4 tubing wall thicknesses. The final wall thickness and concentricity measurements were made using an optical microscope and a 38DLP ultrasonic thickness gauge with a 20 MHz M316-SU F = 0.75" transducer (Olympus NDT, Waltham, MA). The final tubing wall thicknesses, measured at every 90

degrees of cross-sectional rotation, were 0.68 ± 0.02, 0.87 ± 0.02, 1.02 ± 0.02, and 1.21 ± 0.02 mm (mean ± SD, N = 3 tubes).

The extruded tubes were cut to 162-171 mm in length (depending on the tubing wall thickness), and one tubing end was sealed using an induction welder (PlasticWeld Systems Inc., Newfane, NY) equipped with a DuPont 857G (Wilmington, DE) reinforced polytetrafluoroethylene-coated stainless steel bonding die (Industrial Fluoro-Plastics, West Jordan, UT). TFV was mechanically milled using a M20 universal mill (IKA Works, Wilmington, NC) and sieved to less than 425 μm with an average aggregative size between 75-180μm. The TFV, glycerol, and water were mixed at a 65/33/2 wt% ratio using a Hobart mixer with a "B" beater attachment (Troy, OH) and back-filled into the tubing using a semicustomized model 2400 medical-grade high-pressure hydraulic filling system (Dymax, Torrington, CT). The semisolid formulation included 2% water to increase its processable lifetime by reducing its viscosity (i.e., the hydraulic filling system could extrude the water-containing formulation for a longer time after it was prepared). Each tube was filled with 1.4-2.5 g of the semisolid formulation (depending on the extrudate wall thickness and corresponding lumen volume), resulting in 0.9-1.6 g of TFV per IVR (the tubes were weighed before and after filling). Alternatively, the HPU-35 tube lumens with 0.68-mm-thick walls were manually filled and packed with TFV alone to achieve 1.6 g of TFV per IVR. After the lumens were filled with either 100% TFV or the 65/33/2 wt% TFV/glycerol/water mixture, the second end of the tubing was sealed in the same manner as the first and the resultant 5-mm-long hermetically sealed ends were welded together using a second induction welder equipped with a stainless steel split die. The resultant IVR was placed in a custom-machined 12 cavity aluminum

mold to shape-anneal the IVRs into a circular conformation and minimize tube kinking. Water heated to 65°C was circulated through the mold for 15 min, followed by 10°C water circulation for 5 min. After the shape annealing, the IVRs were either tested under various experimental conditions or inserted into foil-lined, moisture-impermeable pouches (LPS Industries, Moonachie, NJ) and heat-sealed using an impulse sealer set at 155°C, recycle setting = 3, and sealing setting = 7 (AIE Inc, Whittier, CA). All of the IVR formulations that were fabricated and tested are listed in Table 5.1.

5.3.3 *In vitro* release and mechanical testing

The *in vitro* TFV release kinetics and the IVR mechanical stiffness were evaluated for up to 90 days as described previously for TFV-matrix IVRs (4, 12). Briefly, IVRs were immersed in 25 mM sodium acetate buffer (pH 4.2) and the volume (50-200 mL) was adjusted as needed to maintain sink conditions (the drug concentration never exceeded one-tenth of the maximum solubility). IVRs were placed in a 37°C orbital shaker set at 80 rpm. The release medium was replaced every 24 h and sampled periodically to quantify the TFV content via HPLC. To calculate the time-averaged steady-state TFV release rates, the amounts of TFV released on days 5, 7, 10, and 14 were averaged because the TFV release kinetics beyond day 3 were generally zero-order. The TFV release rates from the equilibrated HPU-B1 IVRs as a function of wall thickness were estimated using Equation 5.1. Because the variables encompassing the numerator on the right side of Equation 5.1 were constant for HPU-B1, the experimentally determined TFV release rate for the 0.68 mm wall thickness was multiplied by the corresponding $\ln(r_o/r_i)$ to determine the constant. This constant was

then applied to the 0.85, 1.02, and 1.21 mm wall thicknesses to estimate their corresponding TFV release rates. Before and during the release study, the IVR stiffness was measured by compressing the IVRs 10% of their initial outer diameter using a custom-machined aluminum probe and an Instron 3342 uniaxial testing system (Norwood, MA). The force corresponding to 10% ring compression was extracted and compared across all data sets. To predict the theoretical ring compression values, the elastic modulus of HPU-B1 was determined by tensile testing an extruded solid cylinder at 1 mm/sec and obtaining a slope from the 1-5% strain, where $R^2 > 0.995$.

5.3.4 Weld evaluation and joint robustness

To confirm sufficient weld integrity following the IVR fabrication, 100 HPU-B1 rings were hydrated in release media for 7 days and subsequently compressed to 50% of the initial ring diameter for 1000 cyclic fatigue cycles at 1/2 Hz using a custom-made cyclic fatigue instrument controlled by LabView software (National Instruments, Austin, TX). Upon completion of the cyclic fatigue testing, the IVRs were evaluated for joint integrity using the Instron 3342 uniaxial testing system and a modified version of the ASTM D1414 test method, “Standard Test Methods for Rubber O-Rings”. Briefly, rings were elongated at a rate of 5 mm/sec until 100 N of tensile force was reached (approximately 100% elongation). The IVR weld was considered acceptable if joint failure did not occur during the test.

5.3.5 TFV content analysis

Previously reported HPLC methods for determining the TFV concentration were used (4, 12). Briefly, 2 μL of sample were injected into a Phenomenex Luna C18 5 μm, 150 x 4.6 mm column attached to an Agilent 1200 series HPLC with a diode array detector and autosampler. Two separate chromatography methods were used for the release studies and the chemical stability studies. For the release studies, a 15 min gradient method was used to elute the TFV, which began with 100% mobile phase A (potassium phosphate buffer, pH 6.0) and ended with 100% mobile phase B (acetonitrile/potassium phosphate buffer, pH 6.0). The flow rate was 1.5 mL/min, and TFV was detected at 260 nm with an average retention time of 7 min. For the chemical stability studies, similar chromatographic conditions were used, with the exception that a 45 min gradient was run to ensure the separation of potential contaminants or degradation products.

5.3.6 Quantification of IVR TFV content

TFV was extracted from the IVRs for content analysis following fabrication or *in vivo* or *in vitro* release testing. The IVRs were cut into short, 1 cm segments, and all of the segments were placed in a 50 mL volumetric flask with 100 mM phosphate buffer to completely dissolve the TFV. Following the TFV dissolution, the volumetric flask was filled to the 50 mL volume mark, the polymer segments were removed, and an aliquot of the concentrated solution was volumetrically diluted and analyzed for TFV content by HPLC, as described above. The TFV percent recovery was calculated by normalizing the recovered TFV amount by the amount recovered in unused and unstored rings.

5.3.7 Chemical and physical stability testing

IVRs in heat-sealed pouches were placed in a temperature- and humidity-controlled chamber (Caron, Marietta, OH) set at 40°C/75% relative humidity. The IVRs were stored for various durations, after which the chemical and/or physical stability was tested. To test the physical stability, the IVRs were subjected to 14 day release testing as described above to determine any change in the TFV steady-state release values. To test the TFV chemical stability in the formulation, the TFV was extracted from the stored IVRs as described above. To determine whether the TFV release rate changes were caused by changes in the HPU molecular weight, the IVR lumens of the stored IVRs were longitudinally bisected and the lumen contents were physically removed by thoroughly wiping the tubing surface with Kimwipes to remove any surface TFV or glycerol. The HPU molecular weight was then determined as described below.

5.3.8 HPU molecular weight analysis

Approximately 50 mg of HPU sample was weighed, added to a 10 mL volumetric flask, and dissolved overnight in dimethylacetamide with 10 mM lithium bromide (to prevent polymer aggregation). Gel permeation chromatography was performed using an Agilent 1100 series chromatograph equipped with a BI-DNDC refractive index detector (Brookhaven Instruments, Holtsville, NY) and a PLgel 10 μm Mixed-B LS column. The mobile phase consisted of 10 mM lithium bromide in dimethylacetamide, and the flow rate was 0.75 mL/min. The samples were filtered through 0.45 μm PTFE filters and injected using a 100 μL injection volume loop. Monodisperse polystyrene standards were dissolved at 1 mg/mL in 10 mM lithium bromide in dimethylacetamide and used to create

a universal calibration curve with a linearity greater than 0.999. The Mark-Houwink-Sakurada constants of $K = 1.4x10^{-4}$ and $\alpha = 0.68$ were obtained from the literature (6).

5.3.9 HPU IVR swelling measurements

The swelling measurements of the HPU IVRs were made after the completion of the TFV release studies. The IVRs were cut on either side of the joint, and the remaining TFV was flushed from the joint and lumen using deionized water. After 1 week of equilibration in the release medium at 20°C, Kimwipes were used to dry the exterior joint and tubing surface and 60 psi of air was used to remove water from the tubing lumen. Any residual moisture in the joint was removed using cotton swabs. The dried joint and tubing were then weighed, and the percent swelling was calculated using the recorded mass of the tube prior to the IVR fabrication. The tubes were then maintained at 37°C for 1 week to measure swelling at the *in vitro* release temperature using the same procedure as described for 20°C. The drying and weighing procedures were performed in less than 30 sec to avoid temperature- and time-dependent changes in percent swelling.

5.3.10 Differential scanning calorimetry

Differential scanning calorimetry (DSC) scans of hydrated HPU were performed to determine the state of water in the hydrated HPU. Scans were run on a Q200 DSC (TA Instruments, New Castle, DE) equipped with a refrigerator cooling system, autosampler, and 50 mL/min nitrogen purge. Prior to sample analysis, the enthalpy of fusion and peak onset temperature of pure indium were confirmed. The hydrated HPU IVRs that had first undergone various storage conditions, *in vitro* release testing, and subsequent 20°C

swelling measurements (as described above) were scanned by DSC. Biopsy punches (5 mm) were used to cut cylindrical discs from the hydrated tubing. The hydrated HPU discs, ranging from 15 to 20 mg, were weighed and added to aluminum Tzero pans and sealed with hermetic lids. Scans were performed from 25°C to -80°C at 5°C/min. After a 1 min equilibration at -80°C, the temperature was then returned to 25°C at 5°C/min. Universal Analysis software (TA Instruments, New Castle, DE) was used for all parts of the thermogram analysis, including peak integration. The enthalpy of freezing and melting were calculated as joules per gram of dry polymer (the dry polymer mass was back-calculated using the IVR swelling measurements from above).

