

**POLY(ETHYLENE GLYCOL) (PEG) NANOCARRIER-BASED HYDROGELS
FOR THE PREVENTION OF VAGINAL HIV TRANSMISSION**

By

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ABSTRACT OF THE DISSERTATION

Poly(ethylene glycol) (PEG) Nanocarrier-based Hydrogels for the

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Women account for over half the global population with HIV. Given the high rate of new infections, the implementation of female-controlled preventive methods is critical to successfully curb HIV. Toward this end, vaginal microbicides- topically applied, self-administered products that protect against HIV are being investigated. A primary risk factor for cervicovaginal HIV acquisition is bacterial vaginosis (BV). BV is characterized by overgrowth of anaerobic bacteria and decrease in healthy vaginal lactobacilli and acidity. The compromised vaginal environment in women with BV significantly increases their susceptibility to HIV. Thus a microbicide aimed at preventing and treating BV is likely to be effective at HIV prophylaxis.

The overall goal of this thesis is to develop and evaluate poly(ethylene) glycol (PEG) nanocarrier-based hydrogels for application as vaginal microbicides. The hydrogels are formed *in situ* by covalently cross-linking 8-arm PEG-SH and 4-arm PEG-NHS polymers via degradable thioester bonds. First, the vaginal coverage, distribution and retention of the hydrogels in mice were

evaluated using MRI. Hydrogel volumes and surface contact were measured at various times post-dose and compared with hydroxyethylcellulose (HEC) gel. The hydrogels showed significantly longer vaginal retention (12-24 h) than HEC gel (2 h).

In the second part of this thesis, PEG nanocarrier-based hydrogels for the controlled release of lactic acid were developed for maintaining vaginal acidity in women with BV. Nanocarrier-based hydrogels with covalently attached lactic acid showed sustained release (10-14% lactic acid) over several days, while hydrogels with passively entrapped lactic acid showed burst release (90% release within 30 min). Hydrogels with passively entrapped lactic acid inhibited the predominant BV pathogen *Gardnerella vaginalis*. The cytotoxicity of the polymers was also evaluated using vaginal epithelial cells.

Finally, hydrogels for the controlled release of subtilisin, an antimicrobial peptide with activity against *G. vaginalis* was developed. Subtilisin release from the hydrogels was two-phase with initial rapid release (4 µg/hr for 12 h) followed by slow release (0.26 µg/hr from 12-120 h). Subtilisin-containing hydrogels inhibited *G. vaginalis* but did not significantly inhibit healthy vaginal lactobacilli spp. The antiviral activity of subtilisin against HIV was investigated and subtilisin weakly inhibited HIV replication (IC_{50} = 5.8 µM).

DEDICATION

To my husband and best friend Kartik,
for your endless love and support.

To my parents,
for your love, blessings and the many sacrifices you have made
to get me to where I am today

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1. INTRODUCTION AND BACKGROUND

1.1 HIV/AIDS Pandemic and Prevention

At least 34 million people were HIV-positive in 2011, with over 50% being women [1]. Although the rate of new infections has stabilized in the past five years it is still significantly high (2.5- 2.8 million people in 2011), with majority of new infections originating from sub-Saharan Africa. HIV-incidence has also slowly increased in the Middle East, North Africa, Eastern Europe and Central Asia since the early 2000s. Women are still unduly afflicted with HIV, particularly in sub-Saharan Africa and the Caribbean where they comprised 59% and 53%, respectively of total infected adults in 2010 [1].

There has been a significant decline in HIV-related deaths since the 1990s resulting from greater access to antiretroviral (ARV) treatment in sub-Saharan Africa and other countries and the lower rate of new infections. However, alongside improvements in HIV diagnosis, counseling and care there is need for comprehensive prevention programs to halt transmission, especially among high-risk groups. Current prevention strategies include male circumcision, promoting male and female condom usage, preventing and managing sexually transmitted diseases such as syphilis and pre-exposure prophylaxis (PrEP) with ARV drugs.

Four clinical trials performed in the past two years have demonstrated the potential use of biomedical approaches for preventing HIV transmission. The first, in July 2010 showed that a vaginal gel loaded with tenofovir, an anti-HIV drug reduced HIV transmission to uninfected women by 39% [2]. The second, in

November 2010 showed that an oral dosage of tenofovir/emtricitabine reduced HIV transmission by 44% in homosexual men [3]. In 2011, two more trials testing oral PrEP in heterosexual couples showed a significant decrease in HIV acquisition among users. A trial among serodiscordant couples (i.e., only one partner infected with HIV) in Kenya and Uganda showed a 67% reduction in infection in individuals taking oral tenofovir and 73% decline in individuals taking a combination of tenofovir and emtricitabine [4]. A similar trial in Botswana among heterosexual HIV-negative couples showed a 63% reduction in infection [5].

1.2 Sexual Transmission of HIV

Male-to-female transmission of HIV occurs via the vaginal, ectocervical, endocervical and endometrial mucosa [6]. The vaginal mucosa is comprised of multilayered stratified squamous epithelium, which presents a stronger barrier to HIV than the single-layered endocervical and endometrial mucosa. However, the mucosal barrier is often compromised due to traumatic sex (wherein the epithelium gets disrupted), or genital infections such as BV, ulcerative or inflammatory infections, thus increasing exposure of the epithelial and sub-epithelial cells to HIV [7, 8].

HIV transmission during sex has been shown to occur with both cell-free virions and virions secreted from infected donor cells [6]. Several studies using human explants and macaque models of HIV infection have shed light on the chain of events that occur immediately following exposure of the mucosa to HIV. Upon breaching the mucosa, HIV interacts with epithelial cells in a number of

ways (Figure 1.1). Some studies have shown that HIV is endocytosed by epithelial cells and then made available for infection of CD4⁺ T cells [9, 10]. Evidence from other studies suggest that virus particles, especially cell-associated HIV cross the epithelium via transcytosis from the apical to the basal region, although this mechanism may account for transfer of only a small fraction of total virions contacting the mucosa [11-14].

In the epithelial layer, the primary targets for HIV are Langerhans cells (LCs), a type of immature dendritic cells (DCs) and intra-epithelial CD4⁺ T-cells (IELs) (Figure 1.1) [6]. LCs efficiently trap HIV in cytoplasmic organelles, and transfer intact virions to susceptible cells upon migrating from the epithelium. However, the mechanism of viral entry and subsequent infection of LCs remains ambiguous. LCs express the cell-surface receptor CD4 and the chemokine receptor CCR5 that bind the HIV envelope glycoprotein (gp120) to mediate viral fusion with the cell membrane. However, studies by Hladik et al. showed only partial blockage of HIV entry into vaginal LCs by antibodies to CD4 and CCR5 and absence of any productive infection, suggesting an alternate route for viral entry [15]. In addition, vaginal LCs were found to contain intact virions even 60 h post-viral challenge [15]. This is in contrast to epidermal LCs, which quickly degrade virions in Birbeck granules after internalization via the C-type lectin langerin [16]. Thus the exact pathways utilized for HIV entry into vaginal LCs are yet to be elucidated.

The vaginal epithelium also contains a substantial population of CD4⁺ T-cells (IELs), many of which are memory T-cells and reside close to the basal

membrane. Studies in human explants have shown that HIV binds to and productively infects IELs (via CCR5 mediated fusion with the cell membrane), within a few hour of exposure [15, 17, 18]. Similarly, studies in macaques with SIV have shown that early infection of resting $CD4^+$ T-cells contributes significantly to viral dissemination [19, 20].

After crossing the epithelium, HIV reaches sub-epithelial target cells in the cervicovaginal stroma, such as macrophages, dendritic cells (DCs) and T-cells (Figure 1.1). Infection of DCs and T-cells takes place either by transfer of virions through contact with epithelial LCs and/or by internalization of virions that have endocytosed or transcytosed through the epithelium. In addition, HIV can also directly infect stromal DCs, macrophages and T-cells by penetration through the mucosa. The vaginal epithelium is not uniform in thickness and microabrasions in thin areas of the epithelium give easy access to the underlying stroma where T-cells and macrophages cluster, forming foci of target cells for infection. Studies with organ culture models have shown macrophages to be a major sub-epithelial cell type infected with R5-tropic HIV-1, though it is unclear whether macrophages in the vagina express CCR5 [21-23]. Likewise, stromal DCs that mediate viral entry through the C-type lectin DC-SIGN have been shown to contribute to the sexual transmission of HIV.

Local expansion of HIV at infected foci occurs through productive infection of additional activated $CD4^+$ T-cells that are recruited through contact with antigen-presenting DCs or indirectly by chemokine secretion of the inflamed

epithelium. HIV infection then disseminates to the draining lymph node and then the secondary lymphoid organs resulting in systemic infection [24].

1.3 Risk Factors for vaginal acquisition of HIV

Although the intact vaginal mucosa provides high resistance to HIV, several factors have been shown to enhance HIV penetration through the mucosa, significantly increasing risk of infection. HIV incidence is the highest in Africa followed by Asia, reflecting the important role of host factors in the efficiency of transmission. A study among African women found that frequent use of vaginal irritants and the practice of “dry sex”, increased exposure to HIV by causing microtrauma to the vaginal epithelium [25]. Genital ulcerative and inflammatory diseases have been shown to damage the mucosa and enhance the HIV co-receptor CCR5 expression on macrophages, DCs and T-cells in the vagina [8, 26]. Mucosal inflammation also causes recruitment of additional immune cells from the circulation, bringing more HIV target cells to the vaginal tract [8].

Several studies have shown a high correlation between abnormal vaginal microflora and risk of HIV-1 acquisition [27-31]. Bacterial Vaginosis (BV) is a common vaginal pathology characterized by loss of vaginal lactobacilli and subsequently increased vaginal pH. A study investigating the association of BV with HIV-1 infection among female sex-workers in Thailand, found 43% of participants were HIV-1 positive and 33% had BV [27]. A similar study in Uganda showed that >50% of participants had moderate to severe BV, the rate of HIV-1 infection being considerably higher in women with BV (26.7%) than women with

normal vaginal flora (14.2%) [28]. A study among pregnant women in Malawi, found that BV was also significantly associated with antenatal and postnatal HIV seroconversions (14% and 23% risk, respectively) [29].

Viral and bacterial sexually transmitted diseases (STDs) including Herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), *Neisseria gonorrhoeae* and *Trichomonas vaginalis*, are risk factors for HIV [32-35]. A recent study in Zimbabwe and South Africa showed that women infected with *T. vaginalis* had a 2-fold higher risk of acquiring HIV-1 than uninfected women [35]. Another study at three different sites in South Africa- Harare, Durban and Johannesburg highlighted the association of various STDs with HIV-1 infection [33, 34]. Combined analyses of data from all three locations showed that women with prevalent HSV-2, incident HSV-2 and incident *N. gonorrhoeae* had a “2.1- fold, 4.4- fold and 6.9- fold increased risk of acquiring HIV-1, respectively” [33].

Cervical ectopy, a condition in which the single-layered columnar epithelium of the endocervix extends into the ectocervix, often found in younger women and associated with estrogen-based contraceptive use, has also shown to increase risk of HIV infection. In a study conducted in Kenya among concordant and discordant couples where the men were HIV-positive, it was found that women with cervical ectopy had a five-fold greater chance of contracting infection from their spouses [36]. In another study examining HIV-shedding from the genital tract of seropositive women in Kenya, cervical ectopy was associated with a 5-fold higher incidence of HIV in the cervix [37].

1.4 Vaginal Microbicides

Given that women are disproportionately afflicted with HIV, and that infection occurs primarily through sexual transmission, there is a compelling requirement for female-controlled prophylactic methods to prevent vaginal acquisition of HIV. Women are exceptionally susceptible to infection in regions like sub-Saharan Africa where socio-economic factors come into play; women are unable to arrange condom use and are often subjected to non-consensual sex. Vaginal microbicides are topically applied, self-administered products that women can use to prevent HIV acquisition, during sex. It has been estimated that a vaginal microbicide product can prevent as many as 2.5 million infections over 3 years, if adopted 50% of the time by 20% of women at risk [38].

So far, there have been several approaches to designing and developing vaginal microbicide products and include gel formulations, intravaginal rings (IVRs) and nanoparticle-based formulations. 'First-generation' microbicides mainly consisted of non-specific agents such as surfactants, acidifying agents and polyanions that prevented HIV entry by either dissolving/ binding to the viral envelope or enhancing the acidic vaginal milieu. However, none of the microbicides in this category that made it to Phase III trials demonstrated sufficient protection against HIV. The newer microbicide strategies focus on specifically inhibiting viral entry and replication by incorporating ARV drugs either in IVRs or nanoparticle-based formulations. This section reviews some of the first-generation microbicide products, as well as current microbicide strategies and drug candidates.

1.4.1 Semi-solid gel microbicides

The earliest vaginal microbicides for HIV prevention evolved from existing over-the-counter spermicidal and contraceptive products, and were semi-solid gel formulations. These first-generation microbicides comprised non-specific inhibitors such as detergents that disrupt the viral membrane, buffering agents, spermicidal agents and anions that bind the viral envelope. Nonoxynol-9 (N-9), a detergent-like spermicidal agent was the first microbicide to be tested for HIV prevention in clinical trials, because it successfully showed anti-HIV activity *in vitro*. However, the results were disappointing, with multiple trials indicating either no protection or increased risk of infection with frequent use, later shown to be due to toxicity to the cervico-vaginal epithelium [39-42].

Some of the early microbicide formulations included acidifying agents to restore the acidic vaginal pH, which acts as natural barrier to HIV. BufferGel (ReProtect, USA), a polyacrylic acid capable of buffering double its volume of semen to an acidic pH, was shown to be safe for use in phase I trials but did not show efficacy in preventing infection in a phase II study [43]. Acidform (Amphora, USA) another acid buffering gel, was shown to inactivate HSV and prevent genital herpes in a mouse model [44]. Initial phase I studies indicated safety for prolonged use; however a recent study comparing the use of Acidform and K-Y Jelly on diaphragms indicated higher incidence of abnormal pelvic and vaginal events in the Acidform group [45].

Microbicide gels containing anionic polymers that neutralize HIV by binding to the positively charged viral envelope, also failed in phase III clinical trials. Carrageenan (Carraguard, Population Council, USA), a sulfated polysaccharide isolated from seaweed was shown to interrupt viral entry *in vitro* and also prevent infected cells from moving across the vaginal tract.[46] A large phase III in South Africa however, did not indicate any protection over the placebo [47]. Cellulose sulphate (CS, Ushercell, Polydex Pharmaceuticals, Canada), another polyanion microbicide showed good *in vitro* activity against the BV pathogen *Gardnerella vaginalis* and a number of other STD-causing pathogens, including *N. gonorrhoeae*, *C. trachomatis* and HPV [48-50]. Phase I trials of CS gel indicated safety but phase III trials in India and Africa were halted after interim results showed a greater number of infections in the CS group compared to the placebo [51]. Consequently, a parallel phase III trial in Nigeria was stopped even though there was not statistically significant evidence of increased HIV infection in the CS group [52].

First-generation microbicide gels were formulated mainly by dispersing active agents in excipients such as sodium carboxymethylcellulose. A disadvantage of these semi-solid gel microbicides is their low dispersibility and retention in the vaginal lumen. Physical gels have limited ability to spread and cover the mucosal surface once instilled into the vagina (i.e., spreading only occurs at high shear forces, diluting the gel and making it an even less effective barrier) [53]. Dilution of gels in the vaginal fluid leads to poor vaginal retention.

The limited residence time of semi-solid gels necessitates frequent application of the dose leading to low user compliance.

1.4.2 Intravaginal rings (IVRs)

IVRs were first introduced into the market in the early 1990s (Estring®) for the sustained and controlled delivery of steroids to the vagina for contraception [54]. Since then, two more IVRs for contraceptive use have reached the market-Femring® and Nuvaring®. In the context of microbicides, there has been a recent focus on vaginal rings as vehicles for long-term sustained delivery of anti-HIV drugs to the vaginal lumen. Unlike semi-solid gel formulations, IVRs are retained in the upper vaginal tract for several weeks and ring insertion is non-coitally dependant. Hence, participants in recent clinical trials have indicated a preference for IVRs over semi-solid gels and diaphragms [55].

Malcolm et al., initialized research with IVRs in the microbicide field by investigating its potential for the sustained delivery of N-9, a microbicide that was previously formulated as semi-solid gels and abandoned from further development due to cervico-vaginal toxicity (see previous section for details). The study showed that N-9 was released from silicone elastomer rings at very small doses over an 8-day period, the maximum release per day (6 mg) being much lower than the amount shown to cause toxicity [56]. Following this, a number of studies have looked at the use of silicone elastomer rings for delivering dapivirine, a small-molecule antiretroviral (ARV) drug [57-59]. The only microbicide IVRs evaluated in clinical trials to date contain dapivirine with the most recent trial comparing dapivirine release from two different ring

configurations. The study indicated release of larger amounts of dapvirine from matrix rings where the drug was uniformly distributed in the polymer, compared to reservoir rings where the drug core was enveloped in a non-medicated sheath [59].

Though IVRs provide a more controlled release of actives compared to semi-solid gels, they are not without problems. One issue is the difficulty in manufacturing IVRs, including the high temperatures required for melt-extrusion that may cause breakdown of the drug [54]. Another problem is that the drug may be more soluble in the polymer matrix at the high manufacturing temperatures and may form crystals within the polymer matrix (observed with NuvaRing®) or at the surface/air interface upon cooling [60]. In the case of reservoir rings, an initial burst release of the drug is observed for 1- 5 days, due to diffusion of the drug from the reservoir to the sheath during manufacturing [57, 58]. High concentrations of the drug in the vaginal lumen due to burst release may cause toxicity and systemic absorption.

1.4.3 Nanoparticle-based microbicides

The mucosal surface of the vagina represents a complex anatomical barrier, with limited permeability of the epithelium. In addition, the epithelium is protected with a viscoelastic mucus layer that is continuously cleared and replenished. These anatomical attributes make it difficult to deliver drugs to specific target sites in the vagina, which in the case of anti-HIV drugs are target cells (LCs, IELs etc.) in the basal epithelium. Nanoparticles, because of their

small size, might be capable of quickly penetrating the mucosal barrier, efficiently delivering drugs to target sites in the vagina. Another advantage of nanoparticles is that their surface can be tailored to specifically interact with the HIV envelope or cell surface receptors and thus block viral entry into cells. For the above reasons, a few groups have started to look at nanoparticles for development as vaginal microbicides [61-63].

In one of the earliest reports demonstrating the potential of nanoparticle-based microbicides, Hayakawa et al. showed that polystyrene nanoparticles bound to Concanavalin A (Con A) effectively captured HIV-1 virions in solution and reduced viral infectivity by > 3.3 log and 2.2 log at concentrations of 2 mg/ml and 0.5 mg/ml, respectively [62-64]. Con A, a lectin that binds to the heavily mannosylated gp120 portion of HIV with high affinity, was attached to polystyrene nanoparticles using poly(methylacrylic acid) linkers [65]. Intravaginal administration of inactivated HIV-1 bound Con A nanoparticles in mice was found to induce expression of the anti-HIV-1 antibody IgA in the vaginal tract, indicating potential for development as a HIV-1 vaccine [66]. Further studies investigating the prophylactic efficacy of HIV-1 bound Con A nanoparticles in mice via different routes of administration (intravaginal, oral and intranasal) found the intranasal route to induce maximum expression of IgA antibodies in the vagina [67].

Dendrimers, branched macromolecules with unique structural properties have been shown to possess antiviral activity against a number of viruses including HSV and HIV [68]. Dendrimers have a central core from which branching units can be built in three-dimension, and drugs can be encapsulated

into the central core and/or attached to the ends of the branched units [68]. In addition the branches can be modified with functional groups that can complex with viruses or cell receptors, thus interrupting virus-cell fusion. One such class of dendrimers, SPL7013 (Starpharma Inc., Melbourne, Australia), has been given approval as an investigational new drug (IND) by the FDA for development into an HIV microbicide [63, 68]. SPL7013 dendrimers have an amide core with 32 sodium 1-(carboxymethoxy)naphthalene-3,6-disulfonate groups attached to the surface of the branched units, imparting a high anionic surface charge.[68] SPL7013 was shown to be active against HIV type 1 and 2 (HIV-1 and HIV-2), HSV type 1 and 2 (HSV-1 and HSV-2) and human papillomavirus (HPV) [69-71]. SPL7013 was formulated as a water-based polyacrylic acid gel (Vivagel™) and was observed to provide protection against a chimeric simian-human immunodeficiency virus (SHIV89.6P) in a macaque model [72]. Preclinical safety trials with various Vivagel™ formulations (1%, 3% and 5% w/w) showed that the gel was safe for use and a more recent Phase I clinical trial indicated that Vivagel™ was generally well-tolerated, but with a higher incidence of adverse genitourinary symptoms when compared to the placebo group [73, 74].

Recently, Ham et al. have used biodegradable poly(lactide-co-glycolic acid) (PLGA) nanoparticles for encapsulating PSC-RANTES, a chemokine analog that has been shown to block CCR5 expression and provide anti-HIV activity *in vitro* [75]. The PLGA particles provided controlled release of PSC-RANTES for several days *in vitro*. Moreover, in experiments with human cervical explants, the particles penetrated the superficial epithelium and reached the

basal layers, unlike unformulated PSC-RANTES, which remained at the top layers of the epithelium [75].

Alukda et al. synthesized solid lipid nanoparticles (SLNs) functionalized with poly-L-lysine and heparin for encapsulating the anti-HIV drug tenofovir [76]. The encapsulation efficiency of functionalized SLNs was only 8.3% due to the high water solubility of tenofovir; however the SLNs did not show any *in vitro* toxicity in a vaginal epithelial cell line [76]. The same group has also prepared chitosan nanoparticles encapsulated with tenofovir, through ionic cross-linking of chitosan with sodium tripolyphosphate [77]. The particles showed an encapsulation efficiency of 5% to 20%, depending on particle size and larger particles exhibited a controlled release of tenofovir [77]. In another study, tenofovir (TF) and tenofovir disproxil fumarate (TDF) was encapsulated in nanoparticles prepared using a PLGA and methacrylic acid copolymer [78]. The particles showed a higher release of drug at alkaline vs. acidic pH, suggesting the use of these particles for triggered-release of drug in the presence of semen in the vaginal lumen [78].

1.5 Challenges to microbicide development

Microbicides are unique in that they must be available over-the-counter for use by otherwise healthy individuals, and hence pose several challenges from both the drug development and clinical perspective [79-81]. One problem in developing ARV-based microbicides is finding the appropriate formulation type. The choice of gel formulations, tablets, films or IVRs depends on the physicochemical properties of the drug used, including stability at high

manufacturing temperatures (as in the case of IVRs) and storage temperatures (in regions such as sub-Saharan Africa) [81]. Many non-nucleoside reverse transcriptase inhibitors (NNRTIs) have low water-solubility and hence may not be compatible with gel formulations. On the other hand, nucleoside reverse transcriptase inhibitors (NRTIs) such as Tenofovir may be susceptible to systemic absorption, due to high water solubility.

Another problem with regards to product development is estimating the dose of anti-HIV drugs that would be sufficient for prophylaxis. The viral load in semen is highly variable, the peak viral load being much higher in individuals with acute HIV infection than chronic infection [82]. Moreover, some resistant HIV strains may require a 10 to 100-fold higher drug concentration than non-resistant strains. Another factor is that the effective drug concentrations for inhibiting infection in humans may be several orders higher in magnitude ($\geq 10^5$) than inhibitory concentrations found *in vitro* or in animal models [80, 83].

An important requirement for microbicides is that they must be safe for prolonged and repeated usage, and hence present no toxicity to the cervicovaginal epithelium. Several early microbicides have failed in clinical trials due to cervicovaginal toxicity, which in turn enhanced susceptibility to infection [39, 42, 51]. Hence, sufficient *in vitro* and *in vivo* toxicity studies need to be conducted in order to assess microbicide safety, prior to clinical evaluation. This is a significant challenge because there are very few *in vitro* models for microbicide testing and are limited to infection of primary cells (macrophages, DCs, peripheral blood mononuclear cells (PBMCs), etc.) or cervicovaginal

explants [80]. The only animal model that has been extensively used for pre-clinical evaluation of microbicides is viral challenge with SHIV (a chimeric virus containing parts of HIV and simian immunodeficiency virus (SIV)) on rhesus macaques.