5.3.11 *In vivo* IVR evaluation

The final ring composition and design was tested in a sheep model (*Ovis aries*) to determine the ring retention and an *in vivo* TFV release rate. All sheep were housed under appropriate conditions according to the Guide for the Care and Use of Laboratory Animals. The University of Utah animal facility is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC, #A3031-01) and has an assurance on file with the Office for Laboratory Animal Welfare. Five female Columbia crossbred sheep of breeding age (65 - 80 kg) received TFV IVRs under an animal protocol approved by the University of Utah IACUC. The IVRs were placed in the proximal vagina (near the cervix) of the sheep for 29 days, after which the rings were retrieved and residual drug content analysis was performed to determine the amount of drug released *in vivo*.

5.3.12 Statistical analysis

The data were analyzed using one-way ANOVA and unpaired two-tailed Student's t-test. A p-value below 0.05 was considered statistically significant. $R^2$ values were calculated using the Pearson correlation coefficients.

## 5.4 Results

5.4.1 HPU IVR fabrication and weld evaluation

Medical-grade hydrophilic polyurethanes of varying percent swelling were hot-melt extruded to create tubing with a 5.5 mm outer diameter. The tubing lumens were filled with 100% TFV or a semisolid mixture of 65/33/2 wt% TFV/glycerol/water and induction-welded to create 55 mm outer diameter IVRs (Figure 5.1). To confirm that the IVR welding methods were sufficient to prevent joint failure upon HPU hydration and ring mechanical stress, 100 hydrated IVRs underwent mechanical cyclic fatigue testing followed by tensile testing. All of the rings survived 1000 cyclic fatigue cycles and 100 N of tensile force without joint or tubing failure, indicating that the weld failure rate under these rigorous testing conditions was below 3% using a 95% confidence interval.

Qualitatively, the IVRs made from tubing with wall thicknesses less than 0.68 mm demonstrated tubing kinking when only slightly compressed from their original state (less than approximately 35% ring compression), as predicted by a critical bend radius above approximately 20 mm (Figure 5.2). In addition, the welding of tubing with thinner walls proved to be difficult due to insufficient tubing longitudinal stiffness. As a result, 0.68 mm was selected as the lower bound for the tubing wall thickness; 1.21 mm was selected as the upper bound to achieve a minimum TFV loading of 0.9 g per IVR.

5.4.2 TFV release kinetics from unstored IVRs

HPU-35 IVR lumens that incorporated the water-miscible small molecule glycerol established near-steady-state TFV kinetics *in vitro* by day 3 (Figure 5.3A). Conversely, HPU-35 IVRs with 100% TFV in the tubing lumen demonstrated gradually increasing *in vitro* TFV release rates that did not reach steady-state by day 28. The tubing lumen was fully hydrated after 24 h for the glycerol-formulated IVRs, whereas the tubing lumen was still only partially hydrated at day 28 for the 100% TFV-formulated IVRs. As a result, all subsequent IVR testing utilized the lumen formulation of TFV/glycerol/water, whereby the steady-state TFV release rate was found to increase with the HPU percent swelling (Figure 5.3B, t = 0 storage time). However, when the IVRs were first stored at 40°C and subsequently subjected to *in vitro* release testing, they demonstrated lower steady-state TFV release rates that decreased as a function of IVR storage time (Figure 5.3B). The decrease in the steady-state TFV release rates followed exponential decay kinetics, eventually plateauing ($p = 0.09$ between 6- and 8-week storage times for HPU-B1 IVR, Student's two-tailed t-test). The time required to attain equilibrium, related to the decay constant, increased with the elastic modulus of HPU (elastic modulus of HPU-35 > HPU-60 > HPU-B1).

5.4.3 Chemical and physical stability analysis

Several experiments were conducted to investigate whether the diminished TFV steady-state values after IVR storage at 40°C were a result of chemical and/or physical instability in the formulation. The percent recoveries of TFV from IVRs stored for 0, 2, 4, and 6 weeks at 40°C were 100.0 ± 2.1, 98.0 ± 0.4, 99.6 ± 2.1, and 98.2 ± 0.8 percent,

respectively (mean ± SD, N=3 IVRs/time point), indicating no significant TFV chemical degradation ($p = 0.34$, one-way ANOVA). Similarly, there was no considerable change in the molecular weight of HPU after 2 and 8 weeks of IVR storage at 40°C (Table 5.2). Therefore, it was concluded that physical changes occurring in HPU during storage caused the decreased TFV steady-state flux. To evaluate the potential interaction of glycerol with the HPU, the HPU tubing alone (lacking any lumen formulation) and HPU tubing immersed in glycerol (no TFV or water) were stored for various times at 40°C and subsequently filled with the semisolid formulation to test the *in vitro* release of TFV. Both tubing alone and glycerol-immersed tubing demonstrated reduced but equilibrated TFV release rates that were similar to the plateau values observed in Figure 5.3B (data not shown). However, the time to equilibrium was significantly shorter for the glycerol-immersed tubing than for tubing alone, implying that HPU undergoes physical changes during storage that are accelerated by glycerol.

### 5.4.4 HPU swelling and water-state

The percent swelling of the HPU-B1 tubing was measured as a function of the IVR storage time to monitor any potential changes in the bulk swelling which could influence the TFV release kinetics. The swelling of unstored HPU-B1 IVRs at 37°C (after 14 days of *in vitro* release testing) was 65 wt% but dropped to approximately 55 wt% after 8 weeks of IVR storage at 40°C, at which point swelling had equilibrated (Table 5.3, $p = 0.34$ between HPU-B1 IVR 6- and 8-week storage time, 2-tailed Student's t-test). The most significant drop in swelling occurred between 0 and 2 weeks of IVR storage, which paralleled the considerable drop in the TFV release rate. The swelling was

approximately 2-4% higher at 20°C than at 37°C regardless of the IVR storage time. As the state of water in the hydrated HPU (bound, partially bound, or free) may influence the TFV release rate, DSC thermograms were also generated for the hydrated IVRs postrelease which had first been stored for various times at 40°C. Unstored HPU-B1 IVRs exhibited melting endotherm and freezing exotherm water peaks for both free and partially bound water (9, 21). All endotherms and exotherms decreased in height and peak area with increasing IVR storage time (Figure 5.4A and Table 5.3), with peak onsets shifting to lower temperatures. Peak 2, representing the freezing point of partially bound water, changed most dramatically, as it was initially large but was no longer detectable after 6 weeks of storage for the HPU-B1 IVR. The corresponding enthalpy of freezing for peak 2 dropped significantly with increasing IVR storage time and followed exponential decay kinetics that were indistinguishable from those of the steady-state TFV release rate for HPU-B1 (Figure 5.4B, the decay constant for peak 2 and the steady-state TFV release rate were 5.7 and 5.6, respectively). In comparison, peaks 1 and 4, corresponding to the freezing and melting of free water, respectively, showed only slight reductions in the enthalpic peak area as a function of storage time. Peak 3, corresponding to the partially bound water enthalpy of melting, was not calculated because the extremely broad peak shape prevented quantification.

### 5.4.5 Final design selection and *in vitro* characterization

After confirming that equilibrium was achieved for each HPU (i.e., no significant decrease in the TFV release rate or HPU swelling between two storage time points), the TFV steady-state release rate for each HPU IVR was plotted as a function of its

equilibrium swelling percentage (Figure 5.5A). As expected, the TFV release rate increased with the equilibrium swelling percentage, ranging from 3-27 mg/day. However, the curve was nonlinear, following a trend whereby the TFV release rates from the higher-swelling HPUs were disproportionally greater. To understand the potential underlying mechanism, the equilibrated HPU IVRs were also evaluated by DSC. The enthalpic difference of the free water (peaks 1 and 4) between HPU-35 and HPU-B3 was less than 5-fold (Table 5.4) despite their 10-fold difference in steady-state release rate. The free water enthalpy of melting (peak 4) was linearly related to the equilibrium percent swelling (Figure 5.5B). In comparison, the enthalpy of freezing (peak 2) showed significant differences between the HPU swelling types: it was only detectable in the equilibrated HPU-B2 and –B3 IVRs, both of which exhibited much higher TFV release rates than the lower-swelling HPUs.

HPU-B1 was further investigated with various tubing wall thicknesses to determine the TFV release rate dependence on $\ln(r_o/r_i)$ as predicted by Equation 5.1, the IVR mechanical stiffness dependence on $r_i^4$ as predicted by Equation 5.2, and IVR tube kinking dependence on $r_o^2/w$ as predicted by Equation 5.3. The experimental equilibrium TFV release rate (post-IVR storage) followed the general trend predicted by $\ln(r_o/r_i)$ although a slight deviation was observed at the terminal phase (Figure 5.6, $R^2 = 0.98$). To predict the IVR stiffness at 10% ring compression, the elastic modulus of HPU-B1 was measured as 19.3 ± 0.4 MPa (mean ± SD, N = 3). The force required to achieve 10% ring compression for the four different wall thicknesses was then estimated using Equation 5.2. The experimental force values were determined using unfilled HPU-B1 IVRs to eliminate any potential mechanical contribution from the semisolid lumen formulation.

Despite a slight bias, the predicted and experimental force values were comparable with an $R^2$ value of 0.84 (Figure 5.7). IVR tube kinking was qualitatively determined as the exact point of tube kinking is poorly defined and could not be accurately quantified. However, the approximate point of tube kinking for the four wall thicknesses followed the expected trend as predicted in Equation 5.3 whereby the 1.21 mm wall thickness IVR did not kink when fully compressed and the 1.02, 0.87, and 0.68 mm wall thickness IVRs kinked at approximately 100, 75, and 50% ring compression, respectively.

The HPU-B1 IVR with a 0.68 mm wall thickness was selected as the lead prototype as it achieved an equilibrated (poststorage) TFV release rate that was similar to the previous HPU IVR yielding promising results in sheep, possessed the largest core volume to maximize the amount of TFV loaded in the reservoir, and exhibited desirable mechanical stiffness and tube kinking. The 90 day *in vitro* release and mechanical stiffness was tested for these IVRs after 2 weeks of storage at 40°C because this treatment brought the HPU-B1 to near-equilibrium. The TFV release kinetics were time-independent from day 2 through day 90 (Figure 5.8A). In addition, the IVR mechanical stiffness did not change considerably before or throughout the 90 day study period (Figure 5.8B) and the required force for ring compression was approximately 10% higher compared to the empty HPU IVRs previously tested (Figure 5.7 t = 0). The force required for 10% ring compression was approximately 2.4-fold greater than NuvaRing® and only slightly greater than Estring®.