From a clinical standpoint, there is significant difficulty in conducting clinical trials for optimizing dosage and evaluating efficacy of microbicides. Parameters that are normally evaluated in phase II trials for new drug candidates have to instead be evaluated in larger phase III trials for microbicides, due to ethical requirements [84]. Another critical issue that is often faced in clinical trials of microbicides is lack of patient adherence. Since there is no way to accurately determine patient adherence, the low efficacy of microbicide products evaluated so far in clinical trials, can be at least partially attributed to poor patient compliance. New tools for evaluating patient compliance, such as applicators that stain on contact with vaginal mucus during insertion, are being developed [85].

Finally, there is a concern of build-up of drug resistance in HIV-positive individuals, who use microbicides during the time that they are unaware of their status. Topical application of ARV-based microbicides should not lead to significant systemic absorption of the drug, and it is not yet known if low plasma concentrations of ARV drugs will be sufficient to cause development of resistant strains. However there is significant cross-resistance between NNRTIs, so an infected person who is using a particular NNRTI-based microbicide, is capable of transmitting viral strains that are resistance to this NNRTI drug and in addition is unlikely to respond to treatment with other NNRTIs [86]. This is a major concern,

since NNRTI drugs are the primary course of treatment for infected individuals [87].

1.6 Hydrogels as drug delivery systems

Hydrogels are hydrophilic polymer networks that can take up several times of their dry weight in water, and can be formed by either physical or chemical cross-linking of natural or synthetic polymers [88, 89]. Hydrogels have unique physicochemical properties that make them suitable for various biomedical and pharmaceutical applications [89, 90]. The high water-content and three-dimensional structure of hydrogels along with their ability to encapsulate cells, make them a material of choice for constructing scaffolds for tissue engineering [91]. Hydrogels also make excellent vehicles for controlled and sustained delivery of drugs, since active moieties can be encapsulated within or attached to the polymer matrices in a variety of ways [92, 93].

Natural polymers that have been used to form hydrogels include chitosan, hyaluronic acid, collagen, dextran, etc., while synthetic polymers include poly(ethylene glycol) (PEG), poly(acrylic acid) (PAA), poly(hydroxyethyl methacrylate) (PHEMA) etc. Hydrogels can be 'physical gels', wherein the polymer network is held together by weak forces such as molecular entanglements, ionic bonds or hydrophobic interactions [90]. An example of a physical gel is calcium alginate, which is simply formed by combining a polyelectrolyte with a multivalent ion of opposite charge [90]. Physical gels are weaker than chemically cross-linked gels and typically lose their structure when

high mechanical stress is applied to the network. Chemical hydrogels on the other hand, are formed by covalently cross-linking two hydrophilic polymers and such hydrogels exhibit better stability due to the nature of the interactions that form the network. The earliest example of such hydrogels dates back to 1960, when Wichterle and Lim proposed the use of PHEMA hydrogels in contact lenses [94]. Chemically cross-linked hydrogels offer better control of important hydrogel parameters and properties such as gelation time, pore size, degradation time and chemical functionalization, since the cross-linking chemistry can be tailored accordingly. Small-molecule crosslinkers such as genipin have been used for forming hydrogels from a variety of polymers such as amino-terminated PEG, gelatin and bovine serum albumin. However a potential problem with small-molecule crosslinkers is toxicity from any remaining unreacted cross-linker [89]. Another approach is to crosslink polymers that have been pre-functionalized with reactive groups, however this does add significant steps in preparing the functionalized pre-polymers. In addition, the pre-polymers themselves might be toxic and although this will not be a problem during hydrogel formation due to the rapidity of gelation, toxicity might be an issue when the hydrogel degrades back to its constituent polymers [89]. Some cross-linking chemistries that have been extensively explored for hydrogel formation are hydrazone bond formation, Michael addition between amine/thiol and a vinyl group, etc [95-97]. Hydrogels, whether physical gels or chemically crosslinked gels are non-homogeneous systems and usually contain clusters of higher crosslink density (or molecular entanglements/ionically-associated domains) dispersed within the network [90].

When active moieties are incorporated into the hydrogel matrix by admixing or passive entrapment, they are typically released quickly- within a period of hours or days, due to the high water content of hydrogels and hydrophilic nature of polymers used. In order to prolong release, some strategies that have been used to attach drugs to the polymer matrix include ionic interactions and covalent binding of the drug with the polymer. Accordingly, drug release from hydrogels may be diffusion controlled, swelling-controlled or chemically-controlled [93]. Diffusion-controlled release, wherein drug release follows the Fick's law of diffusion is the most common mechanism of release from hydrogels [93]. Release is swelling-controlled when water penetrates the hydrogel matrix and drug molecules are released as the hydrogel transitions from a glassy (dry) state to a rubbery (swollen) state [93]. Swelling-controlled release is non-Fickian due to the slow polymer chain relaxation times in the swollen region [93, 98]. Drug release from hydrogels can also occur due to chemical reactions such as hydrolysis or enzymatic degradation of the polymer chains or other reversible or non-reversible reactions between the polymer and drug. In cases where the drug is tethered to the polymer via a linker, the release is controlled by the kinetics of degradation of the linker, which can be due to hydrolysis or enzymatic degradation. In addition, release may also occur through surface erosion of the polymer or diffusion or a combination of both (reviewed in detail in Lin et al., 2006) [93].

1.6.1. Hydrogels for vaginal drug delivery

Saxena et al. designed a hydrogel comprising of a core of dextran surrounded by sheaths of copolymers of polylactide and caprolactone, for delivering non-hormonal contraceptives [99]. The hydrogel contained ferrous gluconate, a spermistatic agent, and ascorbic acid and polycarboxylic acid to bring vaginal pH close to 4.5. The hydrogel provided sustained release of the agent for several days and eluates from the hydrogels successfully immobilized sperm in semen samples [99]. Aka-Any-Grah et al. examined hydrogels made from block copolymers composed of (ethylene oxide)_a (propylene oxide)_b (ethylene oxide)_a, also known commercially as Pluronics.[100] Hydrogels made from a mixture of two pluronics F127/F128 and hydroxypropylmethylcellulose (HPMC) were found to be mucoadhesive even when diluted, indicating that the gels had suitable physical properties for vaginal administration [100].

Gupta et al. have developed pH and temperature sensitive hydrogels made from a random terpolymer of N-isopropyl acrylamide, butyl methacrylate and acrylic acid [101]. The polymer was shown to undergo a sol to gel transition at 37 °C and pH 4.2 and reverse back to a solution when the pH was brought up to 7.4. Such 'smart' hydrogels were indicated as being useful for the triggered release of microbicides by undergoing a phase transition upon coming in contact with semen in the vaginal lumen. The same group has also developed a pH sensitive hydrogel by covalently cross-linking phenylboronic acid (PBA) with salicylhydroxamic acid (SHA) [102]. The PBA-SHA hydrogel undergoes gelation at the acidic vaginal pH of 4.5-5 and was shown to efficiently trap HIV by impeding its diffusion through the gel network [102].

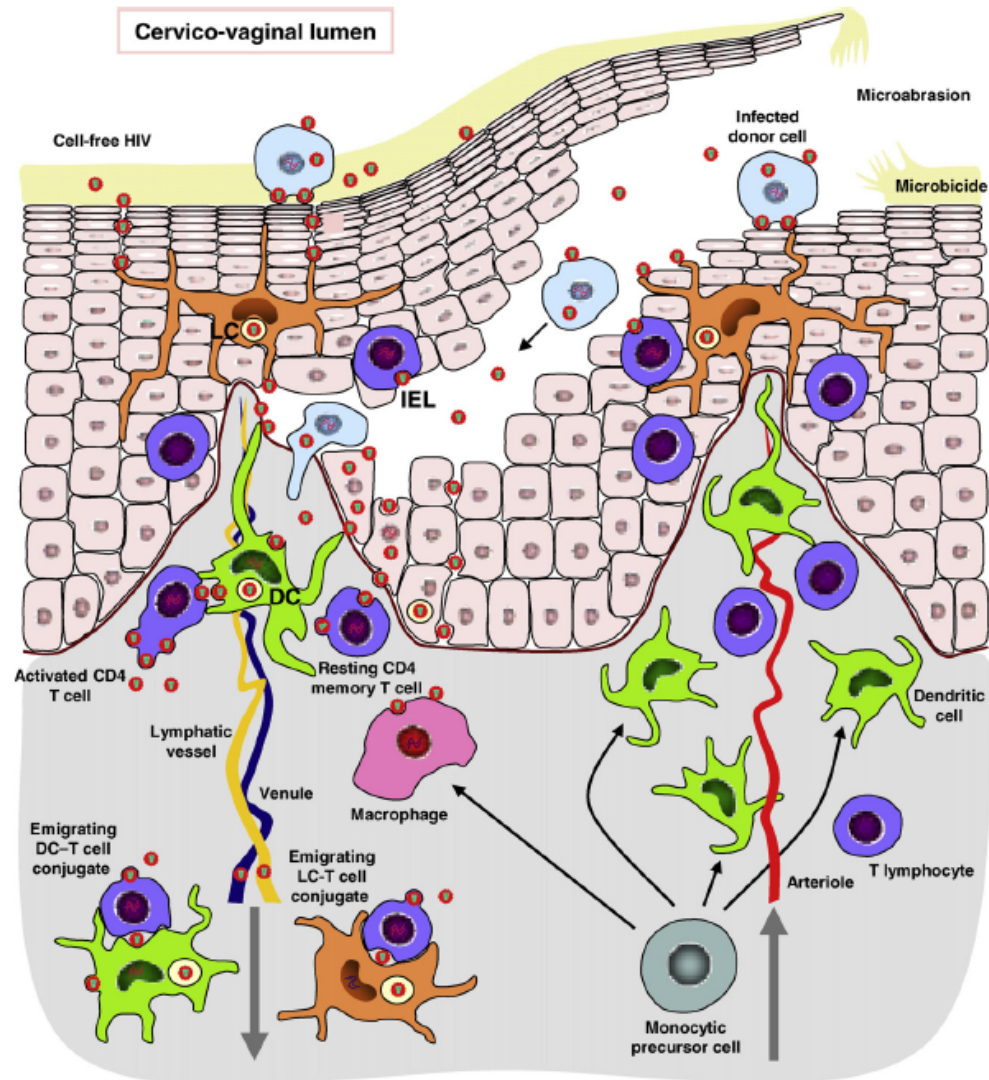


Figure 1.1 Schematic of transmission of HIV across the vaginal mucosa. Used with permission from Elsevier Ltd: Antiviral Res 2010, 88 Suppl. 1, S3. Copyright 2010. HIV penetrates the intact or abraded epithelium via endocytosis or transcytosis. Within the epithelium, HIV targets Langerhans cells (LCs) and intra-epithelial dendritic cells (IELs). Sub-epithelial cells such as dendritic cells (DCs), macrophages and $CD4^+$ T-cells are then infected either through contact with infected LCs and/or by internalizing HIV that has penetrated the epithelium. Expansion of infection takes place by activation of additional $CD4^+$ T-cells through contact with antigen-presenting DCs and LCs and/or chemokine secretion by the damaged epithelium. Infected T-cells, DCs, LCs and their complexes are disseminated to the draining lymph nodes and subsequently into the systemic circulation.

2. SPECIFIC AIMS

The healthy vaginal environment is acidic and is normally resistant to HIV infection. However, the healthy vaginal environment is compromised in bacterial vaginosis (BV), which is characterized by an overgrowth of anaerobic bacteria and a decrease in vaginal acidity. As a result, women with BV have a significantly increased risk of HIV acquisition via sexual transmission. Thus a vaginal microbicide for HIV prophylaxis is likely to be effective if it (1) restores vaginal acidity, (2) treats and prevents recurring BV, and (3) provides good vaginal coverage and retention so as to maintain a sufficient concentration of active agent in the vagina.

In this thesis, the above three functional aims were addressed by designing and developing poly(ethylene glycol) (PEG) nanocarrier-based hydrogels for intravaginal administration. The hydrogels are formed *in situ* by the covalent cross-linking of multi-arm PEG-SH and PEG-NHS polymers. The hydrogels are designed for the controlled release of the active agents lactic acid and subtilisin, both of which will restore the inherent microbicidal environment of the vagina.

The specific aims of the thesis are:

Aim 1: To evaluate the *in vivo* coverage, distribution and retention of the PEG-based hydrogels in the vagina of mice using magnetic resonance imaging (MRI). The hydrogels, which form *in situ* by the covalent cross-linking of multi-arm PEG polymers, will function as a microbicide delivery system for HIV prevention.

Hypothesis: Microbicide gels that have been developed to date are pre-formed semi-solid gels. Semi-solid gels have reduced viscosity on dilution with vaginal fluid resulting in leakage and subsequently poor retention. However, chemically cross-linked hydrogels have higher viscoelasticity when compared to semi-solid gels and resist deformation under applied strain. **Therefore, PEG-based hydrogels might be an improved platform for vaginal drug delivery over semi-solid gels. Since the hydrogels are administered as a viscous liquid they will distribute well within the vagina and provide good mucosal coverage. However, unlike semi-solid gels, the hydrogels rapidly undergo gelation *in situ* to form a covalently cross-linked elastic network on the mucosa. Thus, the hydrogels by virtue of their superior viscoelastic properties will show improved vaginal retention over semi-solid gels.**

Aim 2: To design and develop PEG nanocarrier-based hydrogels for the controlled release of lactic acid in the vagina.

Hypothesis: In women, BV is a primary risk factor for HIV infection via the cervicovaginal route. The normal vaginal flora is composed predominantly of lactobacilli *spp.*, which continuously produce lactic acid that maintains the acidic pH (4.0- 4.5) of the vagina. Lactic acid has broad-spectrum antimicrobial activity against a variety of bacterial and viral pathogens, including HSV-2 and HIV. However, in BV normal vaginal flora is disrupted due to an overgrowth of anaerobic pathogens resulting in reduced vaginal lactobacilli and lactic acid. The decreased vaginal acidity in women with BV greatly increases their susceptibility

to HIV infection. **The sustained release of lactic acid in the vagina might restore vaginal lactic acid levels in women with BV. The broad-spectrum antimicrobial activity of lactic acid will inhibit BV-associated bacteria, thus controlling BV and reducing the risk of HIV infection.**

Aim 3: To develop PEG-based hydrogels for the controlled release of the antimicrobial subtilisin and to investigate the antiviral activity of subtilisin against HIV-1.

Hypothesis: Subtilisin is a bacteriocin produced by both *Bacillus subtilis* and *Bacillus amyloliquifaciens*. Subtilisin has potent antimicrobial activity against BV-associated bacteria, including *Gardnerella vaginalis*. Subtilisin has also been shown to have antiviral activity against herpes simplex virus type 1 (HSV-1). **Therefore, PEG-based hydrogels for the controlled release of subtilisin in the vagina are proposed for the prophylaxis of BV and associated infections including HIV. Subtilisin might also have an inhibitory effect on HIV-1 in light of its antiviral properties against HSV-1.**

3. Evaluation of coverage, distribution and retention of Poly(ethylene) glycol (PEG)-based hydrogels in the vagina of mice using MRI

3.1 Introduction

The past decade has seen an increased effort in the development of vaginal microbicides- topically applied, self-administered products that protect against HIV transmission [79, 81]. The majority of microbicide formulations developed so far have been semi-solid gels, delivered by means of a prefilled vaginal applicator [103, 104]. Nonoxynol-9 (N-9), one of the earliest microbicides to undergo clinical testing, did not protect against HIV and even increased the risk of infection with frequent use [39-42]. Following this, a series of gel-based microbicides (BufferGel, Acidform, Carraguard, etc.) failed to demonstrate evidence of protection in multiple trials, despite showing promising results *in vitro* [45, 47]. It was only recently (CAPRISA 004 trial, July 2010) that a microbicide gel candidate, a 3% tenofovir gel, showed marginal success in prevention, with a 39% reduction in HIV transmission compared to the placebo [2].

Previous microbicide trial failures have helped identify key components that critically impact the performance of vaginal gels [105]. Gel leakage, immediately or within a few hours of application, was one of the most important factors influencing microbicide efficacy [106, 107]. Leakage of gel from the vagina is however, largely dependant on the viscoelastic properties of the gel.

Semi-solid gels, such as those made with hydroxyethylcellulose (HEC) and polyacrylic acid (Carbopol®), have reduced viscosity on dilution with vaginal fluid resulting in leakage and subsequently, poor retention [108, 109]. The limited residence time of semi-solid gels necessitates frequent application of the dose, leading to poor user acceptability and adherence [106, 107]. Thus, there is a clear need for alternative formulations that provide improved vaginal retention (for example, over 8-12 h) so that a microbicide can be administered less frequently and independently of coitus. To this end, novel vaginal drug delivery systems such as temperature and pH sensitive gels, intravaginal rings (IVRs) and nanoparticles are being explored for use as microbicides [58, 75, 101]. Among these, IVRs are currently being developed for the vaginal delivery of antiretroviral drugs. However, with IVRs there are concerns of toxicity and systemic absorption due to initial high concentrations of drug in the vagina [54, 57].

Another major issue in microbicide development is that there is little knowledge of how microbicide gels distribute in the vagina, the extent of their leakage, and spreading (with ambulation and/or sexual intercourse) prior to clinical trials [110, 111]. Understanding the initial deployment of the gel in the vagina, which in turn is dependent on the viscoelastic properties of the gel, is necessary in order to better design microbicide gel formulations [112]. It has been suggested that the inadequate distribution of microbicide gels into vaginal folds, or “rugae”, along the vaginal wall might be a reason for their inefficacy [113, 114]. It is essential that microbicide gels provide maximum surface coverage, since the entire mucosal surface of the vagina is susceptible to

infection [115-117]. However, there is currently a lack of small animal pre-clinical models for evaluating the *in vivo* coverage, distribution and retention of vaginal gels. Although non-invasive imaging techniques such as gamma scintigraphy and magnetic resonance imaging (MRI) have been successfully used in clinical trials for visualizing vaginal gels in women, these techniques are yet to be expanded into pre-clinical development [111, 117-119].

In our laboratory, poly(ethylene) glycol (PEG)-based hydrogels for ocular and dermal wound healing were previously developed [120, 121]. The hydrogels are formed by covalent intermolecular cross-linking of multi-arm PEG polymers with mutually reactive functional groups [120]. We hypothesize that PEG-based hydrogels might be an improved platform for vaginal drug delivery over semi-solid gels for the following reasons: First, hydrogels make excellent vehicles for controlled delivery of drugs, since active moieties can be encapsulated within or attached to the polymer matrices in a variety of ways [120-122]. Second, chemically cross-linked hydrogels have better mechanical strength and are more resistant to applied strain than semi-solid gels, and hence might provide improved retention in the vagina [120-122].

In the current study, MRI was used to determine the surface coverage, distribution and retention of PEG-based hydrogels in the vagina of mice. The PEG-based hydrogels evaluated in this study are formed by covalent cross-linking of 8-arm PEG-SH and 4-arm PEG-NHS polymers via degradable thioester bonds. The hydrogels are administered as a solution into the vagina and undergo rapid gelation *in situ*, within a few minutes. The PEG-based hydrogels are

monitored for at least 24 h following intravaginal administration by obtaining a series of MRI scans at various time points post-dose. We envision that the PEG-based hydrogels, by virtue of their superior viscoelastic properties will provide better surface coverage and retention than conventional semi-solid gels. Therefore, the properties of a HEC gel, used routinely as a “universal placebo” in microbicide trials was compared to the proposed PEG-based hydrogels [123].

3.2 Materials and Methods

3.2.1 Materials

The 8-arm PEG-SH (20 kDa) and 4-arm PEG-NHS (20 kDa) polymers were obtained from NOF Corporation (White Plains, NY). HEC gel was obtained from the NIH AIDS Reagent Reference program (HPTN 035 Study Gel).

3.2.2 Preparation of PEG-based hydrogels

Hydrogels were prepared by mixing varying amounts of 8-arm PEG-SH polymer (4% and 8%, w/v) with 2 equiv. of 4-arm PEG-NHS in sterile sodium phosphate buffer (PB, 20mM, pH = 7.4) at room temperature (RT) (Scheme 1). The time of formation of the hydrogels was determined *in vitro* as follows: The 8-arm PEG-SH and 4-arm PEG-NHS solutions (total volume of 100 μ l) were mixed in a glass vial and the time when the solution in the inverted tube ceased to flow was recorded as the time of formation. The time of formation for the 4% w/v hydrogels was 9.0 ± 0.2 min, and for the 8% w/v hydrogels was 9.1 ± 0.3 min ($n=3$,

mean \pm SD). For the animal study, the polymer solutions were prepared and mixed just prior to dosing so that the hydrogels are formed *in situ*.

3.2.3 Rheological properties of PEG-based hydrogels and HEC gel

The rheological properties of the PEG-based hydrogels and HEC gel were evaluated at 37 °C using a rheometer with parallel plate geometry (plate diameter: 20 mm, gap: 300 μ m). The PEG-based hydrogels (4% and 8% w/v) were allowed to form between the parallel plates at RT, before ramping the temperature up to 37 °C. In the case of the HEC gel, it was directly placed between the plates at RT and the temperature was then raised to 37 °C. The elastic/storage modulus (G') and the viscous/loss modulus (G'') of the samples were measured as a function of frequency by conducting a dynamic oscillatory test. A frequency sweep test over a range of 0.1 – 10 Hz was performed at a constant strain of 1% (within the linear viscoelastic regime).

3.2.4 MR Imaging

All procedures were conducted under protocols approved by the Institutional Animal Care and Use Committee at Rutgers University. Female CD-1 mice (22-24 g, Charles River Laboratories, Wilmington, MA, US) were housed (four per cage) in an environmentally controlled room with 12 h light cycles. All animals were fasted 12-15 h prior to dosing. An equal volume of hydrogel or HEC gel was administered intravaginally to mice (50 μ l per animal, four animals per group) under isoflurane anesthesia. Mice were imaged 10 min post-dose using a

1-Tesla M2™ Compact High-Performance MRI (Aspect Imaging, Toronto, ON, Canada). Images were obtained using a Fast Spin Echo (FSE) 2D sequence (FOV: 64; TR: 3786.69; TE: (80) 73.80; slice thickness: 0.5mm; Image Quality: 256/250; flip angle: 180). The total scan time for the above sequence was 9.51 min. The respiratory rate of all animals was monitored continuously during each dose and scan. To assess the distribution and retention of the hydrogel and HEC gel formulations over time, mice were imaged again at 2, 4, 8, 12 and 24 h post-dose using the sequence mentioned above. All animals were allowed to recover and remain ambulatory between time points.

3.2.5 Data Analysis

MRI images were analyzed using Vivoquant™ pre-clinical image analysis software (inviCRO, LLC). The linear dimensions of the vagina (in mm) were measured using the distance/annotation tool in Vivoquant™ and reported as mean \pm SD. The volume of hydrogels or HEC gel in the vagina was determined by defining a region of interest (ROI) in the images obtained for each mouse. The ROIs were manually defined within the vagina by comparing the images obtained at various time points with the “pre-scan” (image obtained prior to dosing) for each mouse. The ROIs were demarcated using the automatic thresholding tool in Vivoquant™. The initial volume of leakage of the hydrogels and HEC gel was determined similarly, by measuring ROIs outside the vaginal orifice.

“Surface contact” was determined using a method similar to Barnhart et al. [119]. Surface contact was defined as the sum of the linear spread of the gel

into the vaginal fornix (measured from the sagittal section) and the transverse spread of the gel (measured from the axial section) at three different locations along the vagina (2.0, 4.5 and 6.5 mm from the orifice). All measurements were made using the distance/annotation tool in Vivoquant™.

Gel volumes and surface contact were normalized to their initial values and plotted against time using GraphPad Prism software v4.0c (GraphPad Software, Inc., La Jolla, CA). The data are expressed as mean±SD. Statistical analyses were performed using one-way ANOVA with Tukey post-test. A p value <0.05 was considered statistically significant.