5.4.6 *In vivo* TFV release evaluation

The IVRs were subsequently tested in sheep to determine the *in vivo* time-averaged TFV release rate. The IVRs were constructed from HPU-B1 tubing with a 5.5 mm cross-sectional diameter and 0.68 mm wall thickness and stored for 2 weeks at 40°C as the final manufacturing step. There were no ring expulsions over the 29 day study period, and the inspection of the IVRs post-use revealed no issues with the weld integrity. The 29-day average *in vivo* release rate, as determined by residual TFV extraction, was 22.8 ± 7.0 mg/day (mean ± SD, N=5 sheep), approximately 75% higher than the average 13 mg/day *in vitro* release rate for the same lot of IVRs stored for 2 weeks at 40°C (Figure 5.8A).

## 5.5 Discussion

A new reservoir intravaginal ring manufactured from hydrophilic polyurethane tubing achieved time-independent TFV release rates and unchanged mechanical stiffness for up to 90 days. The design offered flexibility in modulating the drug release rate and the ring mechanical properties by adjusting the IVR dimensions, HPU elastic modulus, and HPU percent swelling. Attenuated steady-state drug release rates were observed after the IVRs were stored at 40°C and were primarily attributed to less partially bound water in the hydrated HPU being available for TFV diffusion. The final device design provided a ring mechanical stiffness similar to commercial IVRs and release rates of approximately 10 mg/day for 90 days after the equilibration of the HPU. *In vivo*, no rings were expelled and the mean drug release rate was 75% greater than the *in vitro* rate over the 29-day study duration.

5.5.1 IVR design

We designed a reservoir HPU IVR that can be modulated to provide controlled, milligrams-per-day TFV delivery to offer a long-lasting dosage form as an alternative to the clinically effective short-lasting TFV 1% gel. Because IVR mechanics are likely to play a critical role in ring insertion, retention, and biocompatibility in the vaginal tract (16, 42), we also desired an IVR with time-independent mechanical stiffness that was similar to current commercial IVR products. Moreover, we sought to minimize the IVR cost to ensure affordability in resource-poor regions, where the HIV pandemic is most prevalent. This priority required a ring that could be manufactured for low cost at high throughput capacity and could provide sustained TFV delivery for up to 90 days to amortize cost. Finally, we attempted to minimize the ring volume to reduce the probability of IVR detection during intercourse and the consequent negative impact on user acceptability (33). Marketed IVRs typically possess outer diameters of approximately 55 mm, with cross-sectional diameters ranging from 4.0 mm to 9.0 mm (2). As a result, the IVR outer and cross-sectional diameters in this study were fixed at 55 and 5.5 mm, respectively. In lieu of evaluating various IVR outer and cross-sectional diameters, the HPU swelling and wall thickness were varied to achieve the desired drug release rate, dosage duration, and ring mechanical properties.

IVRs are retained in the vaginal tract via the opposing recoil forces of the compressed elastic ring and distended vaginal musculature, resulting in a slightly ovoid ring conformation of approximately 10% in the case of NuvaRing® (3, 14). If the tubing wall of the HPU IVR were to kink under similar compression values *in vivo*, the IVR recoil force would be diminished and the ring could be displaced or expelled from the

vagina. The tubing critical bend radius is inversely proportional to wall thickness (Equation 5.3); therefore, a tube that possesses a thicker wall will be more kink-resistant but will have a lower lumen volume available for drug loading. We tested HPU tubing with wall thicknesses ranging from 0.68 mm to 1.21 mm, as these constraints allowed for at least 0.9 g of TFV in the lumen yet did not kink under mild ring compression. The force required to compress the IVR to 10% of its initial outer diameter was measured and compared to the commercial IVRs NuvaRing® and Estring®, which have established user acceptability and safety records and therefore serve as benchmarks for mechanical stiffness (30, 31). The 0.68-mm-thick wall of HPU-B1 IVR demonstrated a mechanical stiffness that was most similar to the marketed rings and also possessed the highest lumen volume. When this IVR was filled with the semisolid formulation and shape-annealed in a circular conformation, tube kinking occurred at approximately 75% compression. The cross-sectional collapse of the IVR at or near the maximum ring compression values (i.e., during user insertion of the ring) may be advantageous as it could ease IVR manipulation during insertion yet allow the full recovery of the mechanical stiffness once the IVR is inside the vaginal tract due to the reversibility of the tube kinking in the elastomeric HPU.

The measured ring compression force as a function of the wall thickness of the HPU-B1 IVR was similar to that predicted by Equation 5.2, with deviations likely due in part to geometric inconsistencies in the tubing outer diameter, wall thickness, and IVR concentricity. TFV matrix IVRs were recently manufactured under tight dimensional tolerances by injection molding, and the subsequent predicted and experimental force compression values were nearly identical (4). The predicted force range for the tested

tubing IVR dimensions was quite small (1.4 – 1.8 N), increasing the probability of experimental error and noise during compression testing. Altogether, the mechanical model provided a good estimate of the tubing stiffness for the HPU IVR that may prove useful when prototyping devices. When the TFV/glycerol/water HPU-B1 IVRs were mechanically tested, their force compression values were similar to Estring® and only slightly higher than empty IVRs. Furthermore, the force compression values for HPU-B1 IVR did not change considerably as a function of time during the *in vitro* release testing, in contrast to the matrix IVR, which demonstrated a 3-fold decrease in ring stiffness after 90 days (4). This finding highlights the ability of the semisolid lumen formulation to provide a negligible contribution to the mechanical stiffness of the ring while achieving a high TFV load.

The *in vitro* TFV release kinetics for IVRs with and without the osmotic agent glycerol were remarkably different. Steady-state TFV release kinetics were attained after 3 days with the TFV/glycerol/water semisolid lumen formulation, whereas the 100% TFV lumen formulation did not achieve steady-state after 28 days. The tubing lumens of the glycerol-loaded IVRs were fully hydrated after 24 h in the release medium, whereas the 100% TFV-loaded IVRs were not completely hydrated after 28 days, indicating that the glycerol functioned as an osmotic agent to rapidly draw aqueous media into the HPU lumen and equilibrate the device for steady-state drug release. The measured glycerol absorption in the tubing wall of the stored HPU IVRs was approximately 5 wt%, suggesting that glycerol was able to diffuse through the HPU tubing wall during storage. When the IVR was exposed to aqueous media, a steep glycerol concentration gradient across the tubing wall was immediately established because glycerol and water are

infinitely miscible. This gradient resulted in a significant water flux into the tubing lumen, allowing the subsequent dissolution of TFV and saturation of the lumen aqueous media. The excess of undissolved TFV in the core ensured a fixed TFV concentration gradient was established across the swollen HPU rate-controlling membrane to produce steady-state TFV release kinetics, as described by Equation 5.1. The 100% TFV lumen formulation did not achieve steady-state TFV release after 4 weeks because TFV alone is a poor osmotic agent (~1% w/v solubility in acidic aqueous media) (4). Solid osmotic agents such as sodium chloride demonstrate similar lumen hydration and steady-state TFV release kinetics as glycerol, although the time to steady-state is slightly longer (data not shown). Over 95% of the glycerol was released from the IVR in the first 3 days (data not shown). Earlier work with higher-molecular-weight water-miscible excipients, such as 400-molecular-weight polyethylene glycol, demonstrated a significant ballooning of the tubing cross-section upon *in vitro* and *in vivo* testing (unpublished work), indicating that the swollen effective mesh size of the HPU tested is larger than glycerol but smaller than the hydrodynamic radius of polyethylene glycol-400.

The TFV steady-state release rate increased with the percent swelling of HPU, confirming the previous conclusions that TFV is transported predominantly through the aqueous phase of the swollen HPU because it is virtually insoluble in the hydrophobic polymer phase (4, 12). Equilibrated HPU-B1 IVRs of varying cross-sectional dimensions demonstrated steady-state TFV release rates that were in good agreement with the predicted values. Altogether, these studies demonstrated the role that HPU swelling, tubing cross-sectional dimensions, and HPU elastic modulus play in modulating the TFV release rate and ring mechanical properties.

5.5.2 Assessment of HPU changed during IVR storage

Chemical and physical stability studies are often neglected during IVR development (14), although significant changes that impact device performance may arise as reported herein. The chemical and physical stability of the HPU IVRs was tested by placing IVRs at accelerated 40°C storage for various times. Overall, the steady-state TFV release rates followed exponential decay kinetics as a function of storage time, eventually plateauing. There were no significant changes in the TFV and HPU chemical stability after IVR storage, indicating that physical changes to the HPU were responsible for the decreased TFV flux. As mentioned above, knowledge concerning the microphase separation of polyurethanes has existed for decades (19, 26). However, there has been limited work with polyurethanes in drug delivery applications and the effect of microphase separation on drug release has not been reported in the literature. Furthermore, the chemical composition of polyurethanes is amazingly diverse, as isocyanates (i.e., aliphatic or aromatic), chain extenders, polyols (i.e., hydrophobic, hydrophilic, or amphiphilic), and the molecular weight and weight fraction of hard/soft segments all contribute to the structure-property relationship of polyurethanes (36). As a result, it is difficult to compare the resultant physical properties (i.e., microphase separation) for compositionally different polyurethanes, especially because most literature reports do not utilize commercially available polyurethanes and no studies have been reported with Tecophilic commercial HPU grades, much less regarding drug release from these polyurethanes.

The brand name Tecophilic refers to segmented polyether urethanes composed of the aliphatic 4,4'-dicyclohexylmethane diisocyanate ($H_{12}$MDI) and 1,4-butanediol as the

hard segment and polytetramethylene oxide (PTMO) and polyethylene oxide (PEO) co-polymer as the soft segment (35). The physical properties and morphology of $H_{12}$MDI polyurethanes are generally different from polyurethanes utilizing the more common aromatic isocyanate methylene diphenyl diisocyanate. In particular, $H_{12}$MDI hard segments are inferior at crystallizing and form smaller amorphous or semicrystalline domains (19, 36). Commercially available $H_{12}$MDI is composed of 30% trans,trans, 65% trans,cis, and 5% cis,cis geometric isomers (36). The trans,trans fraction of $H_{12}$MDI influences the degree of crystallinity, as it is the only isomer that can crystallize (27, 38). Polyurethane microphase separation kinetics can be very slow, resulting in significant time-dependent performance, which may influence not only the mechanical stiffness as we have previously reported (5) but also the drug release in a combination drug-device.

Several techniques have been used extensively to characterize polyurethane microphase separation for over four decades, including Fourier transform infrared spectroscopy, differential scanning calorimetry, and small-angle x-ray scattering (18, 26). These three techniques were utilized in an attempt to characterize any physical changes of the HPU as a function of IVR storage time (the IVRs were stored but not hydrated prior to testing). However, no significant changes were observed with any technique (data not shown) even though large decreases in the steady-state drug release rate were evident. The above techniques have primarily been used to monitor the crystallinity of crystallizable hard segments, which increases with the extent of microphase separation. However, $H_{12}$MDI polyurethanes exhibit minimal hard segment crystallinity, as discussed above.

The water molecules present in hydrated hydrophilic polymers have been categorized into three different states: bound, partially bound, and free (9). Bound water is closely associated with the polymer due to strong hydrogen bonding and does not exhibit calorimetric freezing as low as -100°C because its mobility is restricted. Free water is able to diffuse because it is only involved in water-water interactions, crystallizing at approximately -10 to -20°C and melting at approximately 0°C. Partially bound water exhibits both polymer-water and water-water interactions, demonstrating intermediate mobility with phase transition temperatures that are lower than those of free water but observable by calorimetric methods. Drug release from hydrophilic polymers is generally considered to be primarily dependent on the freezable water fraction (partially bound and free), which is mobile and able to dissolve and transport the drug (21, 43). These previous studies demonstrated a modulation of the drug release rate from PEO-containing polyurethanes by varying the percent PEO content, which was correlated with an increase in the free water fraction. Yui *et al.* observed free water by DSC at 21 wt% and greater bulk swelling, which they attributed to a physical transition from dispersed PEO domains in the hard segments and PTMO (thereby consisting primarily of bound water) to dispersed hard segments and PTMO domains in a continuous PEO phase (thereby creating free and partially bound water via the formation of water clusters) (43). The HPUs investigated in this work had aqueous swelling percentages that were significantly higher than 21 wt%, and free water was detected in all swelling grades. The enthalpy of melting for free water was found to increase linearly with the bulk swelling percent from the equilibrated HPU IVRs, in agreement with the literature. However, partially bound water was observed in the higher swelling HPU-B2 and HPU-B3 and was

not detectable in the lower swelling HPUs, which coincided with a disproportional increase in the TFV release rate. These results indicate that both partially bound water and free water contribute to the TFV release kinetics. Unstored HPU-B1 IVRs demonstrated partially bound water peak areas (peak 2), which decreased with storage time and eventually became undetectable when stabilized TFV release rates were achieved. Because the partially bound water peak areas and TFV release rates demonstrated indistinguishable exponential decay kinetics and the free water enthalpic peak areas decreased only slightly with storage time, it appears that the loss in partially bound water was primarily responsible for the decreased TFV flux after IVR storage.

The underlying physical mechanism whereby polyurethane microphase separation induces changes in the bulk swelling and water state is uncertain. The solubility parameters of PTMO and PEO are quite similar at lower temperatures (i.e., during product storage), whereas the solubility parameter of the hard segment differs significantly (43). Although Yui *et al.* did not perform physical stability studies (i.e., microphase separation kinetics), they concluded that the PTMO and/or PEO phase separate from the hard segments but that it is improbable that PEO and PTMO phase separate from each other. We could not discern any change in the soft segment glass transition temperature after storage that would suggest that the PEO and PTMO were phase separating. However, they likely possess similar glass transition temperatures which may be indistinguishable by DSC (32, 43). Therefore, we similarly hypothesize that during hot-melt extrusion, the hard segment and soft segment are miscible and form a homogenous bulk polymer phase, whereby uniform swelling is achieved with little hindrance to the hydration of PEO. However, as a function of cooling and storage time,

PEO (and likely PTMO) phase separates from the hard segments, influencing the hydration of PEO and the overall mass of water absorbed. In turn, this process decreases the total amount of free and partially bound water. Once the microphase separation is complete, the HPU is thermodynamically stable, as indicated by no further changes in bulk swelling, water state, or TFV release kinetics.

The percent swelling of HPU immediately following tubing extrusion was significantly greater when compared to unextruded polymer pellets. ALZA Corporation attempted to reproducibly control the swelling of Tecophilic HPU by subjecting the extrudate to elevated temperatures for approximately 48 h (15). Although we found that similar time-temperature conditions aided in stabilizing the bulk swelling, it was insufficient to fully equilibrate the TFV release rates and the amount of partially bound water. In another attempt to stabilize the HPU rate-controlling membranes, Endo Pharmaceuticals utilized "priming and conditioning," whereby devices were immersed in aqueous solutions and subsequently packaged to allow HPU hydration (17). Their success at achieving stabilized drug release rates after storage was not demonstrated. Also, product hydration is an undesirable manufacturing step because the drug may leach out of the device during manufacturing and storage and polyurethane hydrolytic degradation is probable during storage (36). We thus selected a simple final manufacturing step in which the packaged IVRs are stored for approximately 2 weeks at 40°C to bring the HPU-B1 to near-equilibrium.

The presence of glycerol in the IVR formulation complicated the investigation of the physical instability. There is evidence that low-molecular-weight alcohols accelerate polyurethane microphase separation and impact the polyurethane's morphology (8). In

particular, Meuse *et al.* showed that glycerol enables polyurethane to achieve a higher degree of microphase separation (22). As reported herein, it appears that glycerol accelerates the slow microphase separation kinetics in the hot-melt extruded Tecophilic polyurethanes via a plasticizing effect because the tubing alone eventually attained the same TFV equilibrium release rate but after a significantly longer storage time.

5.5.3 *In vivo* assessment

The sheep vaginal tract is anatomically similar to the human vaginal tract (41) and therefore enables the animal testing of IVR clinical products without needing to alter the device dimensions. *In vivo*, no rings were expelled over the 90 day study and there were no adverse histopathological findings (data not shown). Although clinical testing is needed to confirm the device performance in women, these pilot results indicate that the rings had sufficient mechanical stiffness and critical bend radius to be retained in the vaginal tract yet not cause obvious epithelial tissue damage. The mean TFV release rate was 75% higher *in vivo* than *in vitro*, in agreement with two previous *in vivo* sheep studies with an earlier prototype, where the *in vivo* release rate was approximately 70% higher than *in vitro* (see Chapter 4). This device therefore released a similar daily amount of TFV as that in a previous study, demonstrating that the time-independent *in vivo* vaginal concentrations of TFV were similar to or exceeded the peak concentrations from the TFV 1% vaginal gel. A number of potential explanations exist for the higher *in vivo* release rate, with the most likely being increased TFV solubility in the pH 7 sheep vaginal fluid than in the pH 4.2 *in vitro* release medium that was designed to simulate the normal acidic vaginal pH of women (25). Because the solubility of TFV increases with

increased pH, a higher dissolved TFV concentration in the IVR lumen would establish a higher TFV concentration gradient across the tubing wall, increasing the TFV release rate as described in Equation 5.1. As a result, studies in women are needed to determine an *in vivo*/*in vitro* release correlation. However, any necessary adjustment of the steady-state TFV release rate can be easily accomplished by changing the HPU equilibrium swelling or tubing dimensions, as demonstrated herein.

The vaginal drug delivery field has not met the need for a cost-effective device capable of long-term, controlled delivery of hydrophilic drugs such as TFV. We addressed this need by designing and testing a novel HPU tubing IVR that provides zero-order TFV delivery for up to 90 days. The percent swelling of the HPU and the ring geometry were modulated to achieve equilibrated steady-state release rates ranging from 1 - 30 mg/day, which was approximately 1000-fold greater than other IVR designs recently reported (23, 24). We anticipate that the ring design will serve as a versatile platform for the vaginal delivery of a variety of therapeutic agents (14). The ring design allows for room-temperature, solvent-free manufacturing with drug/excipient loadings up to 3 grams and could potentially expand ring delivery to a diverse spectrum of therapeutics that cannot withstand typical ring fabrication temperatures or cannot adequately diffuse through conventional polymers at the required release rate. Proven manufacturing technologies from the medical device field were utilized to ensure that the device could be scaled to high-volume manufacturing capacity at a low cost to maximize its potential impact in resource-poor regions where such a product is most needed. The steady-state drug release rates decreased exponentially when the IVRs were stored at 40°C, and was primarily attributed to the decreased amount of partially bound water in

the hydrated HPU. After equilibration of the HPU, a final device composition and design was selected that provided a time-independent ring mechanical stiffness similar to marketed rings and TFV release rates of approximately 10 mg/day for 90 days. *In vivo*, the IVRs were well tolerated with no ring expulsions or obvious histopathological findings. In summary, a tunable IVR platform was designed and characterized that demonstrated elevated and zero-order TFV delivery, offering an alternative dosage form to the TFV 1% gel that is controlled and long-lasting.

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Table 5.1. IVR formulation matrix.

| IVR ID | Polymer | Wall thickness evaluated (mm) |
|---|---|---|
| HPU-35 * | HP-60D-35 | 0.68 |
| HPU-60 | HP-60D-60 | 0.68 |
| HPU-B1 | 75/25 HP-60D-60/HP-93A-100 | 0.68, 0.87, 1.02, 1.21 |
| HPU-B2 | 50/50 HP-60D-60/HP-93A-100 | 0.68 |
| HPU-B3 | 25/75 HP-60D-60/HP-93A-100 | 0.68 |

* HPU-35 IVRs with cores containing 100% TFV and 65/33/2 wt% TFV/glycerol/water were tested. All other IVR formulations tested only the TFV/glycerol/water core composition.