To ensure that the number of animals per group (n=4) was sufficient to achieve statistical significance a post hoc power analysis using G*Power 3.1 was performed [124]. Power was determined by performing a post hoc analysis rather than an a priori analysis since there is no literature precedence for estimating effect size for the current study. The difference between the mean gel volumes calculated for the 4% w/v hydrogel group and the HEC gel group at the 2 h time point was used to determine power. Power analysis revealed a power (1-β err prob)=0.834 for α err prob=0.05 and n=4 per group. Since the probability of finding a statistically significant difference was >80% for n=4, the number of animals per group was not increased in the current study.

3.3 Results

An MRI technique to visualize the entire female mouse reproductive tract was developed. A high-resolution T2-weighted FSE scan comprising of 20 sections, with a thickness of 0.5 mm/section to obtain coronal images of untreated mice was used. The coronal images, and the corresponding sagittal and axial images clearly delineated the complete reproductive tract, including the vaginal orifice, clitoral gland, vagina and cervix (Figure 3.1). To obtain baseline dimensions of the vagina, the length (from the orifice to the beginning of the fornix) and width (at 2.5 mm from the orifice) from the coronal images obtained from eight untreated animals ("pre-scan") were measured using the distance/annotation tool in Vivoquant™ software. The length and width of the vagina was found to be 7.0 ± 0.4 mm and 3.6 ± 0.5 mm, respectively. Images were obtained after intravaginal administration of PEG-based hydrogels and the HEC comparator gel using parameters identical to the pre-scans. The PEG-based hydrogels and HEC gel were clearly visible in all three planes with an intensity that was several-fold higher than the background (Figure 3.1). Thus, even without the use of contrast agent the FSE scans were able to distinguish gels within the cervicovaginal tract. The presence of contrast agents may confound the interpretation of the results since they are likely to diffuse out of gels making it difficult to accurately assess gel retention.

To determine the coverage, distribution and retention of hydrogels and HEC gel, a series of MRI scans were obtained at various times post-dose. Mice (n=4 per group) were dosed with hydrogel (4% and 8%, w/v) or HEC gel and imaged immediately, 2, 4, 8, 12 and 24 h post-dose. After initial administration of

the dose, the hydrogels and HEC gel were found to have filled the entire vagina, extending into the fornix (Figure 3.2, “Immediate”). In some cases, the gel appeared to have spread around the clitoral gland (indicated by a circular void in the center slice of the scan). At 2 h post-dose, the distribution of the hydrogels was markedly different from the HEC gel (Figure 3.2, “2 h”). The hydrogels appeared more or less intact, with some spreading of the gel away from the center to the lateral walls of the vagina, possibly as a result of ambulation. However, in the case of the HEC gel, a significant amount of the gel bolus in the center of the vagina had leaked out and the remainder appeared as a thin layer in the upper and lateral walls of the vagina. At 4 h and 8 h post-dose, the hydrogels were found to have further flattened out from the center and distribute to the fornix and lateral walls (Figure 3.2, “4h” and “8h”). Only trace amounts of the HEC gel were detected during the same time period (Figure 3.2, “4h” and “8h”). At 12 h post-dose, hydrogels were detected in all four mice, while the HEC gel had been completely eliminated in two out of the four mice. At 24 h post-dose, the HEC gel was eliminated from the two remaining mice and the 4% w/v hydrogel was eliminated from all but one animal. The 8% w/v hydrogel was still present in all four mice at the 24 h time point. At 48 h post-dose, the 4% w/v hydrogel was completely eliminated from the remaining animal and the 8% w/v hydrogel was eliminated from all but one animal.

Gel volumes were in measured in order to quantify the coverage of the hydrogels and HEC gel at each time point. Regions corresponding to the gel in each slice of the MRI scan were designated as ROIs. ROIs were defined by

thresholding above the background, using the automatic thresholding tool in Vivoquant™. To ensure that only those regions corresponding to the gel were thresholded as ROIs the scans obtained at each time point were compared to the “pre-scan” for the same animal. The resulting ROIs were visualized in 3-D by processing the image as a maximum intensity projection (MIP) (Figure 3.3).

The hydrogels and HEC gel provided similar initial volume of coverage in the vagina (22.7 ± 1.8 , 24.9 ± 3.5 and 23.2 ± 7.2 mm³ for 4% and 8% w/v hydrogel, and HEC gel, respectively) (Table 1). There was some initial leakage of the gels outside the vaginal orifice and was found to be 6.1 ± 4.9 , 4.0 ± 3.5 and 8.1 ± 6.7 mm³ for 4% and 8% w/v hydrogel, and HEC gel, respectively. At 2 h post-dose, there was only a slight reduction (89.3% and 88.7%) in hydrogel volumes (20.3 ± 6.1 and 22.1 ± 2.3 mm³ for 4% and 8% w/v hydrogel, respectively), but a marked decline in HEC gel volume (38.1%, 8.8 ± 2.3 mm³). At 4 h and 8 h post-dose the HEC gel volume reduced to 18.7% and 12.2% of the initial volume, respectively (Table 3.1). On the other hand, the 4% w/v hydrogel volumes remained more or less constant and in the case of the 8% w/v hydrogel, there was a slight increase at 8 h, possibly due to swelling of the hydrogel (see discussion). At 12 h post-dose, the HEC gel was completely eliminated from two out of four animals. The 4% w/v and 8% w/v hydrogels were completely eliminated from the vagina in three out of four animals at 24 h and 48 h post-dose, respectively. Since the HEC gel volume could not be measured at 12 h, gel retention at 12 h and beyond could not be compared. The gel volumes at each time point for the hydrogel and HEC gel groups were normalized to their initial volumes and expressed as a

percentage (Figure 3.4). A one-way ANOVA test was used to determine if the differences in hydrogel and HEC gel volumes at various time points were statistically significant. The hydrogel volumes were significantly greater than the HEC gel volumes at 2-8 h post-dose ($p < 0.01$ for 4% w/v hydrogel and $p < 0.001$ for 8% w/v hydrogel)

Since decreased gel volumes do not necessarily indicate decreased mucosal coverage, “surface contact” for the hydrogel and HEC gel groups were measured at each time point using a method reported by Barnhart et al. with modification [119]. Surface contact was calculated as the sum of the linear spread of the gel into the vaginal fornix as measured from the sagittal section and the transverse spread of the gel as measured from the axial section at three different locations along the vagina (2.0, 4.5 and 6.5 mm from the orifice). The thickness of the gel in the anterior-posterior direction was not taken into account in making these measurements since mucosal surface contact is independent of gel thickness. At the initial time point (i.e., immediately following administration of the gel dose), the hydrogels and HEC gel did not show statistically significant differences in surface contact (11.1 ± 1.6 mm, 12.5 ± 0.5 mm and 10.9 ± 2.0 mm, for the 4% and 8% w/v hydrogels and HEC gel, respectively) indicating similar mucosal coverage. However, at the 2 h time point there were noticeable discontinuities in mucosal coverage with the HEC gel compared to the hydrogels (Figure 3.5). The HEC gel group continued to show a decrease in surface contact at the 4 h and 8 h time points, while the hydrogel groups showed similar or increased values compared to the initial time point. At 4 h post-dose the HEC gel

surface contact declined to 24.7% of the initial value, while the hydrogel surface contact was 97.5% (for the 4% w/v hydrogel) and 93.4% (for the 8% w/v hydrogel) of the initial values (Figure 3.6). At 8 h post dose, the HEC gel surface contact further reduced to 11.5% of the initial value. On the other hand, the hydrogels showed a slightly increased surface contact compared to the initial values (115% and 107% for the 4% and 8% w/v hydrogels, respectively). One-way ANOVA analysis indicated that the hydrogels showed significantly increased surface contact compared to the HEC gel at 2-8 h post-dose ($p < 0.01$ for 4% w/v hydrogel and $p < 0.001$ for 8% w/v hydrogel).

The rheological properties of the PEG-based hydrogels and HEC gel were evaluated by conducting dynamic oscillatory studies using a parallel-plate rheometer. A frequency sweep test was conducted over a range of 0.1-10 Hz at a constant strain of 1%. The variations of the elastic modulus (G') and the viscous modulus (G'') with frequency, for the PEG-based hydrogels and HEC gels are shown in Figure 3.7. The 4% and 8% w/v PEG-based hydrogels displayed an elastic modulus G' that was higher than the viscous modulus G'' , indicating that they are predominantly elastic over the range tested (Figure 3.7). In contrast, the HEC gel displayed a higher viscous modulus G'' when compared to the elastic modulus G' over the entire frequency range (Figure 3.7).

The elastic modulus of the hydrogels and HEC gel at the mid-point of the frequency range tested (1 Hz) was plotted against their retention time, which was defined as the time-point at which ~50% of the gel volumes remained in the vagina (Figure 3.8). The hydrogels, which showed elastic moduli that were four-

fold higher than the HEC gel, were retained in the vagina for a longer time. Thus the *in vivo* retention of the hydrogels and HEC gel was dependent on their *in vitro* rheological properties (Figure 3.8).

3.4 Discussion

Gels are the most common dosage form for vaginal drug delivery [103, 104]. For gels to be effective as microbicides, they must sufficiently coat the vaginal epithelium and provide a physical and/or chemical barrier against HIV in addition to serving as a physical matrix for sustained drug release. Few studies, however, have analyzed *in vivo* gel deployment in the vagina, leakage, spreading and retention over time. The development of a small animal pre-clinical model for quantifying the above parameters is a much needed step in understanding how the viscoelastic properties of gels affect their *in vivo* performance, thus enabling the definition of the optimal specifications for microbicide gels.

In this study, an MRI method for non-invasively imaging vaginal gels in mice over a period of 48 h was developed. This method was used to quantify the *in vivo* surface coverage, distribution and retention of PEG-based hydrogels and the standard HEC comparator gel. A high-resolution T2-weighted FSE scan was found to be sufficient to distinguish the gels from the underlying mucosa with high contrast. On comparing the pre-scan with post-dose images, it was found that the PEG-based hydrogels and HEC gel appeared hyperintense compared to background, allowing for reliable quantification of gel volumes and surface contact at various times post-dose. Thus, with the FSE scan a gadolinium

contrast agent was not required for visualizing the gels in the vagina of mice, unlike in previous clinical studies in women [111, 119].

We hypothesized that PEG-based hydrogels would be an improved platform for vaginal delivery over semi-solid gels for a number of reasons: (1) chemically crosslinked hydrogels have a higher viscoelastic modulus than semi-solid gels and are thus likely to have lower leakage and longer retention in the vagina, (2) controlled release of drug from hydrogels can be achieved by covalent attachment of drug to the PEG polymers via releasable bonds or by passive entrapment within the hydrogel, thus allowing for increased drug concentration in the vagina over a longer duration, and (3) PEG has no reported *in vivo* toxicity and is FDA approved. The *in vivo* performance of two different concentrations of PEG-based hydrogels (4% and 8% w/v) were compared to HEC gel by measuring gel volumes in the vagina and surface contact over a period of 24 h. The results indicate that although the initial volume of coverage for the hydrogels and HEC gel was similar, there was a significant difference in retention. It was found that at 2 h post-dose, there was >50% loss of HEC gel volume from the vagina. Such rapid leakage of semi-solid gels is consistent with previous reports from clinical studies of microbicide and contraceptive gels. In a previous MRI study, Barnhart et al., observed leakage of Gynol II gel in 80% of women at 1 h and 100% at 6 h for an initial dose of 5 ml [111]. Another clinical trial reported a 93% loss of Advantage S, a semi-solid gel contraceptive within 2 h [125]. A study assessing the retention of Replens, a polycarbophil gel using gamma scintigraphy reported clearance of 9-97% of the gel within 2 h [118]. In

contrast to the HEC gel, >90% of the hydrogel volume remained inside the vagina at 2 h. Moreover, at 12 h post-dose, the HEC gel was completely eliminated in two out of four animals, while the 4% w/v and 8% w/v hydrogels were completely eliminated from the vagina only at 24 h and 48 h post-dose, respectively. This represents a 100% and 200% improvement in vaginal residence time demonstrating that the PEG-based hydrogels have superior retention and surface coverage.

Surface contact measurements did not indicate a statistically significant difference between the initial values measured for the hydrogels and HEC gel. However, the HEC gel showed a statistically significant decrease in surface contact at 2-8 h post-dose when compared to the hydrogels. Previous MRI studies on the distribution of Gynol II gel in women showed that while there was a significant decrease in gel thickness, there was an increase in linear coverage and surface contact at 30-45 min and 6 h following administration [111, 119]. One possible explanation for the contrasting results could be the difference in the volume of the dose administered in women and mice. The higher initial dose volume administered in women (3-5 ml) compared to mice (50 μ l) could be the reason for the residual gel observed in the vagina at 6 h, despite noticeable gel leakage within this time period.

Ideally, microbicide gels must have viscoelastic characteristics such that they not only distribute well in the vagina and provide maximum mucosal coverage, but are also retained for a sufficient period of time without leaking [53]. Rheological studies on vaginal semi-solid gels have shown that gels formed by

physical interactions of polymer chains (i.e., chain entanglements) such as HEC and sodium carboxymethylcellulose (NaCMC) gels have less elastic behavior than gels formed by chemical cross-linking, such as polycarbophil and polyacrylic acid (Carbopol®) gels [109, 126]. This means that chemically cross-linked gels will maintain their structure over a wider range of shear rates than physical gels and are thus likely to show better retention in the vagina. Previous dynamic oscillatory studies on HEC gels indicate that they show a behavior similar to that of viscous liquids at higher frequencies [126, 127]. The results from the frequency sweep test in the current study showed that the HEC gels have a higher viscous modulus (G'') than elastic modulus (G') over a frequency range of 0.1-10 Hz confirming the above observations. In contrast to the HEC gels, the PEG-based hydrogels displayed elastic solid-like behavior ($G' > G''$), indicating that these gels have more structural resistance. The differences in vaginal retention of the HEC gel and PEG-based hydrogels are thus due to their markedly different rheological properties. Since the PEG-based hydrogels are administered as a viscous liquid, they distribute well within the vagina and provide good initial coverage, similar to the HEC gel. However, unlike the HEC gel, the hydrogels rapidly undergo gelation *in situ* to form a highly cross-linked elastic network on the mucosa within a few minutes of administration. Thus, while the HEC gels completely leaked out from the vagina within a few hours, the hydrogels continued to be retained inside the vagina.

It must be noted that the PEG-based hydrogels used in the current study are formed by covalent cross-linking of polymers via reversible thioester bonds

and will degrade by hydrolysis or enzymatic cleavage [128]. *In vitro* studies on the above hydrogels have shown that due to their hydrophilic nature, they undergo swelling by absorbing surrounding fluid and reach equilibrium in 12-24 h (depending on the polymer concentration), after which they start degrading (data not shown). Therefore, the slightly increased hydrogel volumes and surface contact measurements observed in our study at the 8 h time point, is most likely due to hydrogel swelling. However, at 12 h, a reduction in hydrogel volumes was observed and the mice were monitored until the hydrogels were completely cleared from the vagina (24 h and 48 h for the 4% w/v hydrogel and 8% w/v hydrogel, respectively).

Previous clinical studies examining the vaginal distribution and retention of microbicide gels in women have indicated that dose volume might be an important parameter affecting the extent of surface coverage. A study examining the distribution of two volumes of cellulose sulfate gel (2.5 and 3.5 ml) found increased linear coverage and surface contact of the gel for a dose volume of 3.5 ml [129]. Another study comparing 3 ml vs. 5 ml of Gynol II gel found greater vaginal coverage with 5 ml of gel at 30 min and 6 h post-dose [111]. In the current study, a dose volume of 50 μ l was used for both the PEG-based hydrogels and HEC gel since this volume completely filled the vagina and provided maximum initial coverage. For the above dose volume, the PEG-based hydrogels formed a “plug” inside the vagina after undergoing gelation *in situ*. The hydrogel “plug” remained as a more or less intact solid mass for several hours post-dose until degradation. Based on the above observations, it is likely that a

lower dose volume of hydrogel compared to semi-solid gels might be sufficient for forming a protective layer on the mucosa and providing maximum surface coverage.

The current study suggests that MR imaging in mice will be a useful pre-clinical model for evaluating the *in vivo* behavior of various microbicide gel candidates for optimizing their formulation properties prior to clinical assessment. To our knowledge, the only animal model that has been developed so far for evaluating *in vivo* coverage of vaginal formulations is sheep [130]. In their study in sheep, Mehta et al. used gamma scintigraphy to assess the vaginal distribution and retention of starch-based pellets and cetomacrogol cream and made a comparison with distribution and retention in women, determined using MRI. The authors observed similar patterns in distribution and retention of the two formulations in both studies, despite having used different imaging modalities. Data from their women study, indicating that most of the cream formulation had leaked out in 4 h, was consistent with their findings from the sheep model (only $31 \pm 15\%$ cream was retained in the vagina at 6 h) [130]. We anticipate that the quantitative measures of vaginal spread and retention obtained using our mouse model can be similarly used to predict distribution and retention of vaginal formulations in women.

3.5 Conclusion

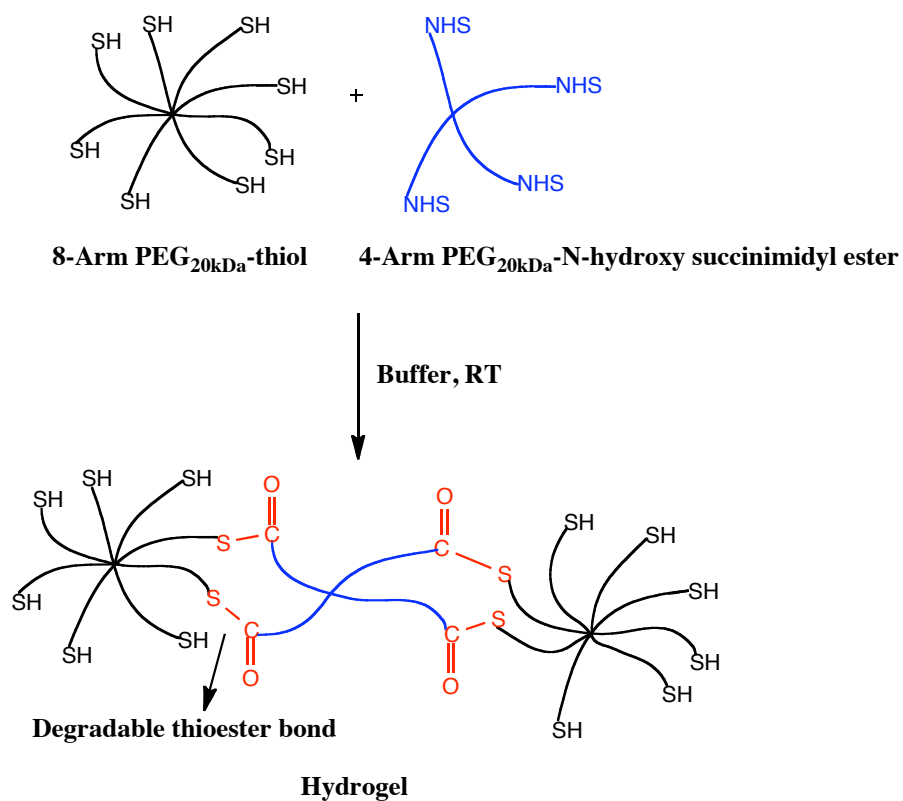
In the current study, the *in vivo* coverage, distribution and retention of PEG-based hydrogels and HEC gel in the vagina of mice were determined using

MRI. A high resolution FSE scan was used to monitor the distribution of the gels in the vagina for up to 48 h post-dose without the use of contrast agent. Hydrogel volumes and surface contact were quantified at each time point and compared to HEC gel. The results demonstrate that PEG-based hydrogels show significantly improved vaginal retention compared to HEC gel, indicating that the hydrogels might be more suitable platform for vaginal drug delivery than semi-solid gels. Rheological measurements using dynamic oscillation showed that the PEG-based hydrogels were predominantly elastic over the frequency range tested, whereas the HEC gel was predominantly viscous. Thus the increased vaginal retention of the hydrogels was due to their superior elastic properties compared to the HEC gel. The above results collectively indicate that MR imaging in mice will be a useful pre-clinical model for evaluating the performance of various microbicide formulations prior to clinical trials.

ACKNOWLEDGEMENT

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FIGURES AND TABLES



Scheme 3.1 Schematic representation of hydrogel formation using 8-arm PEG-SH and 4-arm PEG-NHS polymers.

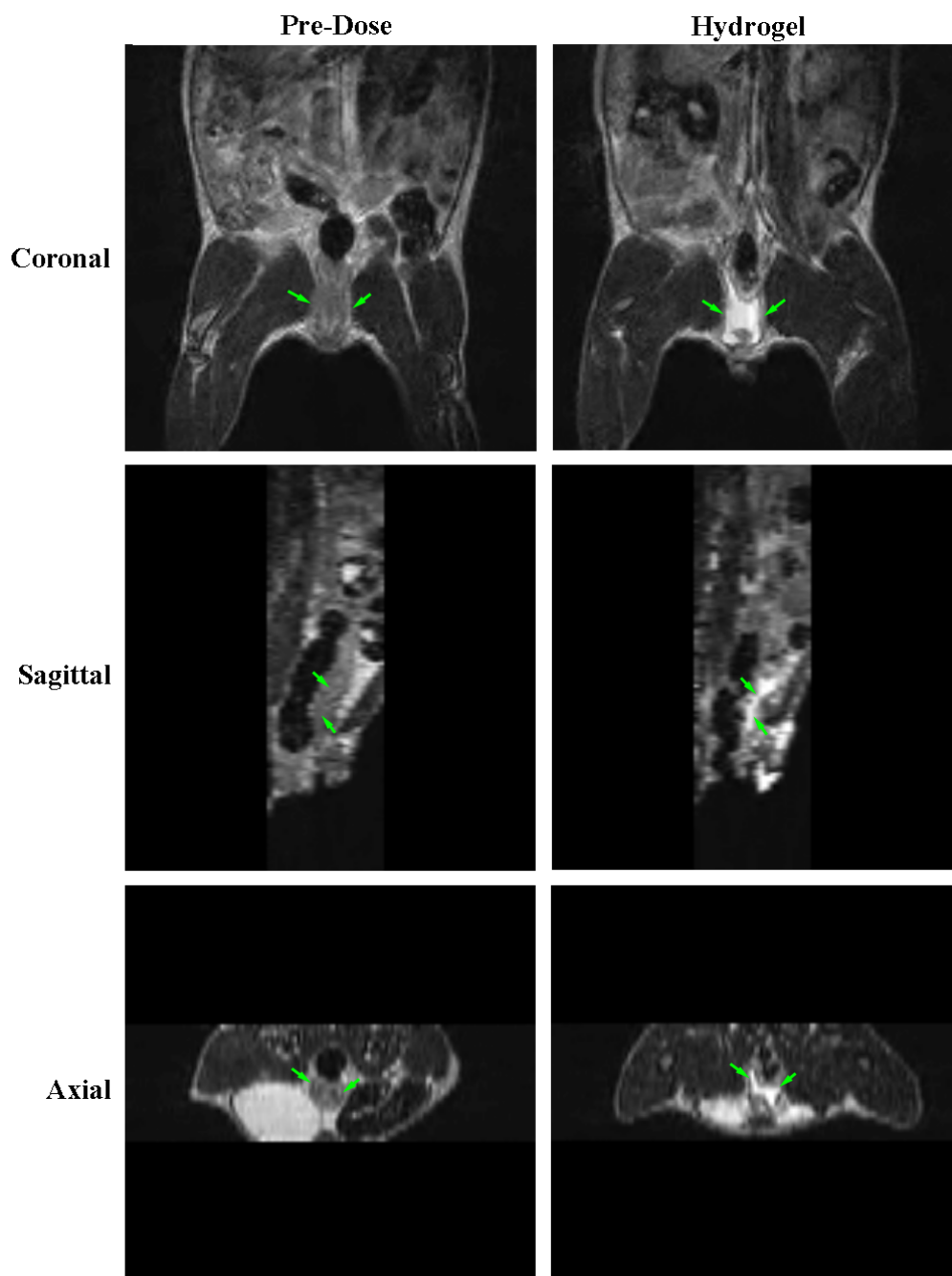


Figure 3.1 FSE scan of female CD-1 mouse prior to dosing, and after administration of 4% w/v hydrogel (FOV: 64; TR: 3786.69; TE: (80) 73.80; slice thickness: 0.5mm; Image Quality: 256/250; flip angle: 180). The green arrows mark the vagina in the coronal, sagittal and axial planes. The hydrogel appears in the scan with an intensity that is several-fold higher than the background.