Table 5.2. HPU-B1 molecular weight as a function of 40°C IVR storage time. Mean of 3 measurements.

| **Time (weeks)** | **$M_n$ (kDa)** | **$M_w$ (kDa)** | **PDI** |
|---|---|---|---|
| 0 | 51 | 127 | 2.5 |
| 2 | 44 | 110 | 2.5 |
| 8 | 45 | 119 | 2.6 |

Table 5.3. The bulk swelling and enthalpic water peak areas of the hydrated HPU-B1 IVRs as a function of 40°C storage time. Mean ± SD, N = 3 IVRs. The peak area is expressed as joules per gram of dry polymer. ND: not detectable.

| **Time (weeks)** | **Bulk swelling at 20°C (wt%)** | **Bulk swelling at 37°C (wt%)** | **-ΔH peak 1 (J/g)** | **-ΔH peak 2 (J/g)** | **ΔH peak 4 (J/g)** |
|---|---|---|---|---|---|
| 0 | 69.4 ± 0.9 | 65.2 ± 0.6 | 12.5 ± 2.5 | 24.6 ± 1.2 | 29.6 ± 2.0 |
| 1 | 64.1 ± 0.3 | 59.8 ± 0.1 | 5.7 ± 1.3 | 7.8 ± 1.0 | 22.9 ± 0.4 |
| 2 | 60.7 ± 0.4 | 57.2 ± 0.1 | 5.9 ± 2.0 | 2.2 ± 0.2 | 20.9 ± 2.6 |
| 4 | 60.5 ± 0.3 | 56.7 ± 0.1 | 6.6 ± 0.5 | 1.2 ± 1.1 | 22.5 ± 1.4 |
| 6 | 58.6 ± 1.0 | 55.8 ± 1.0 | 4.2 ± 0.9 | ND | 16.7 ± 1.1 |
| 8 | 57.9 ± 0.9 | 55.0 ± 0.7 | 6.7 ± 1.1 | ND | 19.4 ± 1.8 |

Table 5.4. The enthalpic water peak areas of the hydrated and equilibrated HPU IVRs. Mean ± SD, N = 3 IVRs. The peak area is expressed as joules per gram of dry polymer. ND: not detectable.

| Polymer | -ΔH peak 1 (J/g) | -ΔH peak 2 (J/g) | ΔH peak 4 (J/g) |
|---|---|---|---|
| HPU-35 | 3.3 ± 1.6 | ND | 9.7 ± 0.8 |
| HPU-60 | 2.7 ± 0.8 | ND | 15.3 ± 0.4 |
| HPU-B1 | 6.7 ± 1.1 | ND | 19.4 ± 1.8 |
| HPU-B2 | 9.4 ± 0.6 | 16.3 ± 3.2 | 24.9 ± 1.3 |
| HPU-B3 | 14.5 ± 2.5 | 37.7 ± 4.3 | 29.8 ± 2.7 |

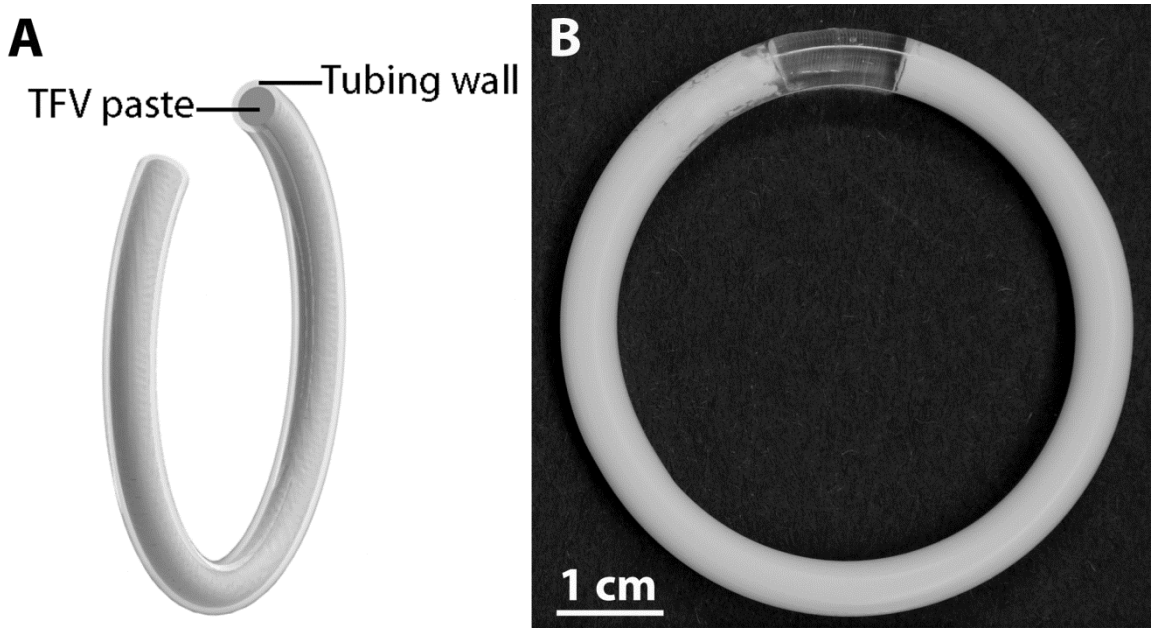


Figure 5.1. (A) Schematic of the HPU tubing IVR and cross-section. (B) Image of an IVR.

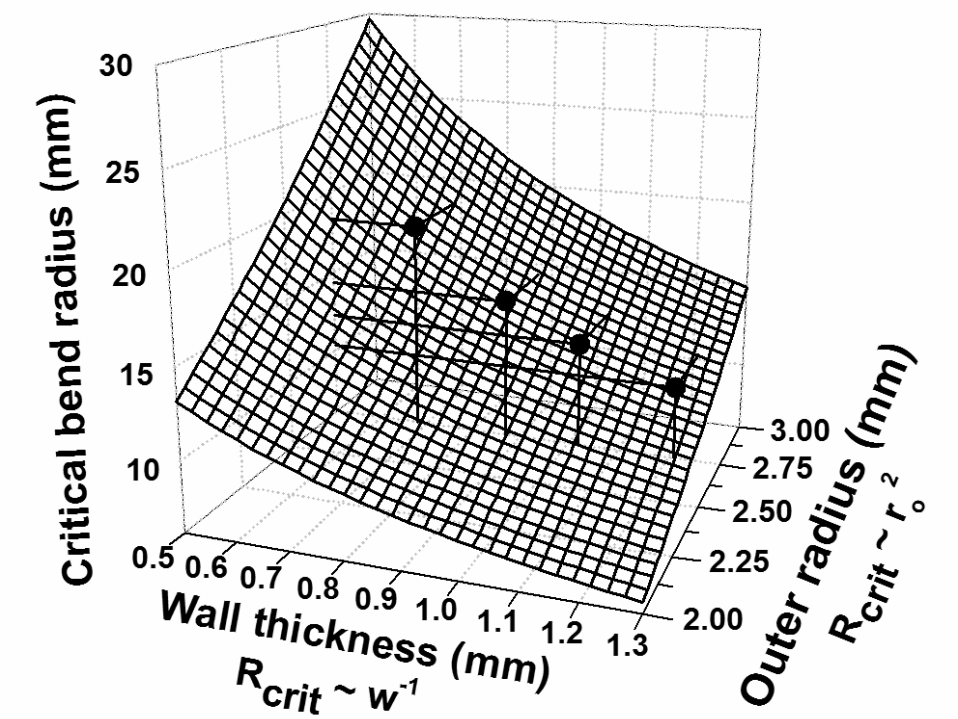


Figure 5.2. The theoretical critical bend radius as a function of tubing wall thickness and cross-sectional diameter. At tubing bend radii below and above the curved surface (i.e., below and above the critical bend radius), the tubing will and will not be kinked, respectively. Black dots indicate the experimentally tested tubing dimensions.

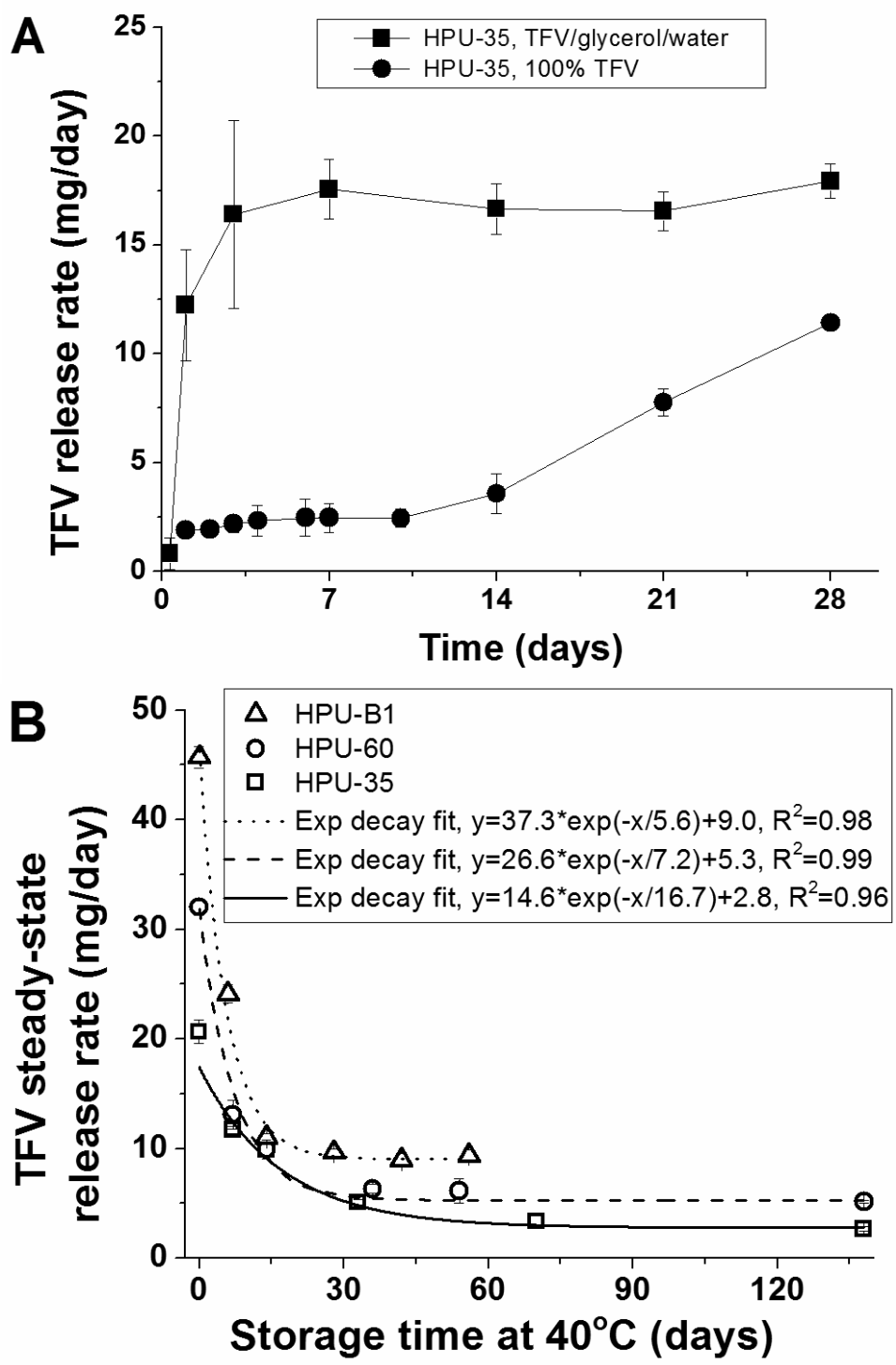


Figure 5.3. (A) *In vitro* TFV release rate as a function of time for unstored HPU-35 IVRs with and without glycerol in the tubing lumen (mean ± SD, N = 3 IVRs). (B) Steady-state TFV release rate as a function of 40°C storage time for IVRs fabricated from HPU-B1, HPU-60, and HPU-35 (mean ± SD, N = 3 IVRs, release rates averaged over days 5-14).

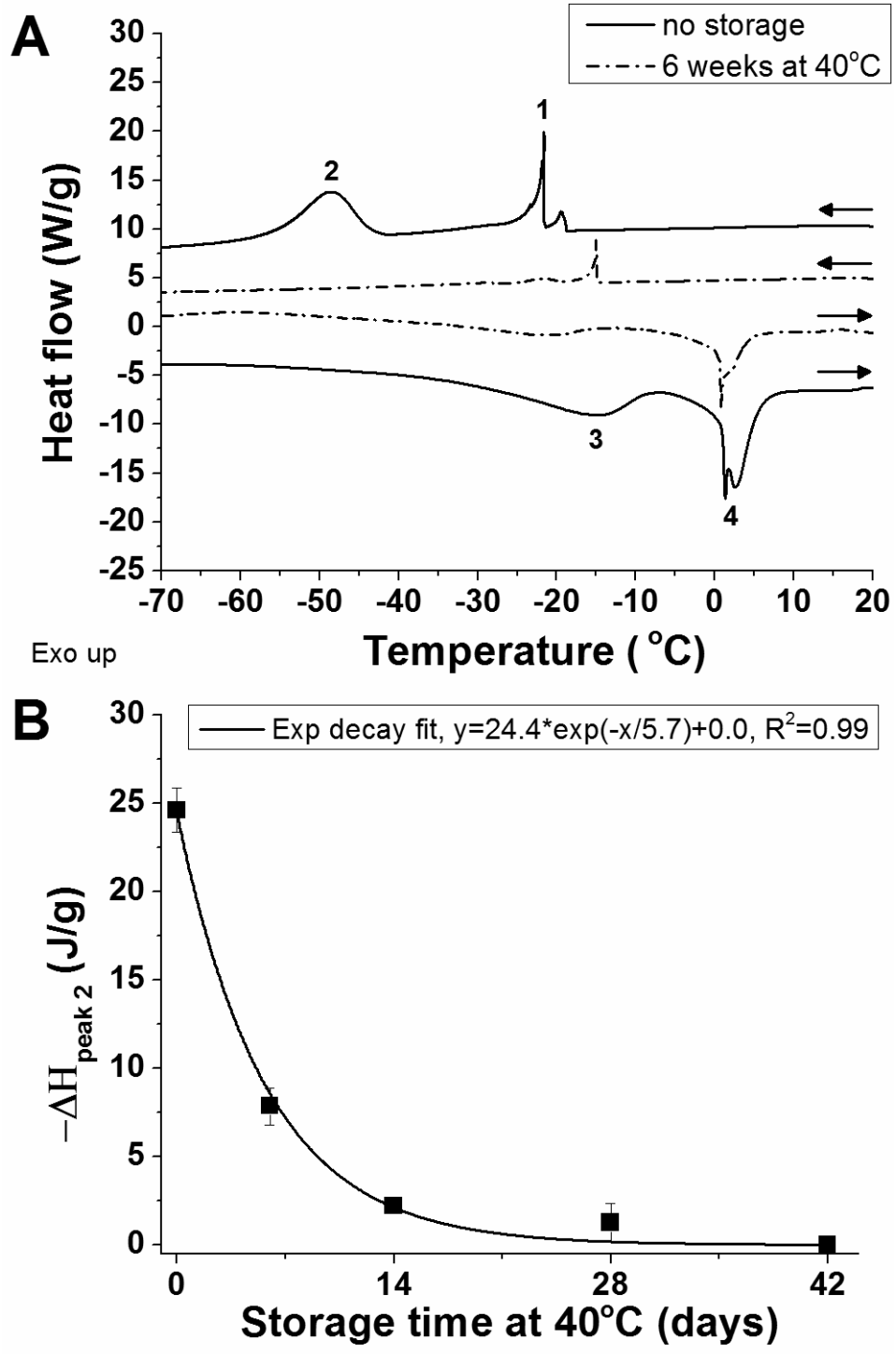


Figure 5.4. (A) Representative DSC cooling and heating curves of hydrated HPU-B1 IVRs before and after IVR 40°C storage. Peaks 1 and 2 represent the freezing point for free and partially bound water, respectively, whereas peaks 3 and 4 represent the melting point for partially bound and free water, respectively. (B) Enthalpy of freezing for partially bound water (peak 2) as a function of 40°C storage time (mean ± SD, N = 3 IVRs). The peak area is expressed as joules per gram of dry polymer.

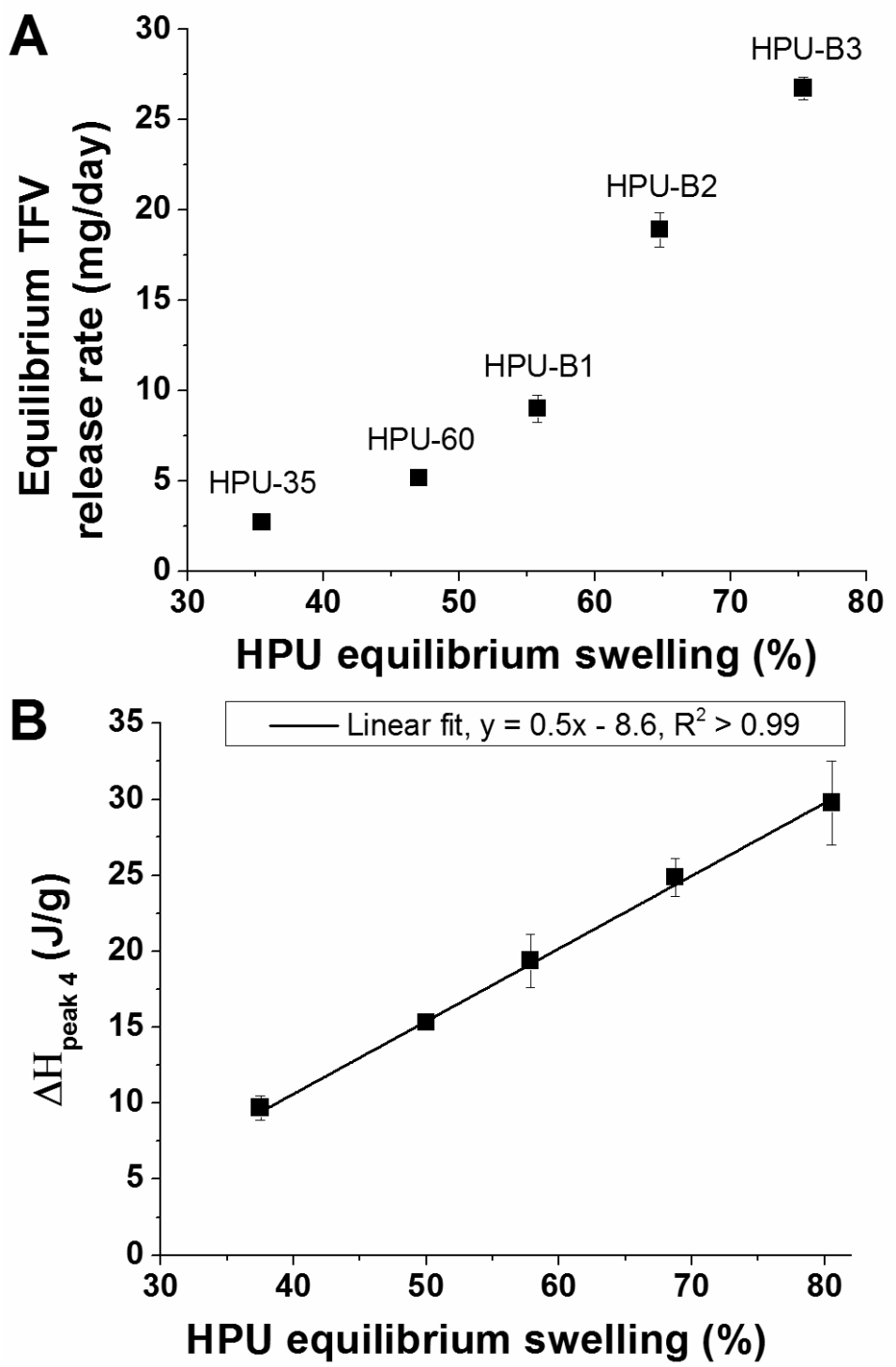


Figure 5.5. (A) Equilibrium TFV release rate as a function of the equilibrium swelling of HPU (mean ± SD, N = 3 IVRs, release rates averaged over days 5-14). (B) Enthalpy of melting for free water (peak 4) as a function of equilibrium swelling of HPU at 20°C (mean ± SD, N = 3 IVRs). Peak area is expressed as joules per gram of dry polymer.

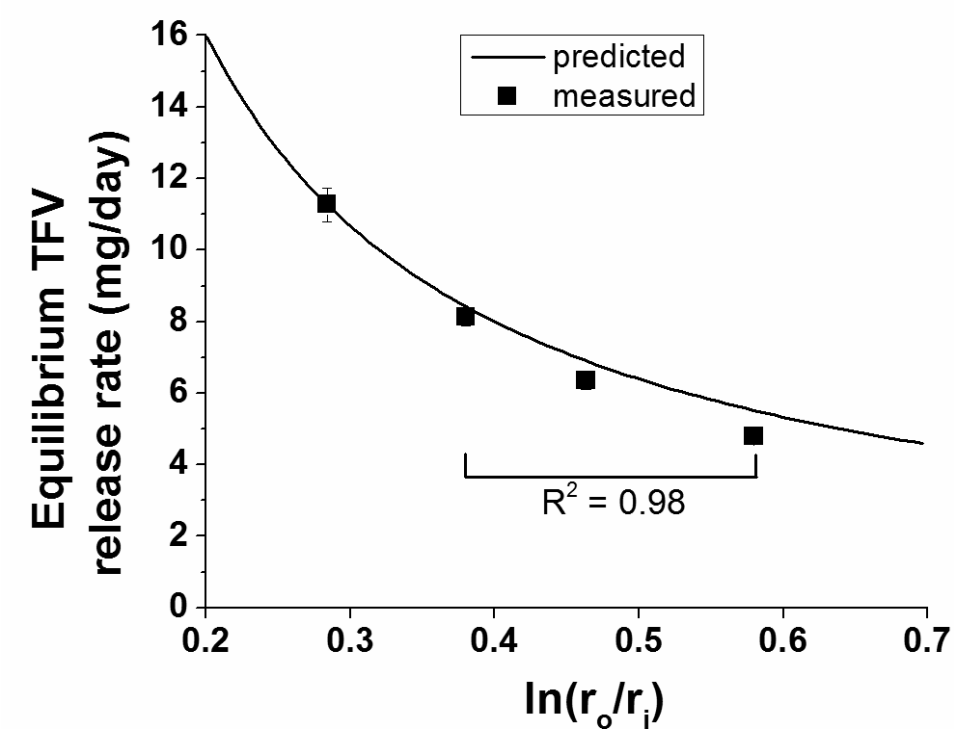


Figure 5.6. Predicted and measured equilibrium TFV release rate as a function of $\ln(r_o/r_i)$ for the HPU-B1 IVRs (mean ± SD, N = 3 IVRs, release rates averaged over days 5-14).