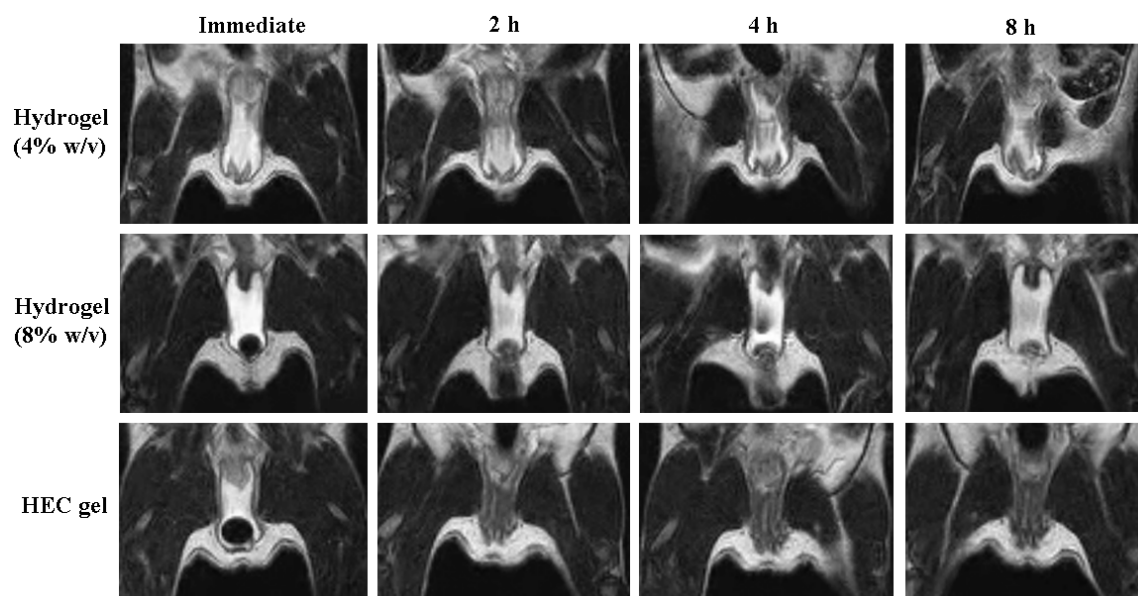


Figure 3.2 Representative FSE scans showing the distribution of PEG-based hydrogels and HEC gel in the vagina, at various time points post-dose. Female CD-1 mice (n=4 per group) were dosed with hydrogel or HEC gel and imaged immediately, 2, 4, 8, 12 and 24 h post-dose (12 and 24 h data not shown).

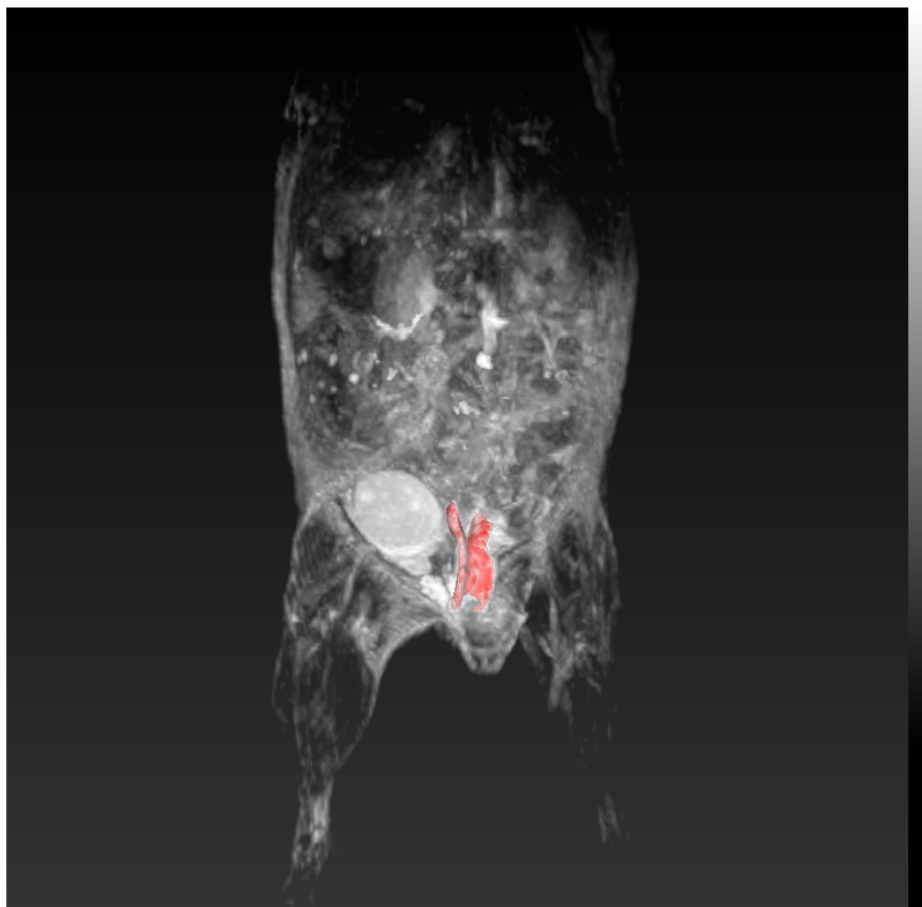


Figure 3.3 Representative MIP of FSE scan, processed using Vivoquant™ software. The above scan was obtained 4 h following hydrogel dose. Gel volumes at each time point were computed by defining ROIs (shown in red) within the vaginal area for each image, using the automatic thresholding tool.

Table 3.1 Gel volumes inside the vagina at various times post-dose; mean \pm SD, n=4

Time (h)	Gel volumes (mm ³)		
	Hydrogel (4%, w/v)	Hydrogel (8%, w/v)	HEC gel
0	22.7 \pm 1.8	24.9 \pm 3.5	23.2 \pm 7.2
2	20.3 \pm 6.1	22.1 \pm 2.3	8.8 \pm 2.3
4	19.3 \pm 5.9	23.7 \pm 3.8	4.3 \pm 2.7
8	20.0 \pm 9.1	31.4 \pm 4.6	2.8 \pm 1.0

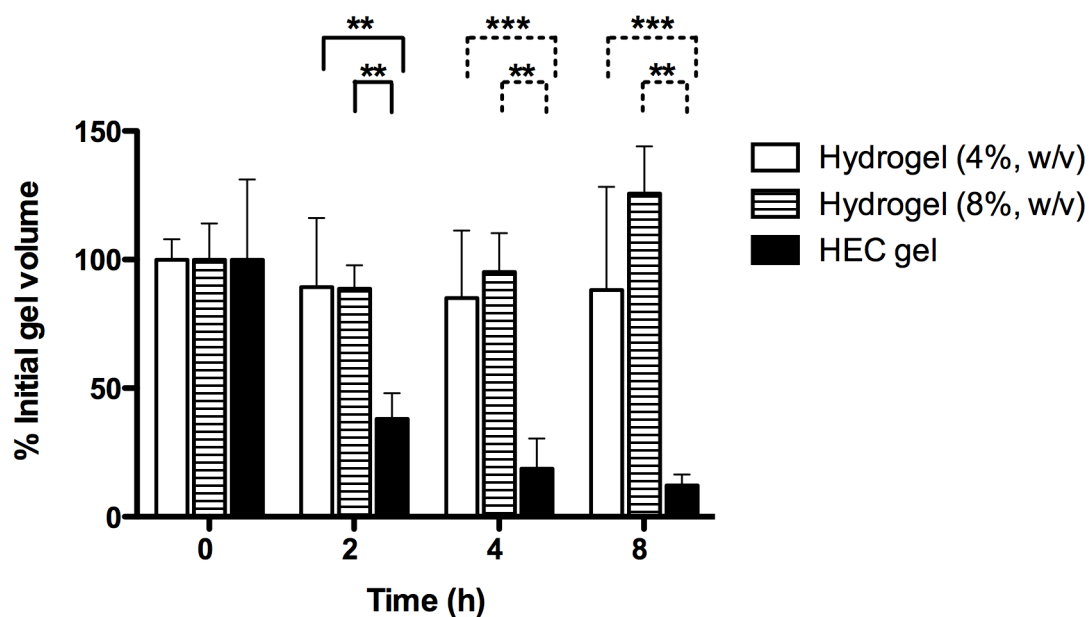


Figure 3.4 Hydrogel and HEC gel volumes inside the vagina, immediately, 2, 4, and 8 h post-dose (n=4). Data are normalized to the initial gel volume for each group and expressed as a percentage; mean \pm SD. Statistical analysis was performed using a one-way ANOVA. ** $p < 0.01$ and *** $p < 0.001$.

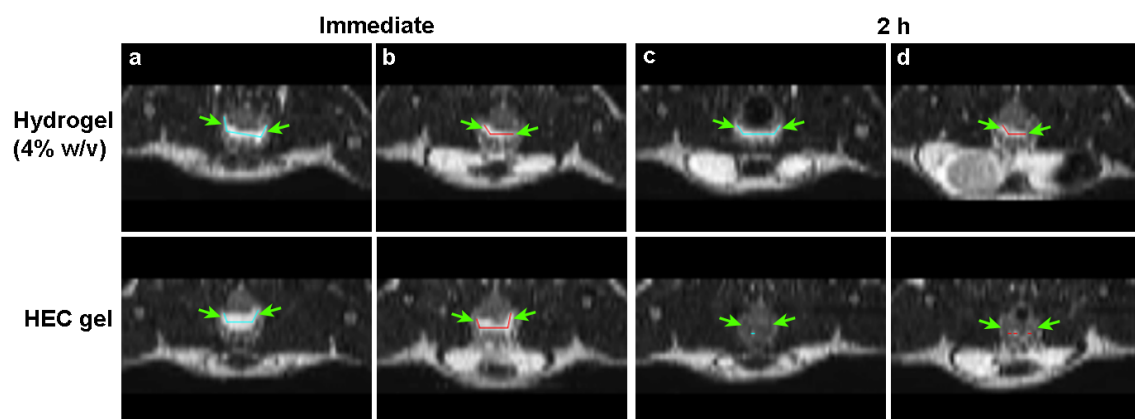


Figure 3.5 Surface contact of the hydrogel (4% w/v) and HEC gel, immediately and 2 h post-dose. Axial sections corresponding to 4.5 mm (a & c) and 6.5 mm (b & d) along the length of the vagina are shown. The green arrows mark the vagina in the axial sections. The hydrogel surface contact (top panel) is unchanged from the immediate to the 2 h time point. The HEC gel surface contact (bottom panel) is significantly diminished at the 2 h time point.

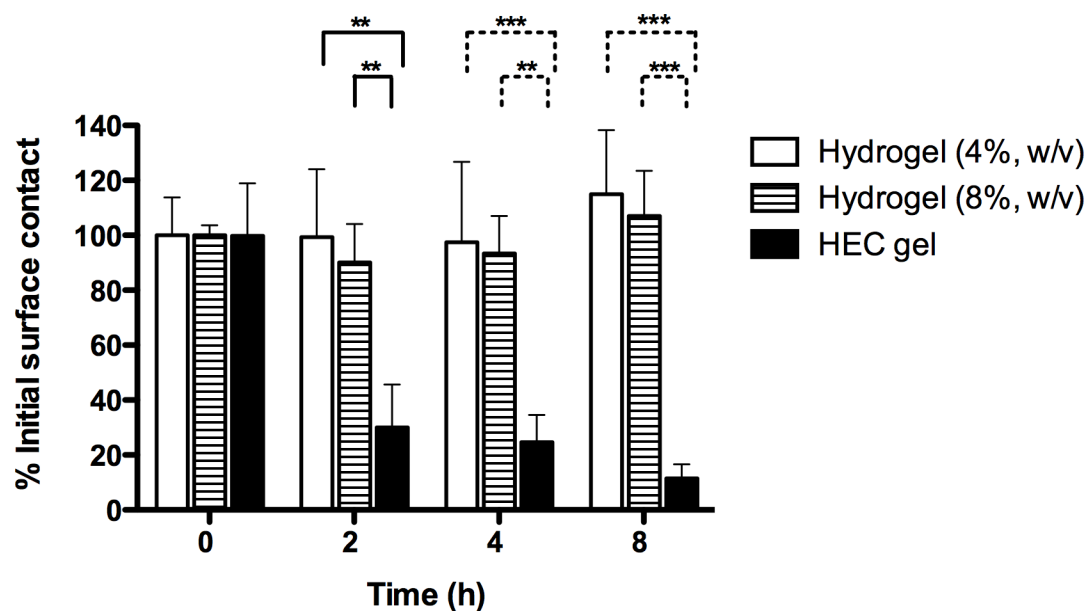


Figure 3.6 Surface contact for the hydrogel and HEC gel groups measured immediately, 2, 4, and 8 h post-dose (n=4). Data are normalized to the initial values of surface contact for each group and expressed as a percentage; mean \pm SD. Statistical analysis was performed using a one-way ANOVA. ** $p < 0.01$ and *** $p < 0.001$.

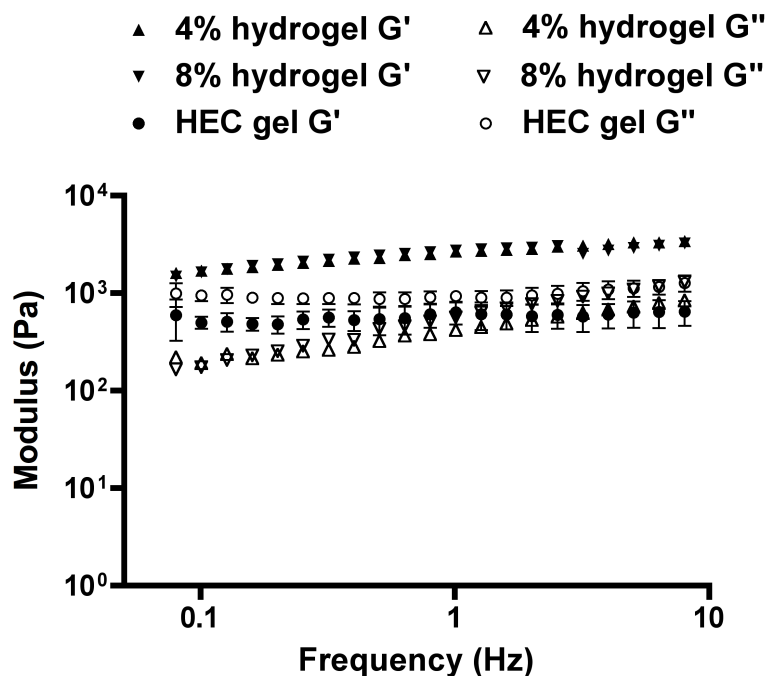


Figure 3.7 Variations of the elastic modulus (G') and viscous modulus (G'') of the PEG-based hydrogels and HEC gel, as a function of frequency. The hydrogels were predominantly elastic ($G' > G''$), whereas the HEC gel was predominantly viscous ($G'' > G'$), over the range tested.

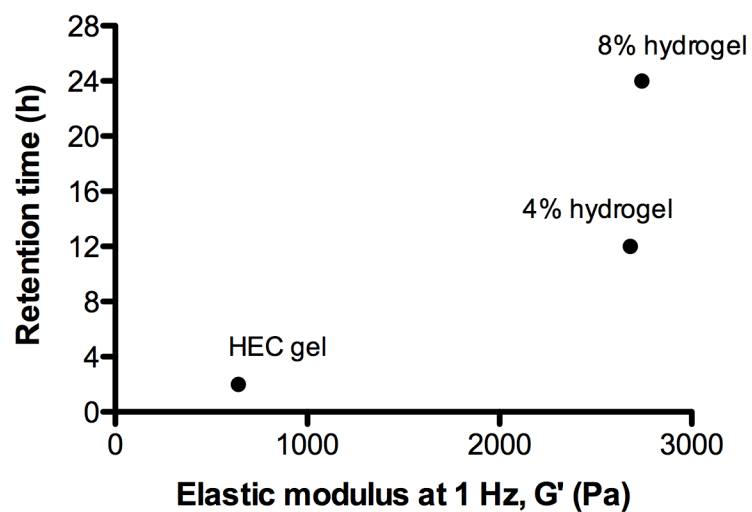


Figure 3.8 Correlation between the rheological properties of the hydrogels and HEC gel and retention time. The elastic modulus at the mid-point of the frequency range tested (1 Hz) was plotted against retention time. The hydrogels demonstrated significantly longer vaginal retention than the HEC gel due to their higher elastic modulus.

4. Poly(ethylene glycol) (PEG) nanocarrier-based hydrogels for the controlled release of lactic acid in the vagina

4.1 Introduction

Around 34 million people are living with HIV worldwide and over half are women [1]. Given the high rate of new infections (2.2-2.8 million in 2011), the development and implementation of female-controlled prophylactic methods is critical in order to successfully curb HIV. Toward this end, the past decade has seen an increased effort in the development of vaginal microbicides- topically applied, self-administered products that protect against HIV transmission [79, 81]. Most microbicides that have been evaluated in clinical trials, however, failed to prevent infection despite showing promising results *in vitro* [42, 47, 131]. It was only recently that a 3% tenofovir gel showed marginal success in prevention (CAPRISA 004 trial results, July 2010) with a 39% reduction in HIV transmission compared to the placebo [2].

In women, a primary risk factor for HIV infection via the cervicovaginal route is bacterial vaginosis (BV) [27-31, 132]. The normal vaginal flora is composed predominantly of *Lactobacilli*, which continuously produce lactic acid that maintains the acidic pH (4.0- 4.5) of the vagina [133, 134]. However, normal vaginal flora is compromised in BV, a common and often recurring infection characterized by overgrowth of anaerobic pathogens such as *Gardnerella vaginalis*, *Prevotella*, *Peptostreptococcus* and *Bacteroides spp.* [135, 136].

Women with BV have reduced vaginal *lactobacilli* and lactic acid and show high vaginal pH (up to 7.0) [137]. The decreased vaginal acidity in women with BV greatly increases their susceptibility to HIV infection [27-29]. A study investigating the association of BV with HIV-1 infection among female sex-workers in Thailand found that among the 43% of participants that were HIV-1 positive, 33% had BV [27]. A similar study in Uganda showed that over half the participants had moderate to severe BV and the rate of HIV-1 infection was considerably higher in women with BV (26.7%) than in women with normal vaginal flora (14.2%) [28]. Thus, it is generally believed that maintaining vaginal acidity and preventing BV infection are critical to HIV prophylaxis.

Previous microbicide gels designed for maintaining vaginal acidity such as BufferGel and Acidform are semi-solid gels consisting primarily of buffering agents (Carbopol 974P for BufferGel and acidifying agents for Acidform) formulated with other excipients [138, 139]. BufferGel was found to be safe for use but did not provide protection against HIV and was discontinued from further development as a microbicide [140, 141]. Acidform was shown to be effective against HSV-2 in a mouse model but is yet to be evaluated for efficacy due to safety concerns [44, 142]. Clinical trials of semi-solid gel microbicides suggest that one of the reasons for their poor *in vivo* performance might be the inadequate retention of the gel itself resulting in insufficient concentrations of active agents in the vagina. Semi-solid gels have been reported to leak from the vagina within a few hours of application [111, 118, 130]. In fact, BufferGel and Acidform are currently being evaluated for contraceptive use and BufferGel has

been shown to be effective only when used in combination with a diaphragm [45, 143]. Hence, there is a need for alternative delivery systems that show improved vaginal retention. Novel vaginal drug delivery systems such as intravaginal rings (IVRs), temperature and pH sensitive gels and nanoparticles are being investigated for use as microbicides [58, 75, 101]. Among these, IVRs are currently being developed for the vaginal delivery of antiretroviral drugs. However, with IVRs there are concerns of toxicity and systemic absorption due to initial high concentrations of drug in the vagina [54, 57].

In the present work, poly(ethylene glycol) (PEG)-based degradable hydrogels were designed and developed for the controlled release of lactic acid in the vagina in order to restore and maintain vaginal acidity in women with BV. The hydrogels are rapidly formed *in situ* by covalent cross-linking of 8-arm PEG-SH and 4-arm PEG-N-hydroxysuccinimide (PEG-NHS) polymers via degradable thioester bonds. In our laboratory, PEG-based hydrogels have been developed for ocular and dermal wound healing [120-122]. The hydrogels formed by covalently cross-linking multi-arm PEG polymers via thioether, disulphide and thioester bonds have good viscoelastic properties and resist deformation under high shear [120-122]. The controlled release of pilocarpine and doxycycline from the above hydrogels was achieved by passive entrapment of the drugs within the polymer network [120-122]. Therefore, the PEG-based hydrogels might be a good platform for vaginal drug delivery due to their viscoelastic properties.

Lactic acid was incorporated as the active moiety in the hydrogels because it is the intrinsic acidifying agent in the vagina [144, 145]. Lactic acid has

broad-spectrum antimicrobial activity against a variety of bacterial and viral pathogens, including HSV-2 and HIV [146-150]. O'Hanlon et al. demonstrated that lactic acid at concentrations of 55-111 mM completely inhibit BV-associated bacteria whereas acetic acid has no effect at these concentrations [146]. In another study, Juarez Tomas et al. showed that lactic acid inhibits uropathogenic *E. coli* at a concentration of 55.5 mM [147]. Recently, Lai et al. have shown that lactic acid has specific activity against HIV when compared to other acids such as hydrochloric acid [150]. Therefore, the controlled release of lactic acid in the vagina might restore normal vaginal lactic acid levels in women with BV and thus strengthen their defense against HIV.

Two types of PEG-based hydrogels were designed in order to achieve the controlled release of lactic acid (Scheme 4.1). The first is a “nanocarrier-based hydrogel”, wherein lactic acid is covalently attached to 8-arm PEG-SH via cleavable thioester bonds to form PEG-lactic acid (PEG-LA) nanocarriers. The nanocarriers are then crosslinked with 4-arm PEG-NHS to form hydrogels (Scheme 4.1A). In the second type of hydrogel, lactic acid is incorporated by passive entrapment within the polymer matrix by mixing lactic acid with the 8-arm PEG-SH and 4-arm PEG-NHS polymers (Scheme 4.1B). It was anticipated that the covalent attachment of lactic acid in the nanocarrier-based hydrogels might provide a more sustained release than passive entrapment since lactic acid is a small molecule (MW: 90 Da) and is highly hydrophilic. Hence, the properties of the nanocarrier-based hydrogels such as gelation time, rheology, swelling and degradation were evaluated under various conditions, with the end goal of

developing these hydrogels for vaginal administration. The release kinetics of lactic acid from the nanocarrier-based hydrogels were examined and compared to the hydrogels with passively entrapped lactic acid. The microbicidal activity of lactic acid and hydrogels with passively entrapped lactic acid were evaluated against the predominant BV pathogen *Gardnerella vaginalis*. In addition, the cytotoxicity of the 8-arm PEG-SH and 4-arm PEG-NHS polymers was determined using a vaginal epithelial cell line.

4.2 Materials and Methods

4.2.1 Materials

The polymers 8-arm PEG-SH (20 kDa) and 4-arm PEG-NHS (20 kDa) were obtained from NOF Corporation (White Plains, NY). Lactic acid, L-Cysteine, N, N'- Dicyclohexylcarbodiimide and 2- (Dimethylamino)pyridine were obtained from Sigma-Aldrich (St. Louis, MO). Ellman's reagent [5,5'-Dithio-bis-(2-nitrobenzoic acid)] and Slide-A-Lyzer® mini-dialysis units (MWCO: 3,500 Da) were obtained from Thermo Fisher Scientific (Waltham, MA). All solvents used were obtained from VWR International (Radnor, PA) or Sigma-Aldrich (St. Louis, MO). Gel permeation chromatography (GPC) was done on a Waters Breeze GPC system (Milford, MA) with dual-absorbance UV and refractive index detectors, using an Ultrahydrogel 1000 Column (12 μ m, 7.8 x 300 mm). Rheological data were obtained using Kinexus Ultra rotational rheometer (Malvern Instruments Inc., Westborough, MA). Lactate assay kit, used to

determine lactic acid concentrations was obtained from BioVision, Inc. (Mountain View, CA).