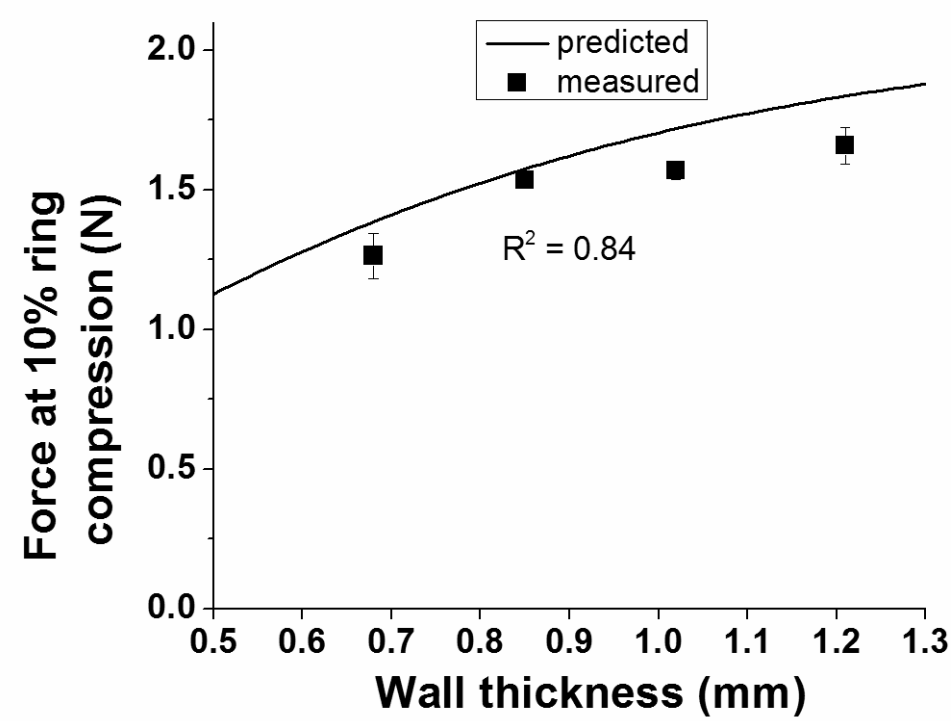


Figure 5.7. Predicted and measured 10% force compression values as a function of the IVR wall thickness for unfilled HPU-B1 IVRs (mean ± SD, N = 3 IVRs, release rates averaged over days 5-14).

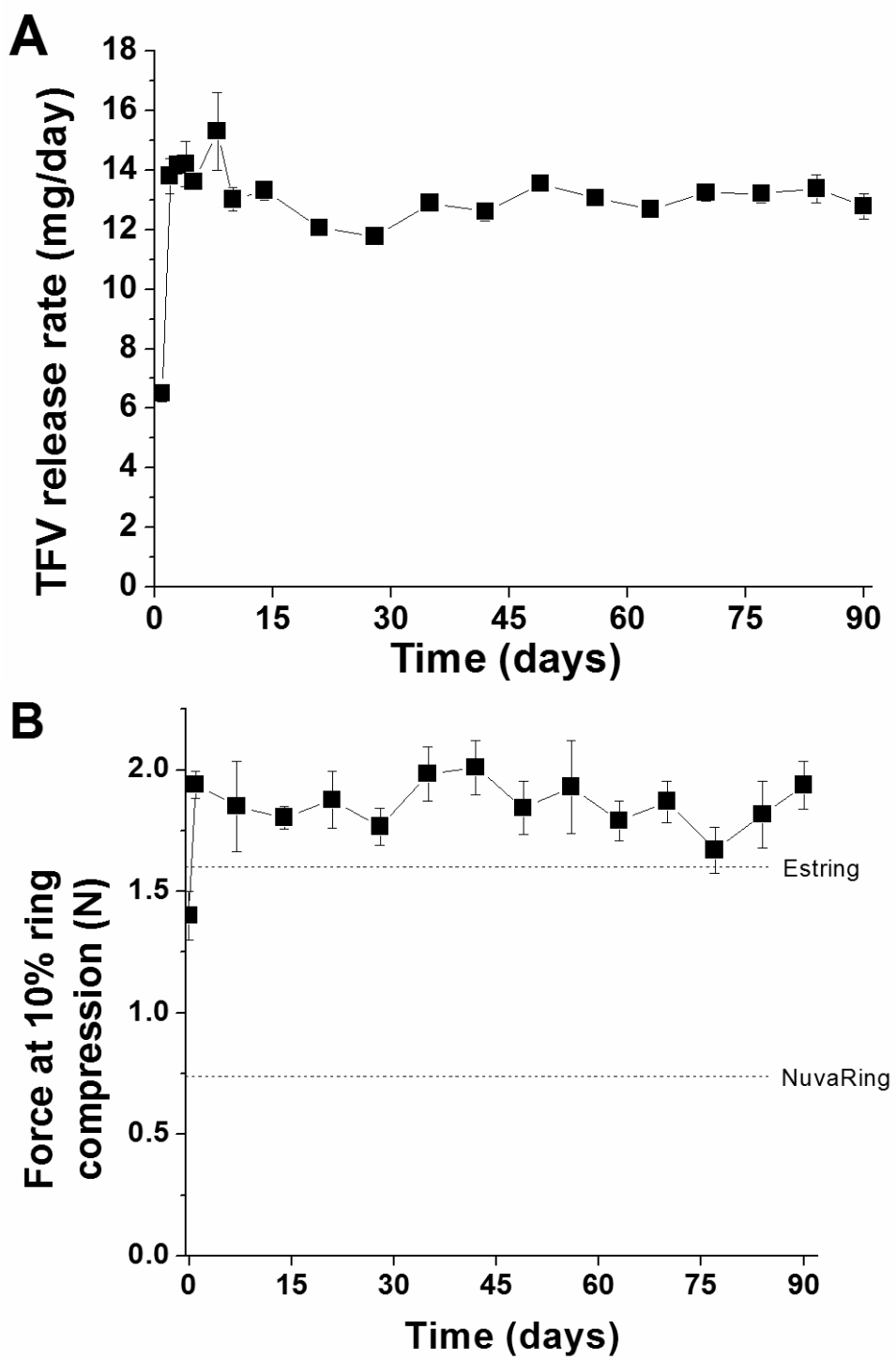


Figure 5.8. (A) TFV release rate and (B) force required for 10% ring compression as a function of time for HPU-B1 IVRs stored for 2 weeks at 40°C (mean ± SD, N = 3 IVRs). Note: TFV IVRs were from a separate manufacturing batch than from previous plots.

# CHAPTER 6

# CONCLUSIONS AND FUTURE RECOMMENDATIONS

This chapter includes excerpts from the manuscript:

STATE OF THE ART IN INTRAVAGINAL RING TECHNOLOGY FOR TOPICAL PROPHYLAXIS OF HIV INFECTION

Patrick F. Kiser, Todd J. Johnson, Justin T. Clark

Aids Reviews 2012;14(1):62-77

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The aim of the work presented in this dissertation was to advance the microbicide IVR field by 1) designing and testing new IVR constructs in which to deliver promising antiretroviral candidates, and 2) increasing the understanding of microbicide IVR performance through new safety and pharmacokinetic animal models. Overall, this dissertation work resulted in new IVR platforms which successfully formulated several diverse antiretrovirals. Furthermore, this work increased the understanding of IVR device design and corresponding *in vivo* safety, vaginal biodistribution, and pharmacokinetics through the development of new animal models. The most recent work with the TFV reservoir IVR resulted in the filing of a patent application as well as the scheduling of a phase one clinical trial in early 2013.

In the following sections, brief conclusions from each chapter will be discussed, followed by recommendations for future research in the microbicide IVR field.

## 6.1 Chapter Conclusions

### 6.1.1 Chapter 2

In Chapter 2 we developed an IVR formulation to deliver the promising small molecule pyrimidinediones which possess nanomolar HIV-1 reverse transcriptase and entry inhibition. Since only a handful of *in vivo* microbicide IVR studies had been reported at the time of publication, a dose-finding study in the pigtail macaque monkey model was performed to determine an IVR drug loading, corresponding vaginal concentrations and pharmacokinetics, and any effects on the vaginal environment. Major conclusions from this chapter are:

1) Nonswellable polyurethanes are an excellent delivery vehicle for hydrophobic small molecules including the pyrimidinediones which are highly soluble in the polymer and thus can establish loading-dependent release rates.
2) Nonsink *in vitro* release conditions accurately predicted the amount of drug released *in vivo* whereas sink conditions over-estimated *in vivo* release. This *in vitro* testing approach may prove relevant for future IVR research of hydrophobic drugs where drug dissolution into the vaginal fluid is likely the rate-limiting step.
3) The 14 wt% pyrimidinedione IVR achieved drug concentrations approximately 1000 times the *in vitro* $EC_{50}$ throughout the vaginal tract over the 28-day period, but caused no obvious vaginal irritation, inflammation, or toxicity as indicated by colposcopies, vaginal microflora levels, and vaginal and systemic cytokine levels.

6.1.2 Chapter 3

In Chapter 3 a dual delivery IVR was developed for the two antiretrovirals dapivirine and tenofovir which display different mechanisms of viral inhibition. Due to their contrasting hydrophilicity, we engineered a multisegment polyurethane ring to optimize release of both drugs wherein dapivirine and tenofovir were delivered from nonwater swellable and water swellable polyurethane segments, respectively. Major conclusions from this chapter are:

1) Water-swellable polyurethanes provide significantly greater TFV flux than hydrophobic polymers conventionally used for IVR formulation.
2) Both antiretrovirals were chemically stable in the multisegment IVR and were released at *in vitro* concentrations expected to be effective against HIV.

3) The ring technology may be useful for other multidrug delivery applications since adjusting the polyurethane hard/soft segment ratio, chemical composition, degree of hydrophilicity, and length of each segment can all modulate drug release rate.