4.2.2 Synthesis and characterization of PEG-LA nanocarriers

Lactic acid (100 mg) was dissolved in Dichloromethane (DCM). N-Hydroxysuccinimide (1.2 eq.), and an excess of N, N'-Dicyclohexylcarbodiimide (DCC) were added under anhydrous conditions and the reaction mixture was stirred at RT for 8 h. The precipitate was filtered using a sintered funnel and the solvent removed using a rotary evaporator. The N-hydroxysuccinimidyl ester of lactic acid was then reacted with 8-arm PEG-SH, to obtain PEG-LA nanocarriers. The 8-arm PEG-SH was dissolved in DCM. The N-hydroxysuccinimidyl ester of lactic acid (8 eq.) was added along with an excess of 4-dimethylaminopyridine (DMAP). The reaction mixture was flushed with argon and stirred at RT for 4 h. Nanocarriers were obtained by precipitation from cold ether followed by drying. The amount of lactic acid bound to the PEG-LA nanocarriers was estimated indirectly using Ellman's assay and Gel permeation chromatography (Supplementary Information).

4.2.3 Release of lactic acid from PEG-LA nanocarriers

The PEG-LA nanocarriers (1 mg/100 μ l) were dissolved in sodium phosphate buffer (PB, 20 mM, pH 7.4). The solution was transferred to Slide-A-Lyzer® mini-dialysis units and dialyzed against phosphate buffered saline (PBS, 10 mM, pH 7.4) or acetate buffer (AB, 20 mM, pH 4.3) at 37 °C, with continuous

stirring. Aliquots were withdrawn from the release medium at pre-determined time intervals and the medium was replenished with an equal volume in order to maintain sink conditions throughout the study. The aliquots were concentrated and the amount of lactic acid determined using a lactate assay kit, as per the manufacturer's protocol (O.D. 570 nm). The above experiments (and subsequent experiments described below) were performed in triplicate and the results reported as mean \pm SEM, unless otherwise mentioned.

4.2.4 Preparation of nanocarrier-based hydrogels

The nanocarrier-based hydrogels were prepared using degradable thioester cross-links as follows: the PEG-LA nanocarriers (4%, 6% and 8%; w/v) were mixed with 2 equiv. of 4-arm PEG-NHS in PB and allowed to stand at room temperature until the hydrogels formed. The PEG-LA nanocarriers formed aggregates upon addition of PB and were sonicated prior to mixing with crosslinker. The time of formation of the hydrogels was determined using the "inverted tube method" and was noted as the time when the solution ceased to flow, upon inversion of the tube.

4.2.5 Rheological characterization of nanocarrier-based hydrogels

Rheological measurements were performed at 37 °C using a rheometer with parallel plate geometry (plate diameter: 20 mm, gap: 300 μ m). Nanocarrier-based hydrogels (4% and 6% w/v) were allowed to form between the parallel plates at RT, before ramping the temperature up to 37 °C. The elastic/storage

modulus (G') and viscous/loss modulus (G'') of the hydrogels were measured as a function of strain and frequency using dynamic oscillatory tests. First, a strain sweep test was performed at a constant frequency of 1 Hz, in order to determine the linear viscoelastic regime. Next, a frequency sweep test (0.1 – 10 Hz) was carried out at a constant strain of 1%.

4.2.6 Swelling and Degradation of nanocarrier-based hydrogels

The swelling and degradation of the nanocarrier-based hydrogels was investigated in both physiological and acidic conditions. Hydrogels were placed in a vial and the initial weight recorded (W_i). Hydrogels were then immersed in 1 ml of PBS or AB, and incubated at 37 °C on an orbital shaker. The buffer was withdrawn at pre-determined time intervals and the vials containing the swollen hydrogels were weighed (W_t). The buffer was replaced after each measurement and the experiment was continued until the hydrogels degraded completely. The swelling ratios were calculated as $\frac{W_t}{W_i} \times 100\%$ and plotted against time. The degradation time was defined as the time taken for the hydrogel to completely disappear ($W_t=0$).

4.2.7 Release of lactic acid from nanocarrier-based hydrogels

The nanocarrier-based hydrogels (4 and 6% w/v) were prepared as described before and the release of lactic acid from hydrogels investigated in physiological and acidic conditions. Hydrogels were placed in a vial and immersed in PBS or AB. The vials were placed on an orbital shaker and

incubated at 37 °C. Aliquots were withdrawn from the release medium at pre-determined time intervals and the medium was replenished with an equal volume in order to maintain sink conditions throughout the study. The amount of lactic acid released was determined using a lactate assay kit (O.D. 570 nm).

4.2.8 Hydrogels with passively entrapped lactic acid

Hydrogels with passively entrapped lactic acid were prepared as follows: The 8-arm PEG-SH (4% and 6%, w/v) was mixed with 2 equiv. of 4-arm PEG-NHS in PB at RT. Lactic acid (0.2 mg) was added to the polymer solutions during mixing. The release of lactic acid was determined as follows: Hydrogels were immersed in PBS (after an initial wash in PBS) and incubated at 37 °C on an orbital shaker. The PBS was withdrawn and replaced at pre-determined time intervals and the amount of lactic acid released determined using a lactate assay kit (O.D. 570 nm).

4.2.9 Death kinetics of *G. vaginalis* in the presence of lactic acid

G. vaginalis ATCC 14018 was the reference BV-associated strain used in these studies. The cells were stored at -80°C in Brain Heart Infusion (BHI) medium (Difco, Sparks, MD) supplemented with 3% horse serum (JRH Biosciences, KS) and 15% glycerol.

G. vaginalis (10^7 CFU/ml) was added to T-25 flasks pre-incubated with BHI medium supplemented with 3% horse serum in the anaerobic chamber, overnight at 37 °C. The initial cell counts in each flask was determined using the

drop plate method, by plating 30 μ l of cell suspension in duplicates on HBT bilayer agar. Lactic acid was added to the medium in the flasks at final concentrations of 0.9 mg/ml, 4.5 mg/ml and 9.1 mg/ml. Flasks with *G. vaginalis* in medium alone was used as the positive control for growth. The flasks were incubated in the anaerobic chamber at 37 °C and cell counts were performed from the flasks at various time points, using the drop plate method.

4.2.10 Growth kinetics of *G. vaginalis*

The growth kinetics of *G. vaginalis* in the presence of lactic acid, and on hydrogels with passively entrapped lactic acid was determined as follows: 4% w/v hydrogels with passively entrapped lactic acid (0.2 mg and 1 mg per 50 μ l of hydrogel), and lactic acid solutions (50 μ l/well in PB, final concentrations of 0.9-4.4 mg/ml per well) were prepared in a 96-well plate. *G. vaginalis* (10^7 CFU/ml, 200 μ l/well) was added to the wells and sterile mineral oil (50 μ l/well) was added on top to each well to facilitate anaerobic growth of *G. vaginalis*. Hydrogels with no entrapped lactic acid and medium alone were used as controls. The growth kinetics was determined by measuring turbidity every hour, over 48 h (O.D. 595 nm). The minimum inhibitory concentration (MIC) of lactic acid was determined as the lowest concentration of lactic acid that completely inhibited *G. vaginalis* growth.

4.2.11 Cytotoxicity evaluation on vaginal epithelial cells

The 8-arm PEG-SH and 4-arm PEG-NHS polymers were evaluated for cytotoxicity on the Vk2/E6E7 human vaginal epithelial cell line. Vk2 cells were seeded on a 96-well plate at a density of 20,000 cells/well in Keratinocyte Serum Free (KSF) growth medium. After overnight incubation, the medium was withdrawn and replaced with medium containing 8-arm PEG-SH and 4-arm PEG-NHS polymers at various concentrations. Nonoxynol-9 cream (N-9, 4% w/v) dissolved in medium was used as the positive control for toxicity and untreated cells (medium alone) were used as the negative control. The cells were grown for an additional 24 h and cell viability was determined relative to the untreated cells using alamarBlue® assay (AbD Serotec, Raleigh, NC). Briefly, the medium containing the various compounds was removed and cells were washed 1x with Dulbecco's Phosphate Buffered Saline (DPBS). The wells treated with 8-arm PEG-SH solution at concentrations above 40 mg/ml showed gelation of the medium above the cell layer and were treated with dithiotreitol (100mM, dissolved in DPBS) for 5-10 min to facilitate removal of the medium. The cells were then incubated with medium containing alamarBlue® (10% v/v; 100 µl/well) for 4h at 37°C. The reduction of alamarBlue® was measured using fluorescence (Ex: 560nm, Em: 590 nm) and the viability calculated as a percentage of the untreated cells. The percentage viability was plotted against polymer concentration, and the EC₅₀ determined by fitting the data with a sigmoidal-dose response curve:

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(LogEC_{50} - X)HillSlope}} ; \text{ where } Bottom = 0$$

4.3 Results and Discussion

4.3.1 Synthesis and characterization of PEG-LA nanocarriers

The PEG-LA nanocarriers were synthesized by attaching multiple copies of lactic acid to 8-arm PEG-SH polymer via thioester bonds. Lactic acid was first activated to form the N-hydroxysuccinimidyl ester of lactic acid and then reacted with 8-arm PEG-SH to obtain PEG-LA nanocarriers (Scheme 4.2). The lactic acid loading efficiency was estimated using Ellman's assay (Supplementary information). The nanocarriers with a loading efficiency of 2% polymer wt., corresponding to four copies of lactic acid per molecule, were used in this study, since the remaining unmodified –SH groups of the polymer were required for further cross-linking. The nanocarriers were characterized using GPC and showed a retention time that was similar to the unmodified polymer (RT ~8.8 min, Supplementary information). In addition, at the nanocarrier peak at 210 nm, the absorbance wavelength of lactic acid was 4-fold higher than the unmodified polymer indicating attachment of lactic acid.

The release of lactic acid from the PEG-LA nanocarriers was determined at 37 ° C in PBS (pH 7.4) and AB (pH 4.3). Since the thioester bonds are hydrolytically labile under both acidic and basic conditions, a sustained release of lactic acid from the nanocarriers was expected under these conditions. The cumulative release of lactic acid from the nanocarriers was plotted against time and the release profile was fit using a one-phase exponential equation:

$$\frac{M_t}{M_\infty} = 1 - e^{-kt} \quad (1)$$

In eq. (1) $\frac{M_t}{M_\infty}$ is the fractional release of the drug and k is the rate constant. A maximum release of 23% of bound lactic acid in PBS and 47% in AB was observed (Figure 4.1A). Since the hydrolysis of esters in buffer is pseudo first-order, the kinetics of nanocarrier hydrolysis were determined by plotting the log of the percentage remaining PEG-LA nanocarriers against time (Figure 4.1B) [151, 152]. The data were fit using linear regression and the rate constant (k) and half-life ($t_{1/2}$) of nanocarrier hydrolysis was determined from the slope. Nanocarrier hydrolysis proceeded at a faster rate in AB ($t_{1/2}$ = 29.5 h) than in PBS ($t_{1/2}$ = 63.1 h). Thus, the increased release of lactic acid from the nanocarriers in AB was due to acid catalyzed ester hydrolysis.

4.3.2 Preparation and characterization of nanocarrier-based hydrogels

The nanocarrier-based hydrogels were prepared by cross-linking the PEG-LA nanocarriers with 4-arm PEG-NHS via thioester bonds (Scheme 4.3). Using this schematic, it was expected that lactic acid will be released from the hydrogels due to thioester hydrolysis and that the hydrogels will undergo degradation also via hydrolysis of the thioester crosslinks. The nanocarrier-based hydrogels were prepared by mixing varying amounts of PEG-LA nanocarriers (4%, 6% and 8%; w/v) with 2 equiv. of 4-arm PEG-NHS in PB at RT. All the hydrogels were formed within 20 min and an increase in nanocarrier concentration from 4% to 8% w/v resulted in faster hydrogel formation (Table 4.1). The 8% w/v nanocarrier-based hydrogels were not evaluated further since

the PEG-LA nanocarriers at this concentration were extremely viscous and mixing the nanocarriers uniformly with the PEG-NHS polymer solutions was difficult.

The viscoelastic properties of the nanocarrier-based hydrogels were evaluated by conducting dynamic oscillatory studies. The viscoelastic properties of the hydrogels are particularly important for vaginal administration because gel leakage and retention in the vagina is largely dependant on these properties [53, 112]. A strain sweep test was conducted in order to establish the linear viscoelastic regime. This was followed by a frequency sweep test. The nanocarrier-based hydrogels displayed an elastic modulus (G') that was 100-fold higher than the viscous modulus (G'') over the entire range indicating that they are more elastic than viscous (Figure 4.2). Moreover, G' increased only slightly with frequency and reached a plateau indicating that the hydrogels can resist structural changes under strain. A transition from the elastic to viscous state (cross-over of G'') was not observed at the higher frequencies tested indicating that nanocarrier-based hydrogels show elastic solid-like behavior at these frequencies (Figure 4.2B) [120]. In contrast to the nanocarrier-based hydrogels, previous rheological studies on vaginal gels formed by physical interactions of polymer chains (i.e., chain entanglements) such as hydroxyethylcellulose (HEC) and sodium carboxymethylcellulose (NaCMC) gels have shown that such gels behave similar to viscous liquids at higher oscillatory frequencies [53, 109, 127]. Therefore, the elastic behavior of the nanocarrier-based hydrogels might result in

improved *in vivo* vaginal retention compared to existing vaginal semi-solid gel formulations.

The swelling and degradation of the nanocarrier-based hydrogels were determined in PBS and AB by measuring hydrogel weights at various time points following immersion in the appropriate buffer. At each time point, the buffer was removed and replaced with an equal volume. The degree of swelling was expressed as a percentage of the initial hydrogel weight and plotted over time. A continuous increase in hydrogel weight was observed within the first 4- 6 h in both conditions due to uptake of the surrounding fluid, and swelling equilibrium was reached within 12 h (Figure 4.3A). The maximum swelling ratio for the 4% w/v hydrogels was $491.6 \pm 22.6\%$ and $379.3 \pm 32.8\%$ in PBS and AB, respectively, and for the 6% w/v hydrogels was $529.2 \pm 70\%$ and $398.4 \pm 15\%$ in PBS and AB, respectively ($n=3$, mean \pm SD). An increase in nanocarrier concentration from 4% to 6% w/v resulted in a larger degree of swelling, in both conditions, due to the increased hydrophilicity of the hydrogels. After 24 h, a decrease in hydrogel weights was observed indicating that hydrogels had started to degrade due to hydrolysis of the thioester crosslinks (Figure 4.3B). Degradation was considered complete when the thioester crosslinks had fully hydrolyzed and the hydrogels had dissolved into the surrounding medium. The hydrogels in PBS were found to degrade within 6 days (Figure 4.3B). However, a slower rate of degradation was observed in AB and the hydrogels degraded completely within 42 days.

The release of lactic acid from the nanocarrier-based hydrogels was determined in PBS and AB at 37 °C. The nanocarrier-based hydrogels showed

controlled release of lactic acid for several hours. Release from 4% w/v nanocarrier-based hydrogels in PBS was first order with a maximum of 10% of bound lactic acid released in 72 h (Figure 4.4A). Release in AB was two-phase, with an initial fast release of 9% of bound lactic acid within 48 h, followed by a slow release phase. A maximum release of 14% of bound lactic acid was observed in AB (Figure 4.4A).

4.3.3 Hydrogels with passively entrapped lactic acid

Hydrogels with passively entrapped lactic acid were prepared by mixing lactic acid with 8-arm PEG-SH (4% and 6%, w/v) and 2 equiv. of 4-arm PEG-NHS in PB at RT. The time of formation for the 4% w/v and 6% w/v hydrogels was 9.8 ± 0.2 min and 6.6 ± 0.2 min, respectively. The hydrogels with passively entrapped lactic acid thus formed faster than the nanocarrier-based hydrogels, due to the availability of more unmodified –SH groups for cross-linking. The release of lactic acid from the above hydrogels was measured in PBS at 37 °C. The hydrogels showed a burst release of lactic acid, with 90% and 80% lactic acid released from 4% w/v and 6% w/v hydrogels, respectively within 30 min. (Figure 4.4B). Thus, the entrapped lactic acid rapidly migrated through the hydrogel network due to its low molecular weight and high hydrophilicity.

4.3.4 Inhibitory effect of lactic acid and hydrogels with passively entrapped lactic acid on *G. vaginalis*

Previously, Atassi et al. demonstrated that the growth of *G. vaginalis* was inhibited within 4 h when incubated with lactic acid at concentrations of 100 mM [153]. More recently, O' Hanlon et al. showed that lactic acid at concentrations of 55-111 mM (corresponding to 5-10 mg/ml) in acidic (pH 4.5) medium killed *G. vaginalis* within 2 h [146]. Because the strains of *G. vaginalis* or the growth conditions in the above studies were different from ours, we first determined the concentration range of lactic acid that inhibits *G. vaginalis*. *G. vaginalis* was incubated with varying concentrations of lactic acid and their death kinetics monitored by performing cell counts at various times following incubation. A concentration of 9.1 mg/ml completely inhibited *G. vaginalis* within 3 h (Figure 4.5).

The growth kinetics of *G. vaginalis* was then monitored in medium with various concentrations of lactic acid (0.9- 4.4 mg/ml) and on hydrogels with passively entrapped lactic acid (0.2 mg and 1 mg lactic acid in 50 μ l hydrogel). The nanocarrier-based hydrogels were not evaluated since these hydrogels released a maximum of only 14% bound lactic acid. The growth of *G. vaginalis* was monitored by measuring turbidity over 48 h (O.D. 595 nm). Complete inhibition of *G. vaginalis* was observed with 3.6 mg/ml of lactic acid and partial inhibition at lower concentrations compared to the medium control (Figure 4.6A). Similarly, hydrogels containing 1 mg lactic acid completely inhibited *G. vaginalis* growth (Figure 4.6B). There was no inhibition of *G. vaginalis* growth on the hydrogels without passively entrapped lactic acid. The above findings thus

indicate that the lactic acid released from the hydrogels has microbicidal activity against *G. vaginalis* suggesting their potential application for BV prophylaxis.

4.3.5 Cytotoxicity evaluation in vaginal epithelial cells

The cytotoxicity of the 8-arm PEG-SH and 4-arm PEG-NHS polymers used to prepare the hydrogels was evaluated using the Vk2/E6E7 vaginal epithelial cell line. Vk2 cells were incubated for 24 h with 8-arm PEG-SH and 4-arm PEG-NHS polymers at concentrations of 2.5-80 mg/ml. N-9 was used as the positive control for toxicity due to its demonstrated *in vitro* and *in vivo* cervicovaginal toxicity [154, 155]. Medium alone was used as the negative control for toxicity. After the incubation period, cell viability assessed using the alamarBlue® proliferation assay. The cells incubated with N-9 (positive control for toxicity) showed a 98% reduction in viability compared to the untreated cells (Figure 4.7). The 8-arm PEG-SH and 4-arm PEG-NHS polymers showed a 50% reduction in cell viability at concentrations of 50.25 mg/ml and 57.45 mg/ml, respectively (Figure 4.7). The cytotoxicity of the individual polymers at high polymer concentrations is most likely due to the -SH and -NHS functionalization of PEG since PEG has no reported cytotoxicity. It is expected that only a negligible amount of unreacted -SH groups will be present in the hydrogels since the 8-arm PEG-SH polymer is cross-linked with an excess of 4-arm PEG-NHS. Moreover, the cytotoxicity study was performed using cell monolayers, which have more sensitivity than intact epithelial tissue [101]. Therefore, the hydrogels are expected to have little or no toxicity *in vivo*. Further testing of the hydrogels

on organotypic or explant models will help evaluate potential *in vivo* toxicity to epithelial tissue [21, 156].

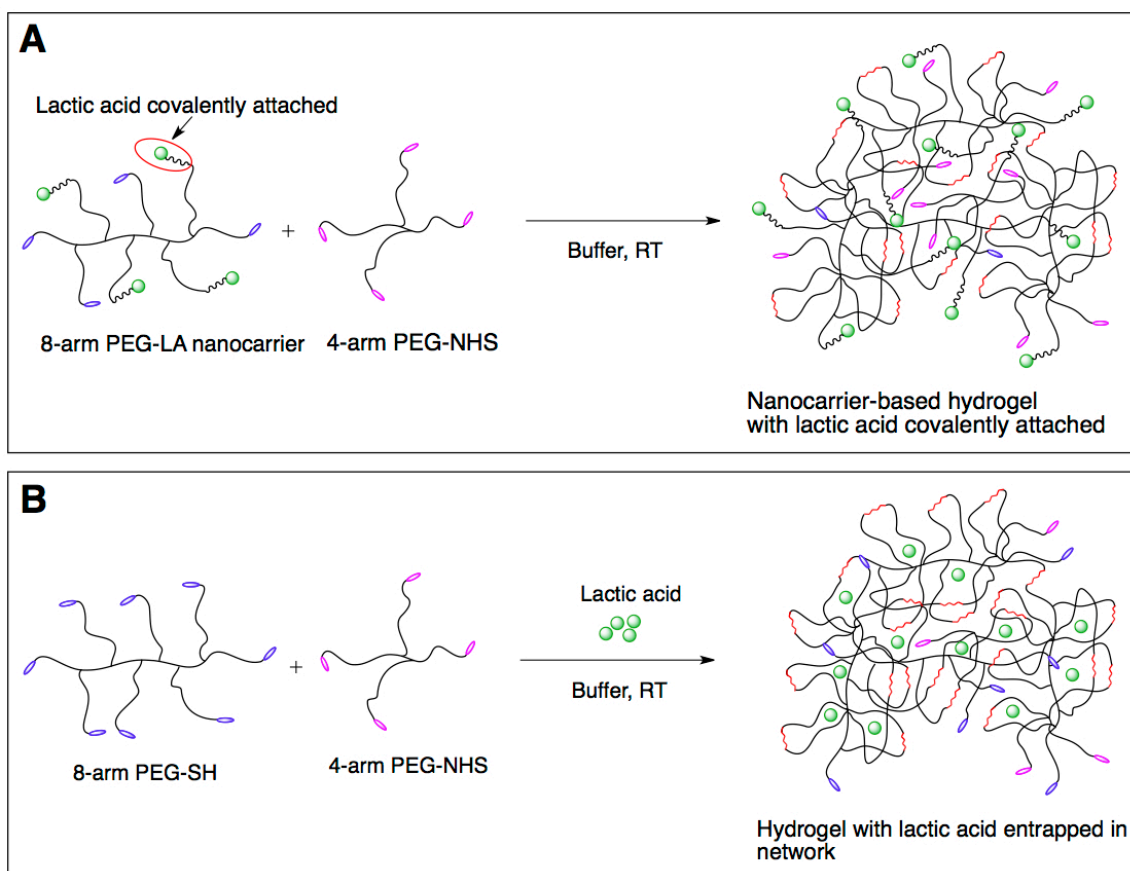
4.4 Conclusions

The goal of the current study was to develop *in situ* PEG-based degradable hydrogels for the controlled release of lactic acid in the vagina with the ultimate aim of restoring vaginal acidity in women with BV. The decreased vaginal acidity and altered vaginal flora in BV significantly increases susceptibility to HIV infection. Thus restoring vaginal acidity and controlling BV-associated bacteria reduces the risk of HIV infection. With the above objectives in mind, hydrogels containing lactic acid incorporated by (1) covalent attachment to the polymer via thioester bonds (nanocarrier-based hydrogels), and (2) passive entrapment within the polymer matrix were designed. The PEG-LA nanocarriers showed a sustained release of lactic acid for 24 h with greater release in acidic conditions due to faster hydrolysis of the thioester bonds. The nanocarrier-based hydrogels formed rapidly (<20 min), showed high mechanical strength and were degradable in both physiological and acidic conditions. Release of lactic acid from the nanocarrier-based hydrogels was sustained for several days; however, a maximum release of only 14% bound lactic acid was observed. In contrast, the hydrogels with passively entrapped lactic acid showed a burst release with 90% lactic acid released in 30 min. Growth of *G. vaginalis* was inhibited with lactic acid (MIC=3.6 mg/ml) and hydrogels containing passively entrapped lactic acid. Collectively, the above results indicate that PEG-based hydrogels are a good

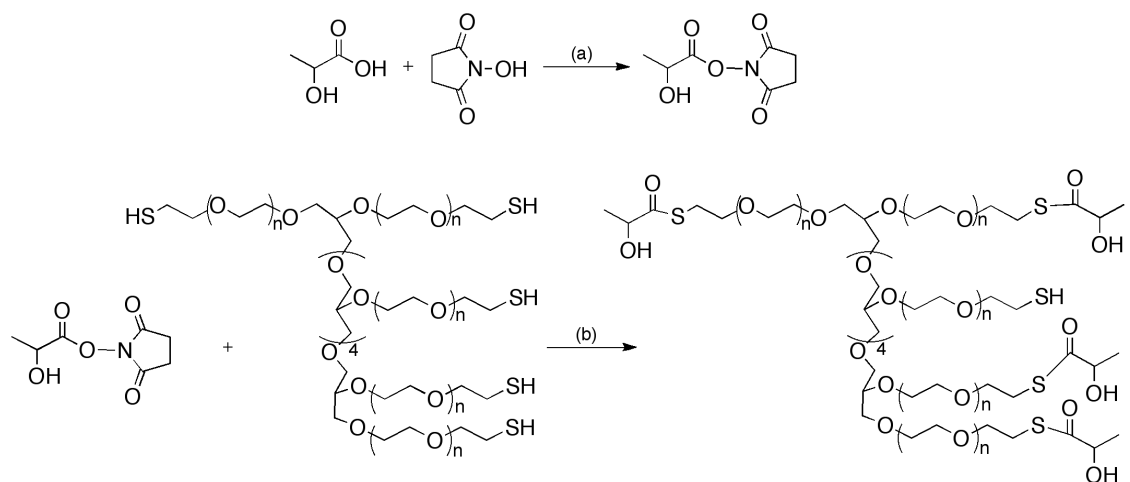
candidate for vaginal drug delivery and hydrogels with lactic acid have potential application as microbicides.