### 6.1.3 Chapter 4

In Chapter 4 we designed and tested a new reservoir IVR platform to address limitations of the TFV matrix IVR reported in Chapter 3. IVRs were made from hydrophilic polyurethane tubing of various equilibrium swelling and tested *in vitro* and in a new sheep model for up to 90 days. Major conclusions from this chapter are:

1) A semisolid tubing lumen incorporating TFV and the water-miscible and membrane-permeable small molecule glycerol enabled rapid equilibration of drug release kinetics and time-independent IVR mechanical stiffness.
2) Hydrophilic polyurethane tubing IVRs demonstrated zero order TFV release rates *in vitro* for up to 90 days which increased with polymer percent swelling.
3) TFV vaginal and systemic concentrations in sheep were near time-independent for 90 days, and were similar to peak concentrations attained with the short duration but clinically effective TFV 1% vaginal gel.
4) Toxicological evaluation indicated that the IVRs were well tolerated for 90 days in sheep, although slight vaginal mucosal irritation was observed.

### 6.1.4 Chapter 5

In Chapter 5 we further designed and characterized a TFV reservoir IVR. IVRs of varying equilibrium swelling and tubing wall thickness were studied for their effect on *in*

*vitro* TFV release kinetics and ring mechanical properties, and chemical and physical stability studies were also performed. Major conclusions from this chapter are:

1) As anticipated, TFV release rates and ring mechanical properties were modulated by varying HPU equilibrium swelling, HPU shore hardness, and/or IVR dimensions. TFV release rates were also dependent on the amount of free and partially bound water as determined by differential scanning calorimetry. IVRs were tested *in vitro* that delivered 1 to 30 mg/day of TFV in a zero order fashion for up to 90 days with time-independent mechanical stiffness.
2) Physical stability testing (i.e., shelf-life experiments) revealed that polyurethane microphase separation occurred over weeks to months at 40°C, resulting in decreased TFV flux through the hydrated polymer due to decreased bulk swelling and the amount of partially bound water.
3) Time to polymer equilibration (i.e., complete microphase separation) increased with polymer shore hardness (elastic modulus). Storing the final formulation for 2 weeks at 40°C brought the ring to near-equilibrium, whereby further storage did not significantly decrease TFV flux or the state of water in the hydrated polymer.
4) The versatile IVR platform may be useful for delivering a wide variety of therapeutics such as biologics and macromolecules which up to now have lacked a sustained vaginal delivery vehicle.

## 6.2 Challenges and Future Directions

In the last several years, intravaginal rings for HIV prophylaxis have undergone significant technological advances and the overall understanding of their *in vivo*

performance has deepened. However, there are still many hurdles and unknowns that need to be resolved relating in part to early formulation work, target product profile, drug selection, dosage duration, multi-drug combinations, and vaginal biodistribution and pharmacodynamics. In the following section, major challenges and unknowns related to microbicide IVR development will be discussed as well as proposed future directions and priorities to ensure that an effective microbicide IVR product becomes readily available and implemented in the fight against HIV.

The academic drug delivery community often conveniently overlooks practical issues such as API chemical and physical stability, cost and reproducibility, and manufacturability, all of which must be seriously considered when designing drug delivery devices for use in the developing world. The majority of HIV infected individuals reside in resource-poor regions of the world, demanding the need for prophylactic devices at a reasonable price point for their region (6). For example, a 10 USD intravaginal ring is considered extremely cost effective in North America but is simply too costly to reach a significant market in the developing world, where a price of approx. 0.50 USD would be a significant expenditure for many if not most users. Complicated systems necessarily may be more desirable, if and only if they measurably perform better than simple devices, show chemical and physical stability, and also meet cost requirements while being made with modern pharmaceutical manufacturing equipment. Weighing these considerations is essential at project initiation to ensure the product has a high probability of reaching the clinic and having an impact on the public health crisis.

As the microbicide field is still emerging with only a handful of clinically evaluated products and only one shown to be clinically effective (1), target product profiles are often vague or even disputed throughout the field. For example, we do not have any design rules for prioritizing the need for time independent drug release versus release that changes with time. Typically, the drug delivery field prefers time-independent release as it threads the needle for products with a narrow therapeutic window between side effects and ineffectiveness. However, a rapid burst or pulsatile release followed by a lower maintenance dose may be sufficient or even advantageous to load up drug in the vaginal tissue and provide near instantaneous protection after insertion. With the proven anti-HIV activity of small molecules used in highly-active anti-retroviral therapies, the microbicide field has generally taken the approach of "the more the better" since little or no side effects have been observed with these small molecules as vaginal microbicides and the vaginal dose attained is significantly less than the oral administration route. Conversely, the long-term effect of locally high concentrations on tissue toxicity or emergence of drug-resistant virus is not known, and the goal of attaining "zero" drug in plasma but "as high as possible" drug in vaginal fluid and tissue is not realistic.

API selection is another important microbicide product consideration, although pharmaceutical company licensing rights often dictate what APIs move forward for microbicide development. API vaginal and/or intracellular half-life, chemical and physical stability, potency, and aqueous and polymer solubility should all be considered and contrasted before moving forward with IVR formulation studies. Target dosage duration is yet another unknown – contraceptive IVRs available currently range from 3

weeks to 1 year duration (2). The primary argument for longer duration is to amortize IVR costs and make the product more affordable, and therefore microbicide IVRs are generally designed for a 1 to 3 month duration (3). The impact of IVR duration on user acceptability, device performance, and biocompatibility has not been comprehensively reported and is worth further evaluation. For example, the user acceptability and willingness to continue using an IVR that is discolored from blood discharge during menses is not known.

Currently, there is significant interest in developing combination IVRs that incorporate multiple antiretrovirals with different mechanisms of HIV inhibition to prevent selection of drug-resistant virus (4, 5). Although this rationale is logical, upon conclusion of the CAPRISA 004 tenofovir vaginal gel clinical trial there were no tenofovir-resistant virus detected in women who contracted the virus while using the gel (1). As regulatory hurdles, formulation complexity, and cost become significantly greater with the incorporation of additional APIs, the necessity of such multi-API formulations should be clinically established. As discussed above, device complexity, manufacturability, and cost play a crucial yet undefined role in the probability of a device being readily available and affordable to the resource-poor regions most in need of HIV prevention technologies. Often, a compromise between achieving target device performance criteria (i.e., time-independent release or device duration) and device cost and manufacturability may be necessary.

Another variable often not considered is the effect of menstrual cycle variation and sexual intercourse on drug release from an IVR. In cases where the flux of a drug from the IVR surface is limited by its solubility in the surrounding fluid, it is conceivable

that changes in the vaginal environment could affect drug release. For instance, drug release could be reduced in the case of peri-menopausal vaginal dryness, or increased upon the introduction of semen to the insertion site due to changes in the available dilution volume. The increase in vaginal pH following intercourse could also result in a temporary modulation of the release rate of drugs which exhibit pH-dependent aqueous solubility. Some IVR dosing regimens currently under consideration involve the user leaving the ring in place for one or more menstrual cycles. During this time drug release rates could be modulated by the composition of the vaginal fluid which may contain various drug-solubilizing factors such as hemoglobin as well as by an increase in the vaginal fluid volume as already mentioned.

As we look ahead to the many challenges facing microbicide IVR development we provide our thoughts as to where future efforts should be focused. The first and most dire need, especially given the results of the VOICE trial, is the need for independent (nonuser reported) measures of adherence to IVR dosage forms. A quantitative technology that can tell clinicians if and when a participant used the IVR device as instructed will be critical in interpreting prevention trial results and in pointing the way forward if they succeed or fail. Without this information the cause of negative clinical outcomes will continue to be uncertain, and the field can only postulate whether ineffectiveness was due to low adherence, an inefficacious product, or some combination of both. Next, the microbicide field needs to significantly better its understanding of *in vivo* pharmacokinetics, vaginal biodistribution and safety and how they relate to efficacy. This is the interdisciplinary junction where drug delivery, immunology, virology, and pharmacokinetics and pharmacodynamics, come together. Expert teams in these areas

need to collaborate with primatologists and clinicians to address these important questions. Increased utilization of animal models at an early stage in IVR formulation development will avoid progressing IVR systems without knowing detailed pharmacokinetics and efficacy, and before spending rare resources on developing an IND, producing clinical supplies, and conducting clinical trials. Employing these pilot animal studies, specific drug delivery performance criteria (e.g., target *in vivo* release rate or vaginal tissue concentration) could then be established early on to guide device design or decide between multiple IVR formulations.

The microbicide IVR field also needs in-depth IVR acceptability studies - where women actually insert and wear IVRs - to provide feedback on IVR design variables such as ring dimensions, stiffness, color, and dosage duration. Clearly, acceptability and adherence are the Achilles' heel of microbicide vaginal gels, and demonstrating improvement of both with an IVR is of utmost importance to give hope for an effective microbicide IVR. IVR dimensions and stiffness are often neglected during early IVR development although several marketed and clinically evaluated rings are known to have significant vaginal expulsion rates. The rationale for microbicide IVR product duration (proposed to range from days to years) is often not explained well, but critical factors considered should include pharmacokinetics/API release rate, cost, safety, and perhaps most importantly acceptability and adherence.

With rapidly emerging macromolecules and biologics possessing microbicide potential, developing suitable yet cost-effective IVR delivery systems is a considerable challenge as conventional IVR designs are inadequate. Achieving greater IVR capability and sophistication with innovative designs does not necessarily denote increased costs,

yet cost must be regarded since an effective yet expensive microbicide may never reach the intended users. Similarly, polymer IVR selection should be considered upon project initiation so as to avoid potential supply issues in clinical trials and beyond. Encouragingly, pharmaceutical companies are becoming more willing to offer anti-HIV APIs for microbicide applications, and as more APIs become available a screening and selection process should be in place to accelerate IVR formulation for the most promising APIs so as to not waste time and resources.

Intravaginal ring technology for microbicide applications has progressed significantly during the last decade. A wide variety of exciting new ring designs show considerable promise for controlled and sustained vaginal delivery of multiple APIs including small molecules, biologics, and synthetic macromolecules. These advances were driven by the international attention and funding directed to the problem of HIV prophylaxis in women. Although incomplete, our understanding of critical device design and performance deepened, and is evident in numerous new IVRs currently in development and under clinical evaluation. Furthermore we are already seeing these technologies disseminate into diverse indications such as vaccines, contraception, menopause and chemotherapy that together show great promise in the often overlooked area of women's health. The accelerating development and utilization of animal models for IVR safety and drug pharmacokinetic evaluation has helped broaden our understanding of the relationship of IVR device design and *in vivo* performance. Non-human primate models will continue to play an increased role in designing, characterizing, and screening IVR products prior to clinical trials. The effectiveness demonstrated in the CAPRISA 004 clinical trial with the tenofovir vaginal gel is recent

proof of progress in HIV prevention science and offers hope backed by solid clinical science for new and improved technologies and methodologies to reduce HIV transmission in women.

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