TABLES AND FIGURES**Table 4.1 Time of formation of PEG-LA nanocarrier-based hydrogels; mean \pm SD, n=3**

8-arm PEG-LA (mg/0.05 ml)	4-arm PEG-NHS (mg/0.05 ml)	Time of hydrogel (0.1 ml) formation (min)
4	8	16.6 \pm 0.2
6	12	16.5 \pm 0.2
8	16	10.4 \pm 0.4



Scheme 4.1 Schematic of PEG-based hydrogel formation. Hydrogels are formed by (A) covalent attachment of the lactic acid to 8-arm PEG-SH to form PEG-LA nanocarriers, followed by cross-linking with 4-arm PEG-NHS, and (B) passive entrapment of lactic acid within the hydrogel network by admixing with the 8-arm PEG-SH and 4-arm PEG-NHS solutions



Scheme 4.2 Preparation of PEG-lactic acid (PEG-LA) nanocarriers (a) DMF, DCC, room temperature, stir for 8 h (b) DMF, DMAP, room temperature, stir for 4 h.

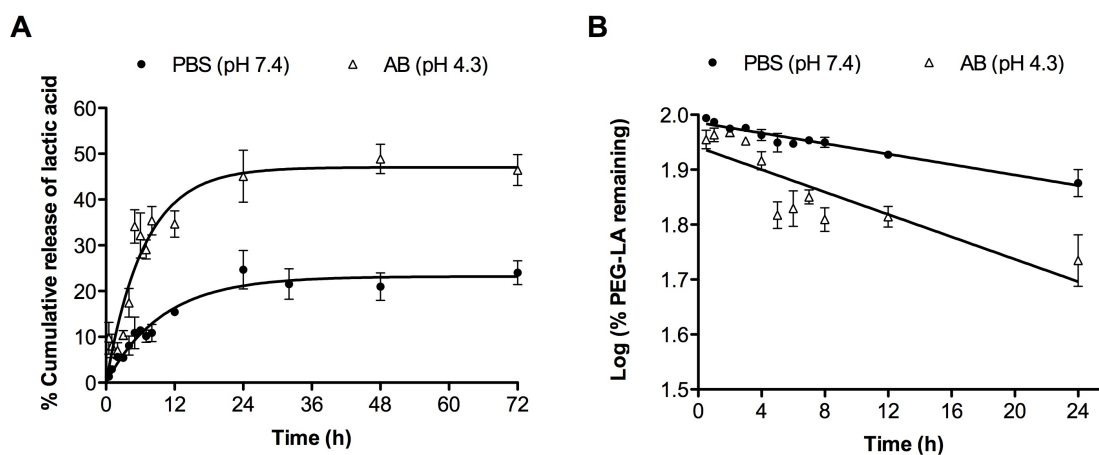
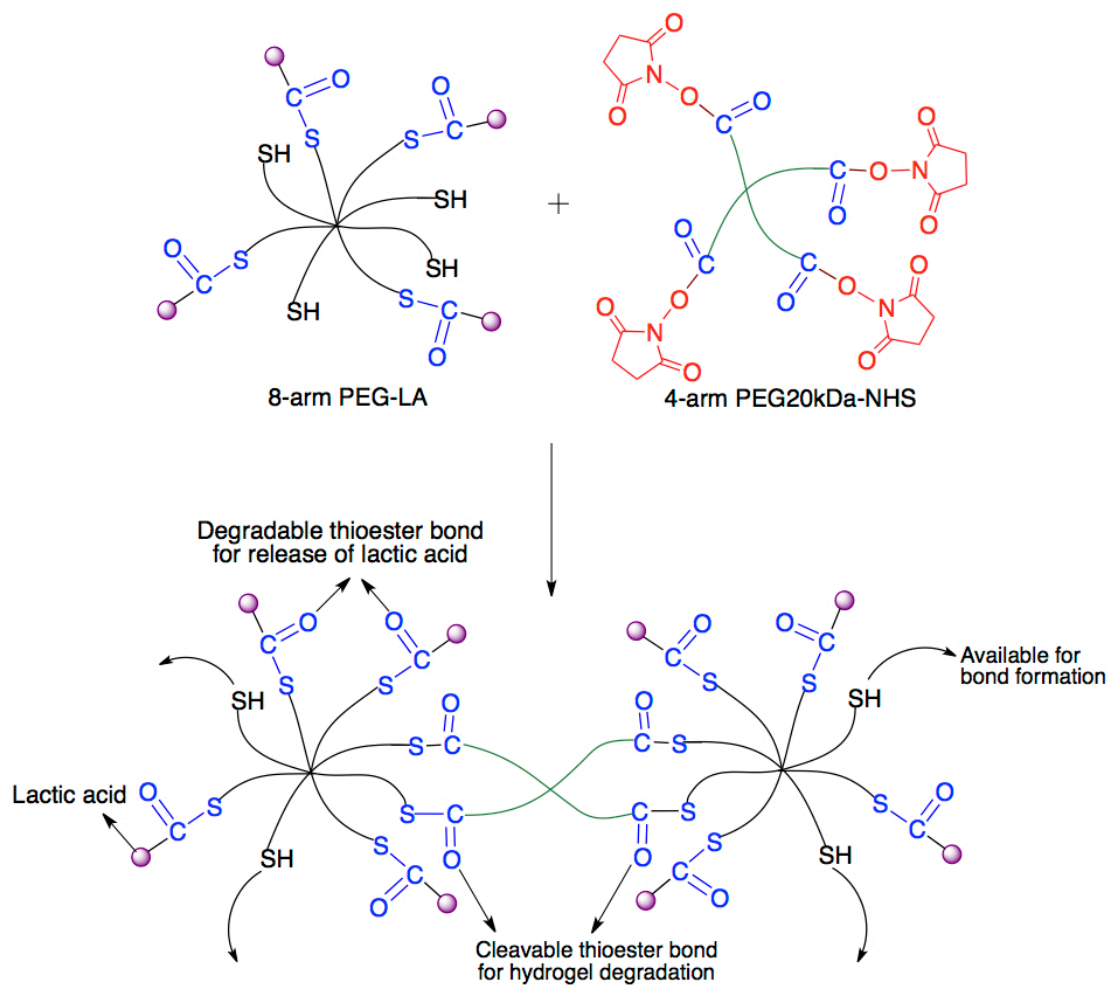


Figure 4.1 Release of lactic acid from PEG-LA nanocarriers, $n=3$, mean \pm SEM. (A) Cumulative release of lactic acid in PBS and AB (pH 4.3). The release was first order with a maximum release of 23% in PBS and 47% in AB. (B) Kinetics of PEG-LA nanocarrier hydrolysis in PBS and AB. The hydrolysis of the PEG-LA nanocarriers was pseudo-first order with a $t_{1/2} = 63.1$ h in PBS and 29.5 h in AB.



Scheme 4.3 Nanocarrier-based hydrogel formation using PEG-LA nanocarriers and 4-arm PEG-NHS. The release of lactic acid and degradation of the hydrogel is via hydrolysis of the thioester bonds.

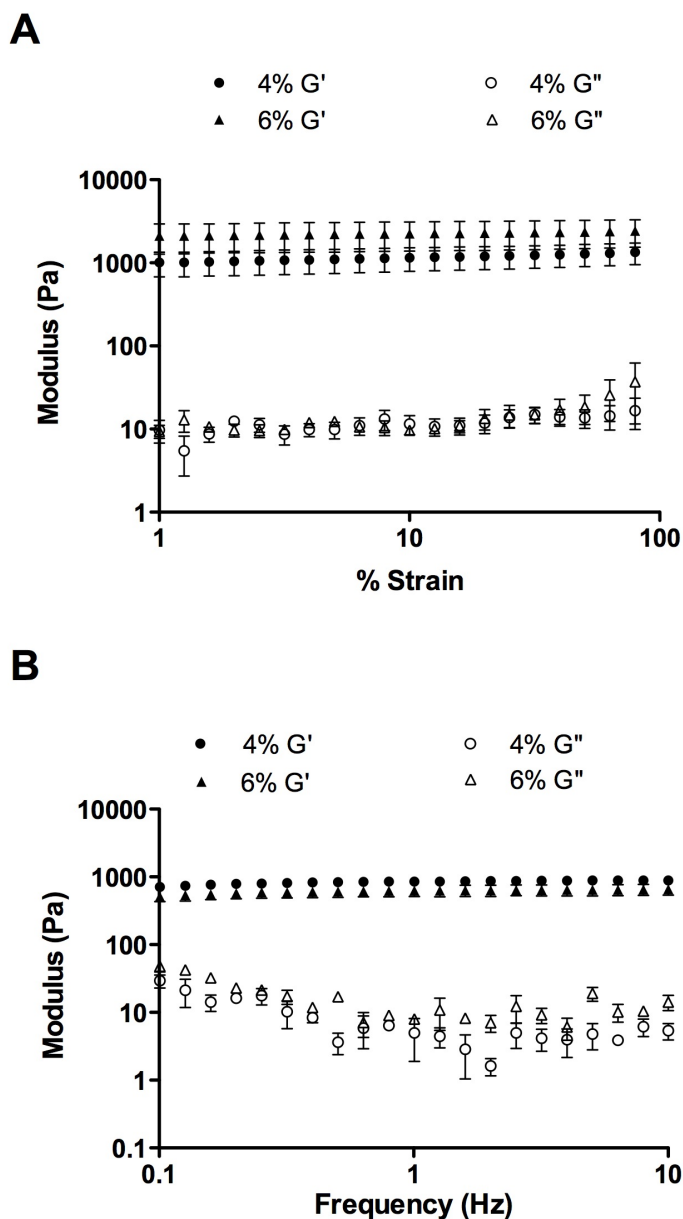


Figure 4.2 Dynamic oscillatory measurements on 4% and 6% w/v nanocarrier-based hydrogels, $n=3$; mean \pm SEM. (A) Strain sweep test for determining the linear viscoelastic regime (LVE). The elastic modulus (G') was greater than the viscous modulus (G'') of the hydrogels over the entire range tested. (B) Frequency sweep test over a range of 0.1-10 Hz, at a constant strain of 1%. The hydrogels showed $G' > G''$ even at the higher applied frequencies.

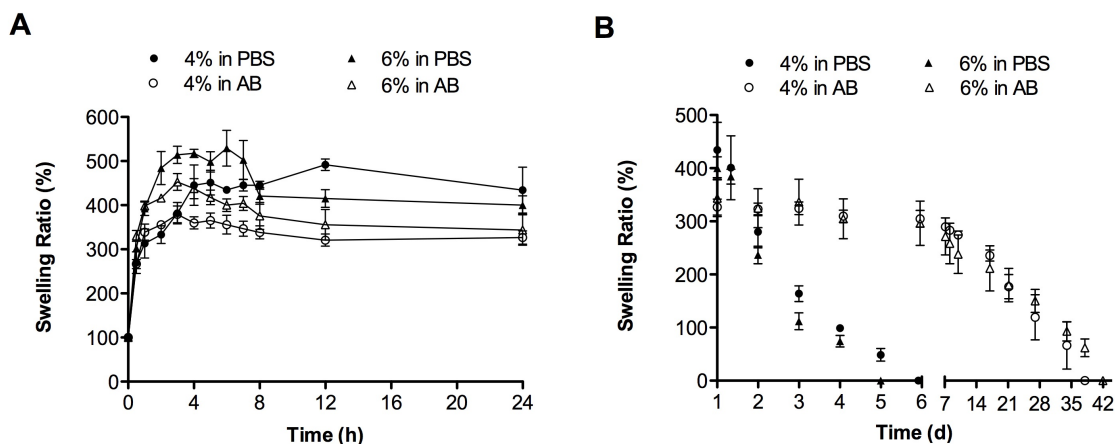


Figure 4.3 Swelling and degradation profiles of nanocarrier based hydrogels in PBS (pH 7.4) and AB (pH 4.3) at 37 °C, n=3; mean±SEM. (A) Swelling ratio of 4% and 6% w/v nanocarrier-based hydrogels in PBS and AB, until equilibrium was reached. (B) Degradation profiles of 4% and 6% w/v hydrogels determined by measuring swelling ratios from equilibrium until the hydrogels completely dissolved into the surrounding medium. The hydrogels degraded completely in 6 days in PBS and 42 days in AB.

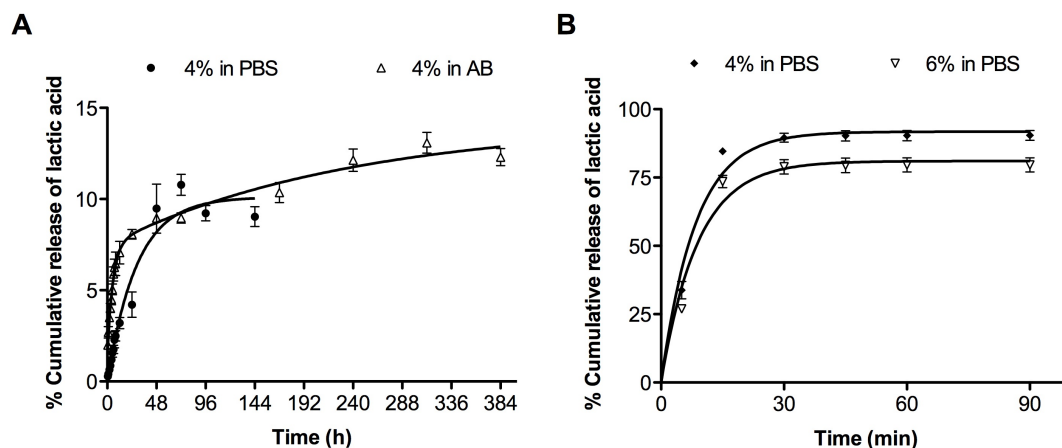


Figure 4.4 Release of lactic acid from (A) nanocarrier-based hydrogels, and (B) hydrogels with passively entrapped lactic acid at 37 °C, $n=3$; mean \pm SEM. (A) The nanocarrier-based hydrogels showed a controlled release of lactic acid with maximum release of 10% in PBS and 14% in AB. (B) The hydrogels with passively entrapped lactic acid showed a burst release of lactic acid in PBS with 90% and 80% lactic acid released from 4% w/v and 6% w/v hydrogels, respectively within 30 min.

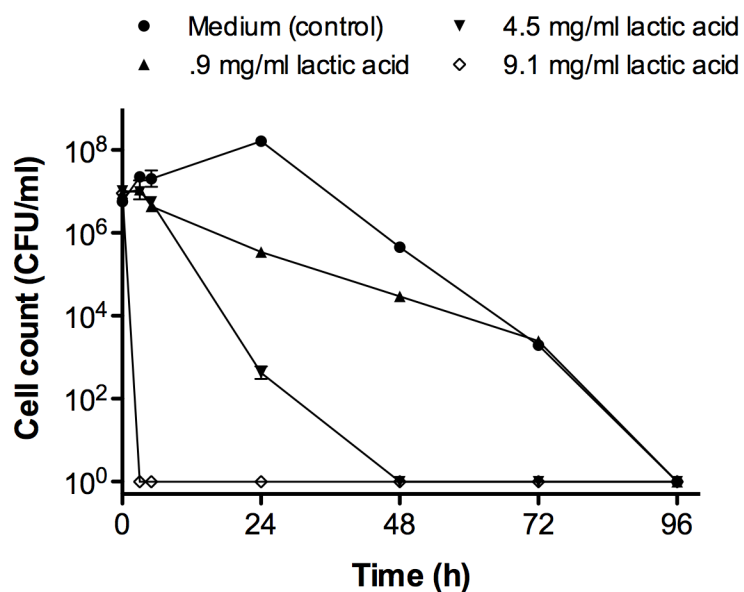


Figure 4.5 Death kinetics of *G. vaginalis* in the presence of lactic acid. *G. vaginalis* was incubated in flasks with medium alone (positive control for growth) or medium with various concentrations of lactic acid (0.9-9.1 mg/ml). The flasks were incubated in the anaerobic chamber at 37 °C cell counts were performed from the flasks at various time points, using the drop plate method. n=2, mean \pm SD.

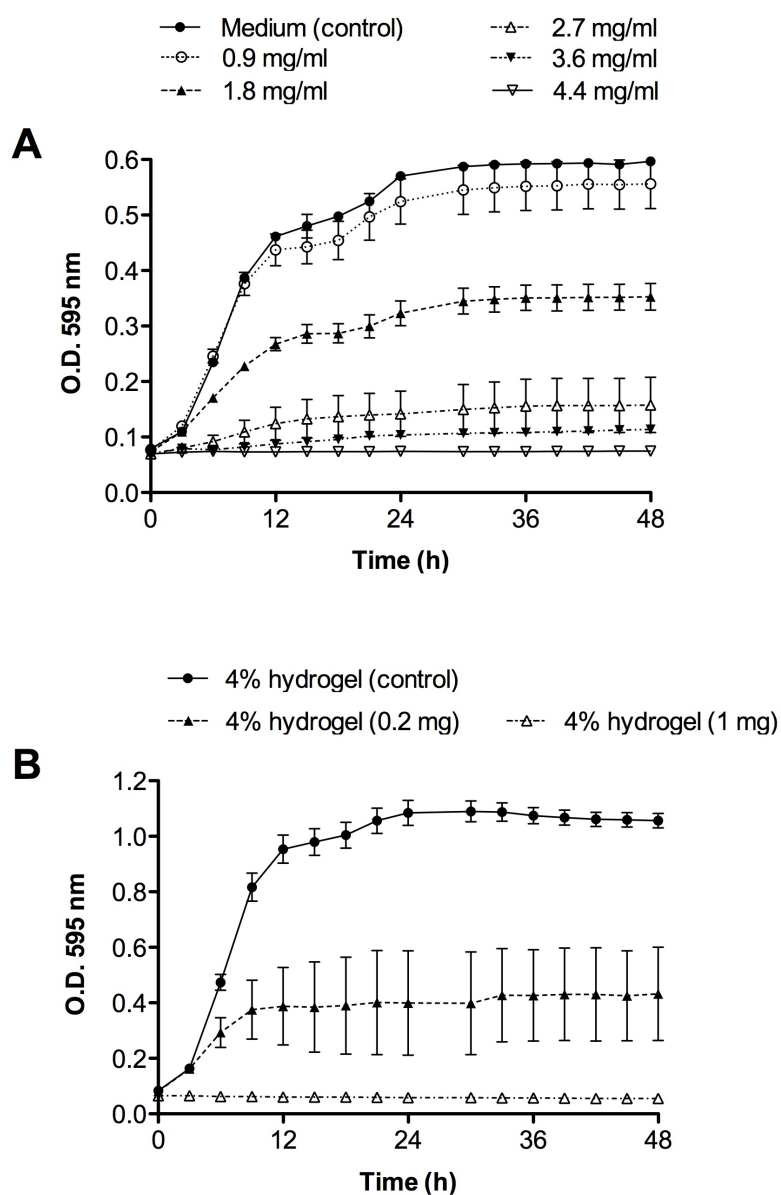


Figure 4.6 Growth kinetics of *G. vaginalis* in the presence of lactic acid (0.9- 4.4 mg/ml), and on hydrogels with passively entrapped lactic acid (0.2 and 1 mg per 50 μ l of hydrogel), $n=3$, mean \pm SEM. The growth kinetics was determined by measuring absorbance every hour, for 48 h (O.D. 595 nm). For the sake of clarity, the readings obtained every 3 h are shown in the above figure. The MIC of lactic acid was found to be 3.6 mg/ml.

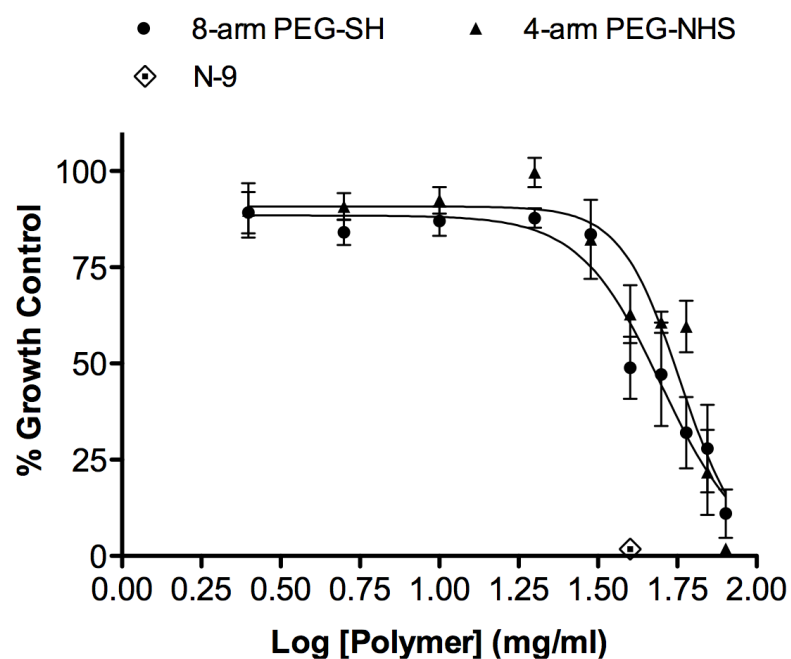


Figure 4.7 Cytotoxicity of the 8-arm PEG-SH and 4-arm PEG-NHS polymers on Vk2 cells, determined using the alamarBlue proliferation assay. Cells were incubated with 8-arm PEG-SH and 4-arm PEG-NHS at concentrations from 2.5- 80 mg/ml, for 24 h. N-9 was used as the positive control for toxicity and medium was used as the negative control. The data are plotted as a percentage of the medium control, $n=6$, $\text{mean} \pm \text{SEM}$. The EC_{50} values for the 8-arm PEG-SH and 4-arm PEG-NHS polymers were found to be 50.24 mg/ml and 57.45 mg/ml respectively.

Supplementary Information

S4.1 Ellman's assay for estimating the amount of lactic acid bound to PEG-LA nanocarriers

Ellman's reagent was prepared by dissolving 4 mg of Ellman's reagent in 1 ml of reaction buffer (0.1 M sodium phosphate, containing 1 mM EDTA, pH 8). The 8-arm PEG-SH polymer and PEG-LA nanocarriers were dissolved in reaction buffer. Ellman's assay was carried out by adding either 8-arm PEG-SH or nanocarrier solution to the reaction buffer, along with Ellman's reagent. The mixture was incubated for 15 min at RT and absorbance measured using Ellman's reagent as the blank (O.D. 410 nm). The free sulfhydryl concentration was obtained by interpolation from a standard curve generated using L-cysteine, and subsequently the concentration of bound lactic acid estimated (Table 1).

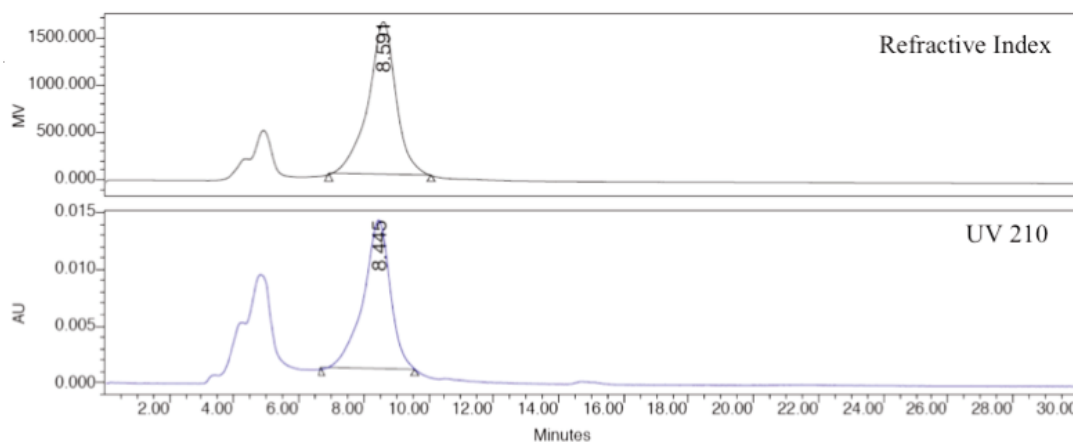
Table S4.1 Estimation of thiol groups in PEG-LA nanocarriers using Ellman's assay; mean \pm SD, n=3

Polymer	Lactic acid ester (eq.)	O.D. 410 nm	Conc. of free thiol (mM)	Conc. of lactic acid (mM)
8-arm PEG _{20kDa} -SH	0	0.991 \pm 0.02 4	0.808 \pm 0.019	-
8-arm PEG _{20kDa} -SH	8	0.484 \pm 0.02 6	0.405 \pm 0.020	0.403 \pm 0.024
8-arm PEG _{20kDa} -SH	0	0.967 \pm 0.12 4	0.789 \pm 0.099	-
8-arm PEG _{20kDa} -SH	8	0.404 \pm 0.05 1	0.341 \pm 0.041	0.448 \pm 0.119

S4.2 Gel permeation chromatography of PEG-LA nanocarriers

Gel permeation chromatography was done using a Waters Breeze system with an Ultrahydrogel 1000 Column (12 μ m, 7.8 x 300 mm). The 8-arm PEG-SH polymer and PEG-LA nanocarriers were injected at a concentration of 2 mg/ml. Water was used as the eluent at a flow rate of 1 ml/min. The GPC profiles of the 8-arm PEG-SH polymer and PEG-LA nanocarriers are shown in Figure S4.2.

A



B

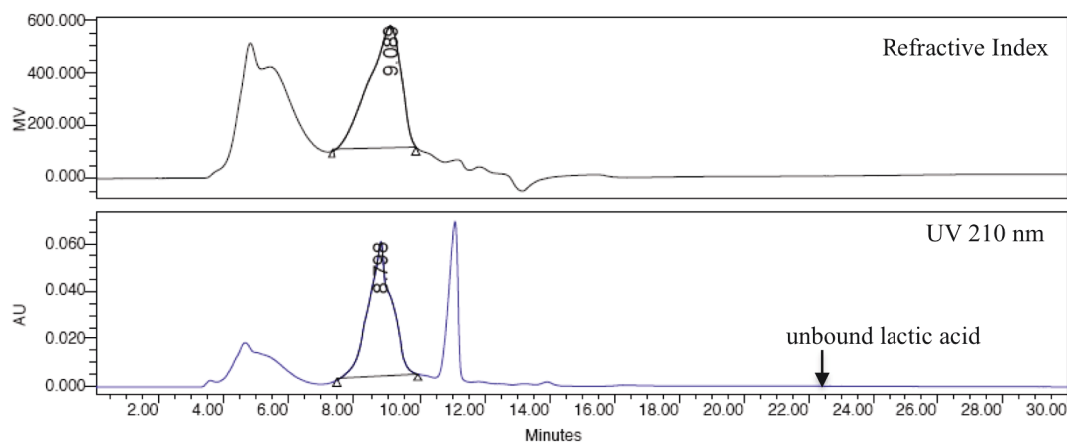


Figure S4.2 GPC profiles of (A) 8-arm PEG-SH polymer (B) PEG-LA nanocarriers.

5. Poly(ethylene glycol) (PEG)-based hydrogels for the controlled release of the antimicrobial subtilisin, for prophylaxis of bacterial vaginosis (BV) and HIV

5.1 Introduction

Bacterial vaginosis (BV) is a common and often recurring vaginal pathology in women of childbearing age [135, 136]. BV is characterized by overgrowth of anaerobic pathogens such as *Gardnerella vaginalis*, *Prevotella*, *Peptostreptococcus* and *Bacteroides spp.* [135, 136]. Women with BV display reduced vaginal lactobacilli *spp.* and consequently high vaginal pH (up to 7.0) [137]. The disrupted vaginal microbiome in women with BV make them highly susceptible to HIV infection [157-160]. Several clinical studies have shown a significant correlation between abnormal vaginal flora, BV and HIV-1 infection [27-29]. A study investigating the association of BV with HIV-1 infection among female sex-workers in Thailand found that among the 43% of participants that were HIV-1 positive, 33% had BV [27]. A similar study in Uganda showed that over half the participants had moderate to severe BV and the rate of HIV-1 infection was considerably higher in women with BV (26.7%) than in women with normal vaginal flora (14.2%) [28].

The current recommended treatment regimen for BV is oral or vaginal administration of metronidazole or clindamycin [161, 162]. However, the

recurrence rate for BV is high with cure rates of only 60-70% after a month of treatment [163-166]. A concern with antibiotic treatments for BV is the development of resistance of the target microbes due to repeated exposure to the same drug. While antimicrobial resistance is not common with metronidazole, recent reports have suggested the development of resistance with clindamycin treatment [167-170]. Prolonged treatment with antibiotics might also result in problems such as systemic toxicity and inhibition of healthy vaginal lactobacilli [171]. Therefore alternative options are being explored for the prophylaxis and treatment of BV such as oral/vaginal administration of probiotics and vaginal acidification [172-176]. In addition, vaginal microbicide formulations designed for the prevention of HIV are currently being examined for their inhibitory effect on BV-associated pathogens due to the significant correlation between BV and HIV incidence [177-179]. The Phase I safety trial of a microbicide candidate, BufferGel showed a reduction in the incidence of BV from 30% at enrollment to 6% at one week [177]. Another study examining the effect of polystyrene sulfonate (T-PSS) and cellulose sulphate (Ushercell) on BV-associated bacteria showed that these compounds inhibit several anaerobes commonly associated with BV [179]. Currently, a Phase 3 trial for assessing the efficacy of 1% SPL7013 Gel (VivaGel) for the treatment of BV is underway (ClinicalTrials.gov Identifier NCT01577537).

Recently, bacteriocins have been suggested as potential alternatives to antibiotics for the treatment of BV [180-185]. Unlike broad-spectrum antibiotics, bacteriocins selectively target pathogenic microorganisms without disturbing

healthy vaginal flora. One such bacteriocin subtilosin A (referred to hereafter as subtilosin) has demonstrated antimicrobial activity against BV-associated pathogens including *Gardnerella vaginalis* [183, 186, 187]. Subtilosin is a cyclic anionic peptide produced by both *Bacillus subtilis* and *Bacillus amyloliquifaciens* [182, 188, 189]. Torres et al. have recently shown that subtilosin also has antiviral activity against herpes simplex virus type 1 (HSV-1) [190]. The antimicrobial and antiviral properties of subtilosin make it a good candidate for development as a vaginal microbicide for the prophylaxis of BV and potentially HIV. Therefore, the feasibility of incorporating subtilosin in poly(ethylene glycol) (PEG)-based hydrogels for vaginal administration is investigated in this study. In addition, the inhibitory effect of subtilosin on HIV-1 is examined given its demonstrated *in vitro* antiviral activity against HSV-1.

In the current study, PEG-based hydrogels were developed for the sustained release of subtilosin. The hydrogels are formed *in situ* by the covalent cross-linking of 8-arm PEG-SH and 4-arm PEG-NHS polymers. Subtilosin is incorporated into the hydrogels by passive entrapment within the polymer matrix. The release profile of subtilosin from the hydrogels was investigated in physiological conditions. The antimicrobial activity of the subtilosin-containing hydrogels on the growth of the predominant BV pathogen *G. vaginalis* was evaluated using an endpoint assay. The effect of the subtilosin-containing hydrogels on the growth of four reference strains of healthy human vaginal lactobacilli was also examined. The inhibitory effect of subtilosin on HIV-1 was evaluated by conducting a virucidal assay using the MT-2 cell line.

5.2 Materials and Methods

5.2.1 Materials

The polymers 8-arm PEG-SH (20 kDa) and 4-arm PEG-NHS (20 kDa) were obtained from NOF Corporation (White Plains, NY). The micro-BCA protein assay kit was obtained from Thermo Fisher Scientific Inc. (Rockford, IL) and the Bio-Rad protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). Clindamycin was obtained from Tokyo Chemical Industry (Tokyo, Japan). The p24 protein assay kit was obtained from Advanced BioScience Laboratories, Inc. (Kensington, MD).

5.2.2 Production of subtilisin

Subtilisin was isolated and purified through fermentation of *Bacillus amyloliquefaciens* KATMIRA1933, as previously described [182, 186]. Briefly, the cell-free supernatant was collected, filter-sterilized and then purified using ammonium sulfate (30%) precipitation and affinity chromatography with a C18 column. The concentration of subtilisin determined using the micro-BCA protein assay kit (as per the manufacturer's instructions) was found to be 3.14 mg/ml. The subtilisin solutions were stored in sterile distilled water at 4 °C, until further use.

5.2.3 Stability of subtilisin

The stability of subtilisin was determined at pH 7.4 and 9.0 using the following procedure. Aqueous subtilisin solutions were lyophilized using a centrifugal evaporator and re-suspended in sodium phosphate buffer (PB, 20 mM, pH 7.4 and 9.0). The solutions were then diluted to 200 µg/ml in the appropriate buffer and incubated at 37 °C. Aliquots were withdrawn at pre-determined time intervals over a period of two weeks and subtilisin concentration was analyzed by HPLC using a Waters XSELECT™ HSS T3 2.5 µm (3.0 x 50 mm) column. The mobile phase consisted of water with 0.05% TFA (solvent A) and acetonitrile with 0.05% TFA (solvent B). A gradient from 5% to 100% B was applied over 6.5 min, at a flow rate of 0.5 ml/min. The subtilisin concentration at each time point was expressed as a percentage of the initial concentration (t=0 min) and plotted over time. The experiment was performed in triplicate and the data expressed as mean±SEM.

5.2.4 Preparation of PEG-based hydrogels with subtilisin

The PEG-based hydrogels were prepared by passively entrapping subtilisin within the polymer matrix. Hydrogels were prepared by mixing varying amounts of 8-arm PEG-SH (4%, 6% and 8%; w/v) with 2 equiv. of 4-arm PEG-NHS and subtilisin in PB (pH 7.4) at RT. The amount of subtilisin loaded into the hydrogels was verified by Bio-Rad protein assay. The time of formation of the hydrogels was determined using the “inverted tube method” and was noted as the time when the solution ceased to flow, upon inversion of the tube [120-122].

5.2.5 Release of subtilisin from hydrogels

The release of subtilisin from the hydrogels was determined in phosphate buffered saline (PBS, 10 mM, pH 7.4). The hydrogels with passively entrapped subtilisin were placed in vials and immersed in PBS. The vials were incubated at 37 °C on an orbital shaker. At pre-determined time intervals, the PBS was removed and replaced with an equal volume of PBS in order to maintain sink conditions throughout the study. The amount of subtilisin in the release medium was determined using the Bio-Rad protein assay as per the manufacturer's instructions (O.D. 595 nm). The cumulative release of subtilisin was expressed as a percentage and plotted over time (n=3, mean±SEM).

5.2.6 Bacterial strains and growth conditions

Gardnerella vaginalis ATCC 14018 was the reference BV associated strain used in these studies. The cells were stored at -80°C in Brain Heart Infusion (BHI) medium (Difco, Sparks, MD) supplemented with 3% horse serum (JRH Biosciences, KS) and 15% glycerol. For *in vitro* studies, cells from frozen stocks were cultured on human blood bilayer-Tween (HBT) agar (Remel, Lenexa, KS) and grown at 37 °C in 5% CO₂ and 2.5% H₂ for 48 h using EZ Anaerobe Container System GasPaks (Becton, Dickinson and Co., Sparks, MD). Colonies were inoculated in BHI medium supplemented with 3% horse serum for 24-48 h and then serially diluted and plated until counts were observed at 10⁸ CFU/ml. These cells were then subcultured at least twice before use. Briefly, the overnight culture was transferred to fresh BHI medium supplemented with 3% horse serum

and incubated anaerobically at 37°C. All media and agar were pre-incubated for at least 24 h in the above-mentioned anaerobic conditions to remove oxygen-associated stress.

The four reference strains of healthy human lactobacilli used in this study are *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus gasseri* ATCC 33323, *Lactobacillus plantarum* ATCC 39268 and *Lactobacillus vaginalis* ATCC 49540. The lactobacilli *spp.* were stored at -80°C in DeMan, Rogosa and Sharpe (MRS) broth (Oxoid, Hampshire, England) containing 15% glycerol. The cells were cultured on MRS agar and grown aerobically at 37°C. Single colonies were inoculated in MRS broth and grown aerobically for 24 h with agitation. These cells were subcultured at least twice before use. Briefly, the overnight culture was transferred to fresh MRS broth and incubated aerobically at 37°C.

5.2.7 Growth of *G. vaginalis* on subtilisin-containing hydrogels

The growth of *G. vaginalis* on hydrogels with varying amounts of passively entrapped subtilisin was evaluated by endpoint analysis. Hydrogels (4%, w/v) with varying amounts of subtilisin (8, 12, 15 and 20 µg per 50 µl of gel) were prepared in a 96-well plate (n=3, per condition). In addition, hydrogels with no entrapped subtilisin were prepared in order to evaluate the effect of the hydrogel alone on *G. vaginalis* growth. *G. vaginalis* (10⁸ CFU/ml) was added to the wells (200 µl/well) and incubated in anaerobic conditions at 37 °C for 48 h. Medium alone was used as the positive control and clindamycin (100 µg/ml) was used as the negative control for growth. Following the incubation period, growth was

evaluated by measuring turbidity (O.D. 595) and by performing cell counts using the drop plate method [183, 191]. Cells were extracted from the wells with hydrogels by slicing the gels open. The cell counts were performed from two wells per condition (duplicate counts per well). *G. vaginalis* growth in the presence of subtilisin alone (without hydrogel) was evaluated by incubation in the same medium supplemented with pre-determined concentrations (8-20 µg/ml) of subtilisin using the procedure described above.

5.2.8 Growth of lactobacilli spp. in the presence of subtilisin-containing hydrogels

The growth kinetics of four strains of vaginal lactobacilli (*L. acidophilus*, *L. gasseri*, *L. plantarum* and *L. vaginalis*) in the presence of subtilisin, and subtilisin-containing hydrogels was evaluated as follows: hydrogels (4% w/v) with subtilisin (8, 12, and 20 µg per 50 µl of hydrogel) and subtilisin solutions (50 µl/well in PB, final concentrations of 8- 20 µg/ml per well) were prepared in a 96-well plate. In addition, hydrogels with no entrapped subtilisin were prepared in order to evaluate the effect of the hydrogel alone on *lactobacilli* growth. The selected lactobacilli spp. were added to the wells (200 µl/well) and incubated in anaerobic conditions at 37 °C for 48 h. Medium alone was used as the positive control and clindamycin (100 µg/ml) was used as the negative control for growth. The growth kinetics of *lactobacilli* spp. was evaluated by measuring absorbance (O.D. 595) every hour, for 48 h. The data are represented as the mean±SEM of two experiments performed in triplicate (a total of n=6).

5.2.9 Inhibition of HIV-1

The inhibitory effect of subtilisin on HIV-1 was determined by performing a virucidal assay using MT-2 cells. HIV-1_{IIIB} (m.o.i= 0.03) was pre-incubated with varying concentrations of subtilisin for 90 min and then MT-2 cells (5×10^3 cells/well) were added and further incubated for a period of 5 days. The final concentration of subtilisin in the wells after the addition of MT2 cells ranged from 3-24 μ M (10-80 μ g/ml). Cells with medium alone were used as the control. At the end of the incubation period, HIV-1 replication was assessed using the p24 assay. In addition, cytotoxicity was assessed using MTT assay. The inhibitory concentration of subtilisin that reduced HIV-1 replication by 50% (IC_{50}) was determined by fitting the data with a sigmoidal-dose response curve:

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(LogEC_{50} - X)HillSlope}} ; \text{ where } Bottom = 0$$

The cytotoxic concentration of subtilisin that reduced cell viability by 50% (CC_{50}) was also similarly determined by fitting the data with the dose response curve mentioned above.

5.3 Results and Discussion

5.3.1 Stability of subtilisin

Subtilisin is a cyclic peptide with a molecular weight of 3.4 kDa with unique post-translational modifications (Scheme 5.1) [192]. Subtilisin is a relatively hydrophobic peptide with an isoelectric point (pI) of 4.03. Since our goal was to incorporate subtilisin within PEG-based hydrogels prepared in buffer at or close

to physiological pH (7.4), the stability of subtilisin was investigated at pH 7.4 and 9.0 at 37 °C. Subtilisin solutions (200 µg/ml) were prepared in PB at pH 7.4 and 9.0 and incubated at 37 °C. Subtilisin did not completely dissolve in buffers over a pH range of 4.0- 5.0 since its pI value is within this range. Hence its stability was not investigated under these pH conditions. The concentration of subtilisin was determined at various time points over a period of 2 weeks using HPLC. A change in subtilisin concentrations was not observed indicating that subtilisin is stable under these conditions (Figure 5.1). Understanding the stability of peptide drugs is especially important because peptides often undergo conformational changes, hydrolysis and oxidation when incorporated in a formulation [193, 194]. This can affect the activity of the peptide and thus the efficacy of the formulation. The stability of subtilisin at pH 7.4-9.0 allows the possibility of formulating the peptide over this range of pH.

5.3.2 Preparation of PEG-based hydrogels and release profile of subtilisin

The PEG-based hydrogels were prepared by cross-linking the 8-arm PEG-SH and 4-arm PEG-NHS polymers via thioester bonds. Subtilisin was loaded into the hydrogels by passive entrapment within the polymer matrix (Scheme 5.2). The concentration of subtilisin was determined using both micro-BCA assay and Bio-Rad protein assay (based on the Bradford method) before incorporation in the hydrogels. The micro-BCA assay was found to interact better with subtilisin given the anionic nature of the peptide. However, unlike the Bio-Rad protein assay, the micro-BCA assay was found to interfere with the PEG-SH

polymer. Hence, the Bio-Rad protein assay was used to determine subtilisin concentration before incorporation into hydrogels and in the release medium. The hydrogels were prepared by mixing 8-arm PEG-SH (4%, 6% and 8%; w/v) with 2 equiv. of 4-arm PEG-NHS and subtilisin in PB (pH 7.4) at RT. The hydrogels formed within 30 min and increasing the polymer concentration did not significantly alter the gelation time (Table 5.1).

The release of subtilisin from the hydrogels (4% and 6%, w/v) was determined in PBS (10 mM, pH 7.4) at 37 °C. The amount of subtilisin released at each time point was determined using the Bio-Rad protein assay and percentage cumulative amount released plotted over time. The release of subtilisin from the hydrogels was two-phase with an initial rapid release phase (47% and 42% release in 24 h from the 4% and 6% w/v hydrogels, respectively), followed by a slow sustained release phase (Figure 5.2).

5.3.3 Growth of *G. vaginalis* on hydrogels with subtilisin

The antimicrobial activity of subtilisin against *G. vaginalis* was previously investigated and a minimum inhibitory concentration (MIC) of 7.2-9.2 µg/ml was observed depending on the growth conditions [183, 186]. The MIC of subtilisin for the growth conditions used in our study was therefore determined and observed to be 12 µg/ml.

The inhibitory effect of the hydrogels prepared with varying amounts (8-20 µg per 50 µl of hydrogel) of entrapped subtilisin on *G. vaginalis* growth was determined using endpoint analysis. Growth was evaluated by measuring absorbance (O.D.

595 nm) and by performing cell counts before and after the incubation period of 48 h. Inhibition of *G. vaginalis* growth was observed on hydrogels containing 12-20 µg of subtilisin with absorbance values comparable to the negative control (clindamycin) (Figure 5.3). Cell counts indicated partial inhibition (reduction in cell counts from 10^8 to 10^5 CFU/ml) of *G. vaginalis* growth on hydrogels with 12 µg of subtilisin and complete inhibition on hydrogels with 15 and 20 µg of subtilisin (Table 5.2).

5.3.4 Growth of lactobacilli spp. in the presence of subtilisin-containing hydrogels

The growth kinetics of four vaginal lactobacilli spp. (*L. acidophilus*, *L. gasseri*, *L. plantarum* and *L. vaginalis*) in the presence of subtilisin (8-20 µg/ml), and subtilisin-containing hydrogels (8-20 µg per 50 µl of hydrogel) were evaluated. The growth kinetics was determined by measuring absorbance every hour, over 48 h (O.D. 595 nm). No inhibition of the lactobacilli spp. was observed in medium with subtilisin (Figure 4). This is consistent with the earlier observed values of MIC for subtilisin (>100 µg/ml) against *L. vaginalis*, *L. gasseri* and *L. plantarum* [186]. A slight decrease in lactobacilli growth was observed on the hydrogels (33% reduction in O.D.), compared to the medium control (Figure 5.4).

5.3.5 Inhibitory effect of subtilisin on HIV-1

The inhibitory effect of subtilisin on HIV-1 was determined by performing a virucidal assay using MT-2 cells. HIV-1_{BaL} (m.o.i= 0.03) was pre-incubated with

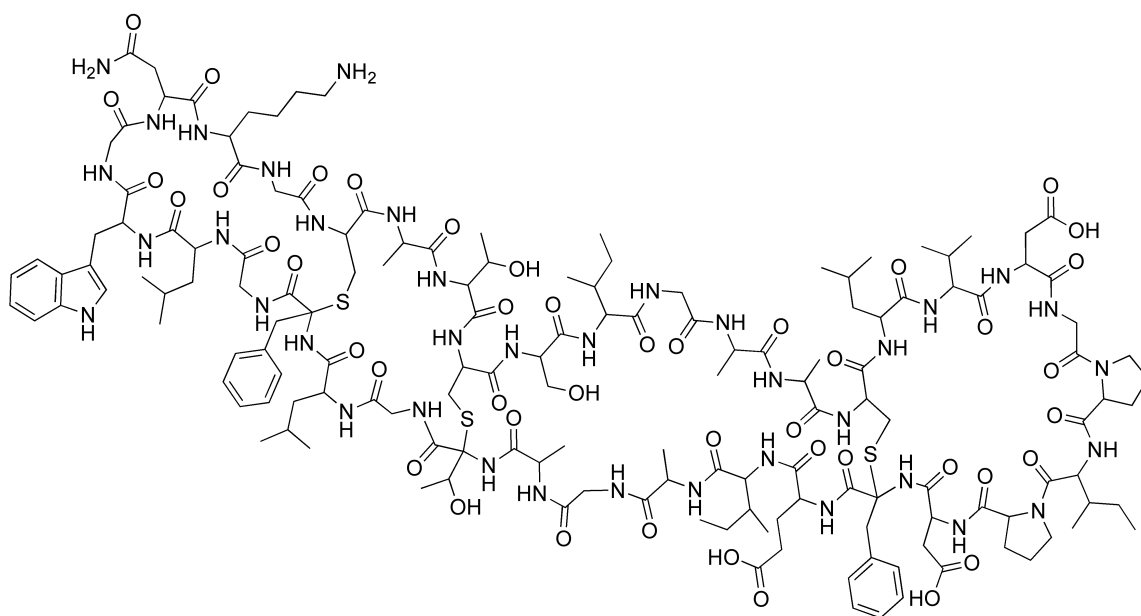
varying concentrations of subtilisin for 90 min, following which MT-2 cells were added to wells. HIV-1 replication was assessed after a further incubation period of 5 days using the p24 assay. The cytotoxicity of subtilisin to MT-2 cells was determined using the MTT assay. Subtilisin was found to inhibit HIV-1 with a 50% reduction in HIV-1 replication (IC_{50}) at a concentration of 5.8 μ M (19.7 μ g/ml) (Figure 5.5A). The MTT assay indicated a 50% decrease in cell viability (CC_{50}) at 19.8 μ M (67.3 μ g/ml) (Figure 5.5B).

Recently, natural antimicrobial proteins and peptides from vaginal fluid have been identified to have anti-HIV activity [148, 195, 196]. Furci et al. have shown that among these classes of peptides, α -defensin-5 binds to the HIV-1 envelope with an IC_{50} of 3.67 μ M (for R5 virus) [197]. Another class of cyclic antimicrobial peptides θ -defensins, which are found in non-human primates have antiviral properties against HIV-1 [198]. Synthetic human θ -defensins known as retrocyclins (cyclic 18 res. peptides) are currently being explored for use in microbicides due to their anti-HIV activity [199, 200]. The retrocyclin RC-101 has been shown to inhibit HIV-1 at concentrations of 10-20 μ g/ml [199]. In the current study, subtilisin was found to directly inactivate HIV-1 and inhibit viral replication with an IC_{50} (19.7 μ g/ml), which is close to the observed range of inhibitory concentrations for defensins. In addition, subtilisin has antiviral activity against HSV-1 with an IC_{50} of 9.6 μ g/ml [190]. The activity of subtilisin against multiple bacterial and viral vaginal pathogens associated with HIV infection suggests the potential application of subtilisin-containing hydrogels for BV and HIV prophylaxis.

5.4 Conclusion

In this study the feasibility of incorporating the antimicrobial peptide subtilisin in PEG-based hydrogels for vaginal drug delivery was demonstrated. The hydrogels with passively entrapped subtilisin showed a two-phase release of subtilisin with an initial rapid phase (48% release in 24 h), followed by a sustained release phase over several days. Subtilisin released from the hydrogels retained activity against the primary BV pathogen *G. vaginalis*. The hydrogels not significantly affect the growth of four strains of healthy human vaginal lactobacilli *spp.* In addition, subtilisin was found to inhibit HIV-1 *in vitro* with an IC_{50} of 5.8 μ M. Thus, the above results indicate potential application of PEG-based hydrogels with subtilisin as vaginal microbicides for BV and HIV prophylaxis.

TABLES AND FIGURES



Scheme 5.1 Structure of the cyclic peptide subtilisin A. Three cross-links exist between the cysteine residues and the two phenylalanines and threonine residues.

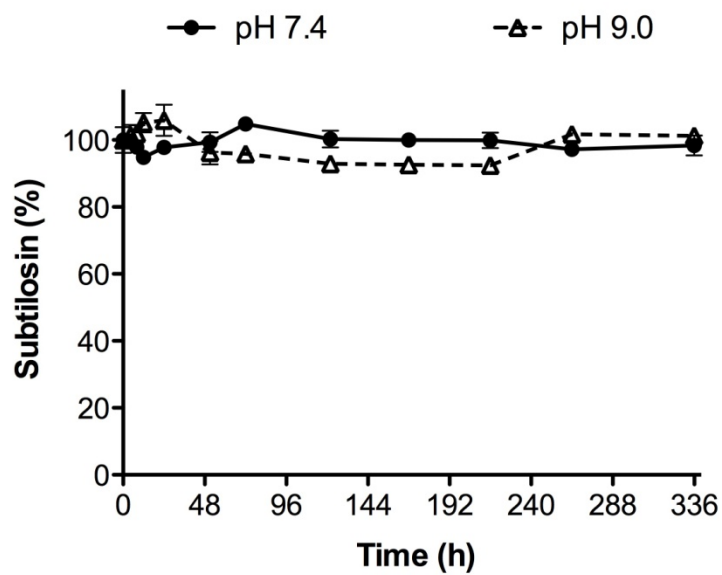
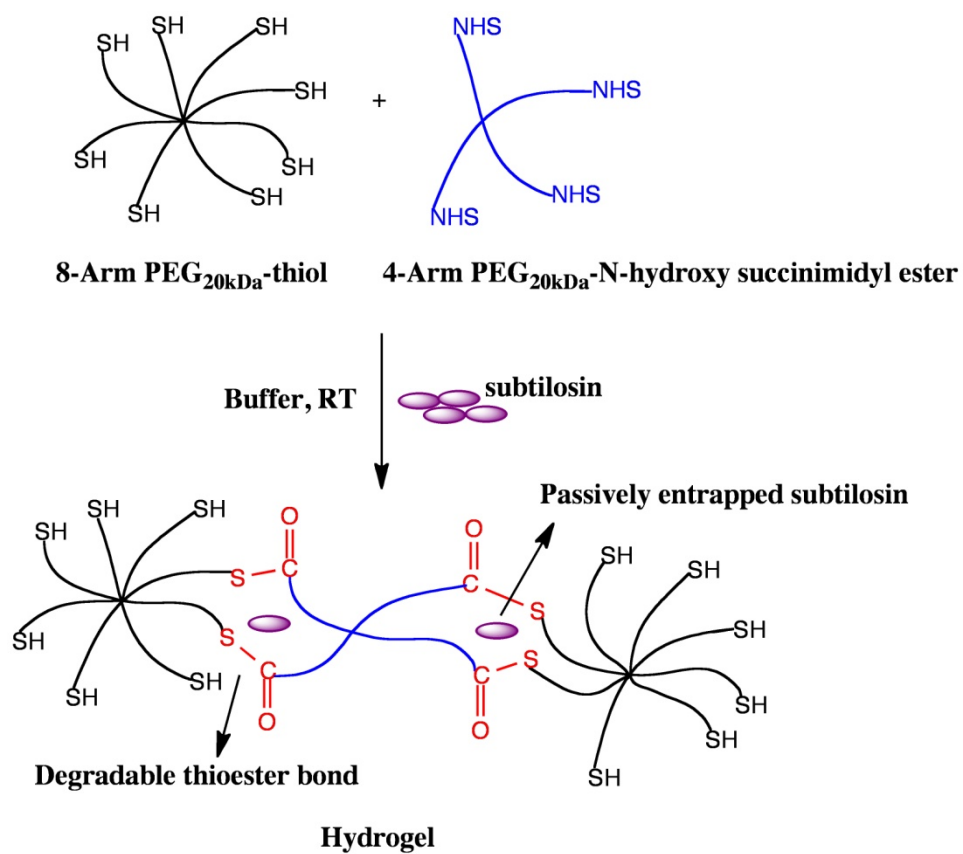


Figure 5.1 Stability of subtilisin at 37 °C in PB (pH 7.4 and 9.0); mean \pm SEM, n=3.

Subtilisin concentration at each time point was determined using HPLC. Subtilisin was found to be stable under both pH conditions over a period of two weeks.



Scheme 5.2 Schematic representation of subtilisin-containing hydrogel formation using 8-arm PEG-SH and 4-arm PEG-NHS polymers

Table 5.1 Time of formation of hydrogels with passively entrapped subtilisin; mean \pm SD, n=3

8-arm PEG-SH (mg/0.05 ml)	4-arm PEG-NHS (mg/0.05 ml)	Time of hydrogel (0.1 ml) formation (min)
4	8	26.7 \pm 1.6
6	12	25.6 \pm 0.3
8	16	25.6 \pm 1.1

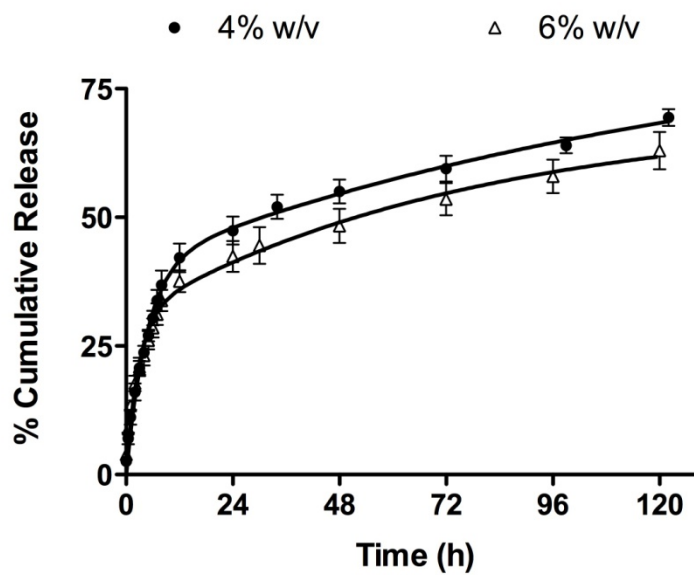


Figure 5.2 Release of subtilisin from 4% w/v and 6% w/v PEG-based hydrogels in PBS at 37 °C, n=3; mean±SEM. The release of subtilisin from the hydrogels was two-phase, with an initial rapid release phase (47% and 42% release in 24 h from the 4% and 6% w/v hydrogels, respectively), followed by a slow sustained release phase. The average release rate for the first 12 h was 3.96 and 4.04 $\mu\text{g/hr}$ for the 4% and 6% w/v hydrogels, respectively. The average release rate from 12-120 h was 0.28 and 0.24 $\mu\text{g/hr}$ for the 4% and 6% w/v hydrogels, respectively.

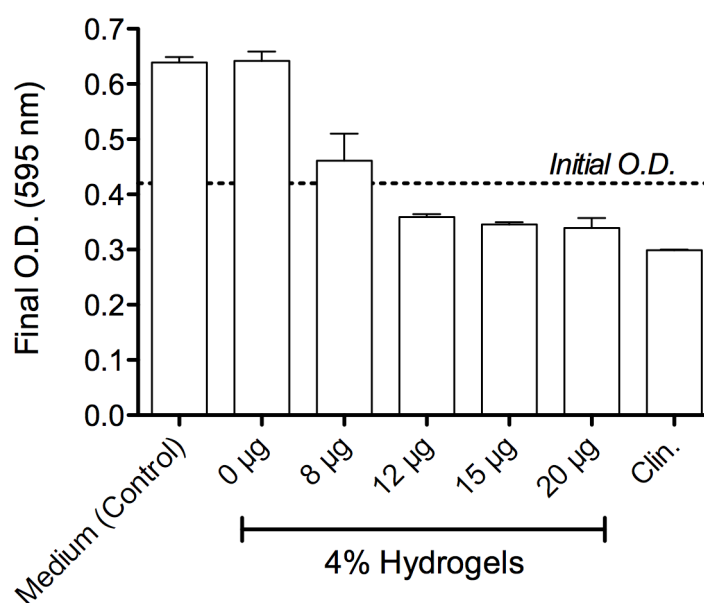


Figure 5.3 Growth of *G. vaginalis* on hydrogels with varying concentration of subtilisin, determined by endpoint analysis, $n=3$, mean \pm SEM. *G. vaginalis* was plated on wells containing 4% w/v hydrogels with 8-20 μ g entrapped subtilisin (per 50 μ l of gel). The plate was incubated at 37 °C for a period of 48 h. Medium was used as the positive control for growth and clindamycin (100 μ g/ml) was used as the negative control.

Table 5.2 Final cell counts of *G. vaginalis* on hydrogels with subtilisin (n=2, mean±SD)

Condition	Final cell counts (CFU/ml)
Medium (Control)	$0.97 \pm 0.06 \times 10^8$
Hydrogel (0 µg)	$1.02 \pm 0.01 \times 10^8$
Hydrogel (8 µg)	$4.9 \pm 0.35 \times 10^6$
Hydrogel (12 µg)	2.33×10^5
Hydrogel (15 µg)	0
Hydrogel (20 µg)	0
Clin. (100 µg/ml)	0

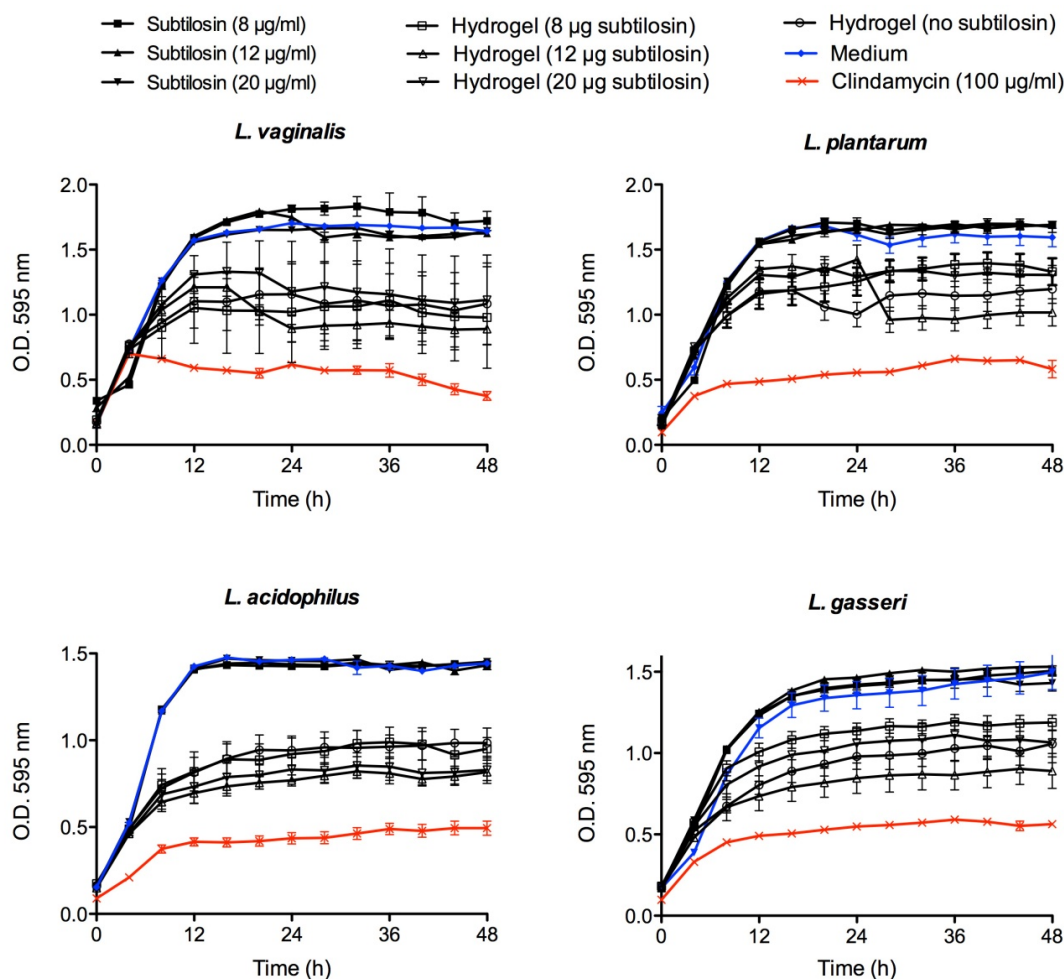


Figure 5.4 Growth kinetics of lactobacilli spp in medium with various concentrations (8-20 µg/ml) of subtilisin, and in the presence of 4% w/v hydrogels with 8-20 µg passively entrapped subtilisin (per 50 µl of gel), n=3, mean±SEM. The growth kinetics was determined by measuring absorbance every hour, for 48 h (O.D. 595 nm). For the sake of clarity, the readings obtained every 4 h are shown in the above figure.

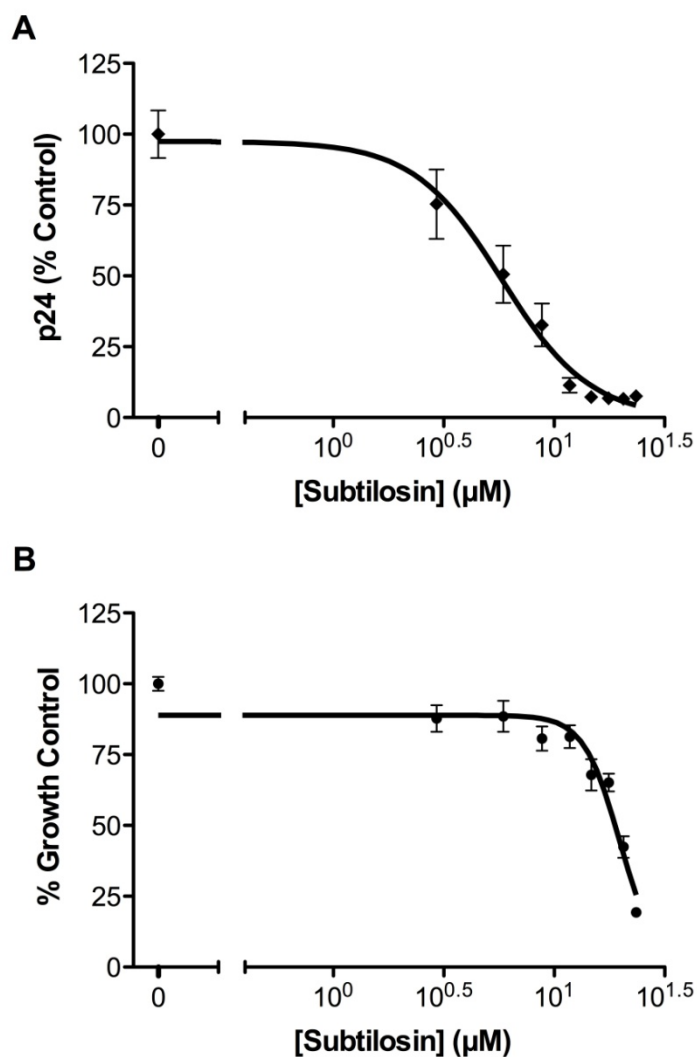


Figure 5.5 (A) Inhibitory effect of subtilosin on HIV-1, determined using p24 assay; mean \pm SEM, $n = 18$. The concentration of subtilosin that inhibits p24 production by 50% (IC₅₀) was found to be 5.8 μ M. (B) Cytotoxicity of subtilosin on MT-2 cells, determined using MTT assay; mean \pm SEM, $n = 12$. A 50% decrease in cell viability (CC₅₀) was observed at 19.8 μ M.

6. Conclusions and Future Directions

6.1 Conclusions

The current work explores the feasibility of using PEG-based hydrogels for sustained vaginal drug delivery. PEG-based hydrogels formed via degradable thioester bonds were developed and their properties examined, with the end goal of using the hydrogels as vaginal microbicides for the prevention of HIV transmission. Infection with bacterial vaginosis (BV) has been shown to significantly increase the risk of vaginal HIV acquisition due to altered vaginal flora and reduced vaginal acidity. Therefore, PEG-based hydrogels for the controlled release of lactic acid and the antimicrobial peptide subtilisin were developed, both of which will prevent and treat BV and thus reduce the risk of HIV infection. In addition, the antiviral effects of subtilisin against HIV were investigated given its antiviral activity against HSV-1.

There is currently a lack of animal models for quantitatively analyzing the *in vivo* performance of vaginal microbicides prior to clinical trials. Therefore, an MRI method for evaluating the coverage, distribution and retention of the hydrogels in the vagina of mice was developed. The PEG-based hydrogels showed a significantly longer retention time in mice (12-24 h) when compared to hydroxyethylcellulose (HEC) gel (2 h) indicating that the hydrogels might be an improved platform for vaginal drug delivery over existing semi-solid gel formulations. The increased retention time of the hydrogels was found to

correlate with their superior viscoelastic properties when compared to the HEC gel.

PEG nanocarrier-based hydrogels were developed and evaluated for the controlled release of lactic acid in the vagina. The nanocarrier-based hydrogels provided sustained release of lactic acid for several hours via thioester hydrolysis and prevented the burst release of lactic acid seen with passive entrapment. Thus nanocarrier-based hydrogels are a good strategy for achieving sustained vaginal drug delivery. In addition, lactic acid demonstrated antimicrobial activity against the predominant BV pathogen *G. Vaginalis* (MIC= 3.6 µg/ml).

PEG-based hydrogels with passively entrapped subtilisin provided a two-phase release of subtilisin with an initial rapid phase (release rate of 4 µg/hr), followed by a slow sustained release phase (release rate of 0.25 µg/hr). Subtilisin-containing hydrogels inhibited the growth of *G. vaginalis* but did not significantly inhibit healthy vaginal lactobacilli *spp*. In addition, subtilisin was found to weakly inhibit HIV-1 replication with an IC₅₀ of 5.8 µM (19.6 µg/ml). The multiple inhibitory effects of subtilisin against BV-associated pathogens, HSV-1 and HIV suggest its potential application for BV and HIV prophylaxis.

6.2 Future Directions

6.2.1 Evaluating the *in vivo* performance of various vaginal microbicide formulations in mice using MRI

In this thesis, a pre-clinical mouse model for quantitatively evaluating the *in vivo* performance of vaginal gels using MRI was developed. This model was then used to evaluate the vaginal coverage, distribution and retention of PEG-based hydrogels and HEC gel. The hydrogels, which have a higher elastic modulus than the HEC gel showed significantly longer vaginal retention. A logical next step would be to use this model for evaluating the *in vivo* performance of various microbicide formulations and understanding how the viscoelastic properties of the formulations influence vaginal retention. It would be particularly interesting to examine formulations made with varying amounts of polyacrylic acid (Carbopol®) polymers, which have a range of elastic moduli between the HEC gel and PEG-based hydrogels [53, 201].

6.2.2 Increasing the lactic acid loading of PEG nanocarrier-based hydrogels

The PEG-LA nanocarriers developed in this thesis were prepared by covalent attachment of lactic acid to 8-arm PEG-SH polymer via thioester bonds. The nanocarrier-based hydrogels provided sustained release of lactic acid when compared to hydrogels with passively entrapped lactic acid. However, only a small fraction of bound lactic acid (10-14%) was released possibly due to steric hindrance of the polymer chains in the covalently cross-linked hydrogel. One way of increasing the lactic acid loading of the nanocarriers would be to attach polylactic acid to the 8-arm PEG-SH polymer. Nanocarriers with lactic acid oligomers attached to each arm of the PEG-SH polymer might provide greater release of lactic acid when compared to nanocarriers with a single copy of lactic

acid attached to each arm of the PEG-SH polymer. An alternative strategy to increase loading would be to passively entrap polylactic acid acid within PEG-based hydrogels.

6.2.3 Evaluating the antiviral activity of subtilisin-containing hydrogels against HIV

In this thesis, we investigated the antiviral activity of subtilisin against HIV and found subtilisin to inhibit HIV-1 replication with an IC_{50} of 5.8 μ M. The next step would be to evaluate the inhibitory effect of subtilisin-containing hydrogels on HIV transmission. A major challenge to this is the development of an appropriate model to evaluate the antiviral properties of subtilisin in combination with the barrier properties of the hydrogel. Recently, transwell models have been developed for evaluating the cytotoxicity and barrier properties of microbicide gels [202-204]. However, developing a similar model for the current study using V_k2 cell monolayers proved to be extremely challenging. An optimization of experimental parameters such as membrane pore size and material of the transwells, gel thickness and incubation time with virus is required in order to successfully use this model for evaluating the antiviral activity of subtilisin-containing hydrogels.

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