LEVERAGING MUCOADHESIVE THIOMERS AND PLA-BASED POLYMERS TO DESIGN BIODEGRADABLE EXTENDED RELEASE VAGINAL FILMS FOR HIV PREVENTION

by

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LEVERAGING MUCOADHESIVE THIOMERS AND PLA-BASED POLYMERS TO DESIGN BIODEGRADABLE EXTENDED RELEASE VAGINAL FILMS FOR HIV PREVENTION

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HIV is one of the most serious health issues in the world. Women are disproportionally affected by sexually transmitted HIV-1 infection. Without effective vaccine in hand, topical microbicides are developed to prevent the HIV-1 incidence. Tenofovir gel and dapivirine vaginal ring were shown to have some effectiveness in the clinic. However, it is also noticed that low user adherence is one of the major reasons for inconsistent effectiveness across different clinical trials and age groups. The approaches that do not require daily or coitally-dependent use could potentially improve user adherence. This dissertation describes our efforts in the design and characterization of extended-release microbicides through modification of film matrix with functional polymers or nanotechnology to improve user adherence in HIV prevention.

To achieve the extended-release profile of the vaginal film, prolongation of the film mucosal residence is one of the promising strategies. Thiolated chitosan was synthesized and incorporated into poly(vinyl alcohol) based film. This bioadhesive film performed one-week tissue adhesion in pigtailed macaques and showed the feasibility of delivering non-nucleoside reverse transcriptase inhibitor dapivirine and progestin levonorgestrel simultaneously. To further sustained the release profile of bioadhesive film, poly(lactide-co-Ɛ-caprolactone) (PLACL) were synthesized and incorporated into the bioadhesive film matrix for delivering an integrase inhibitor MK-2048. We demonstrated the sustained release profile, excellent safety and *in vitro* effectiveness of this PLACL bioadhesive film.

Nanoparticles have been well developed for controlled or sustained release, which is very promising to be applied for coitally-independent vaginal microbicide to improve the user adherence. We developed a thiomer coated poly(lactic-co-glycolic acid) nanoparticle loaded film with prolonged mucoadhesion and enhanced tissue penetration of MK-2048. This delivery system demonstrated sustained release profile over 30 days in vaginal fluid simulant, improved tissue permeability and potent anti-HIV activity.

Collectively, these studies demonstrate the safety and efficacy of three novel sustained release polymeric film platforms, which can potentially improve the user adherence and provide more options for women to protect themselves from HIV infection.

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AIDS: Acquired Immunodeficiency Syndrome; **APIs**: Active Pharmaceutical Ingredients; **CC_{50}**: 50% Cytotoxic Concentration; **CL**: Ɛ-caprolactone; **CMC**: Carboxymethyl Celluloses; **CYP450**: Cytochrome P-450; **CYP3A4**: Cytochrome P-450 Isoenzyme 3A4; **DAPI**: 4',6-diamidino-2-phenylindole; **DCM**: Methylene Chloride; **DMEM**: Dulbecco's Modification of Eagles Medium; **DPBS**: Dulbecco's Phosphate-Buffered Saline **DPV**: Dapivirine; **DSC**: Differential Scanning Calorimetry; **EC_{50}**: 50% of Effective Concentration; **EDS**: Energy Dispersive X-ray Spectroscopy;**%EE**: Encapsulation Efficiency; **EVA**: Poly(Ethylene Vinyl Acetate); **FBS**: Fetal Bovine Serum; **FDA**: Food and Drug Administration; **FITC**: Fluorescein Isothiocyanate; **FTIR**: Fourier-Transform Infrared Spectroscopy; **GPC**: Gel Permeation Chromatography; **HA**: Hyaluronic Acid; **H&E**: Hematoxylin and Eosin; **HEC**: Hydroxyethyl Celluloses; **HIV**: Human Immunodeficiency Virus; **HLA-DR**: Human Leukocyte Antigen - Antigen D Related; **^{1}H-NMR**: Proton Nuclear Magnetic Resonance; **HPC**: Hydroxypropyl Celluloses; **HPLC**: High-Performance Liquid Chromatography; **HPMC**: Hydroxypropylmethyl Celluloses; **IC_{50}**: Half Maximal Inhibitory Concentration; **IN**: Integrase; **IPM**: International Partnership for Microbicides; **IVR**: Intravaginal Ring; **LA**: L-lactide; **LCs**: Langerhans Cells; **LC-MS/MS**: Liquid Chromatography–Mass Spectrometry/Mass Spectrometry; **LLOQ**: Lower Limit of Quantification; **LNG**: Levonorgestrel; **M_n**: Number Average Molar Mass; **MPT**: Multipurpose Prevention Technology; **MSM**: Men Who Have Sex with Men; **MTBE**: Methyl Tert-Butyl

Ether; **MTN**: Microbicide Trials Network; $\mathbf{M_w}$: Mass Average Molar Mass; **MWCO**: Molecular Weight-Cutoff; **PAA**: Poly (Acrylic Acid); $\mathbf{P_{app}}$: Apparent Partition Coefficients; **PAS II**: Product Acceptability Study; **PBS**: Phosphate Buffered Saline; **PCL**: Poly(Caprolactone); **PD**: Pharmacodynamic; **PdI**: Polydispersion Index; **PEG**: Polyethylene Glycol; **PEG-8000**: Polyethylene Glycol 8000; **PEG-PLGA**: Polyethylene Glycol-Poly(Lactic-Co-Glycolic Acid); **PEO**: Poly(Ethylene Oxide); **PK**: Pharmacokinetics; **PLA**: Poly-Lactide; **PLACL**: Poly(lactide-co-Ɛ-caprolactone); **PLGA**: Poly(Lactic-Co-Glycolic Acid); **PNP**: PLGA Nanoparticle; **PrEP**: Pre Exposure Prophylaxis; **PVA**: Polyvinyl Alcohol; **SD**: Standard Deviation; **SDS**: Sodium Dodecyl Sulfate; **SEM**: Scanning Electron Microscopy; **TEER**: Transepithelial Electrical Resistance; **T film**: Thiomer Film; $\mathbf{T_g}$: Glass Transition Temperature; $\mathbf{T_m}$: Melting Temperature; **TP film**: Thiomer PLACL80 Film; **TNP**: Thiomer Coated PLGA Nanoparticles; **UV**: Ultraviolet; **VFS**: Vaginal Fluid Simulant; **WHO**: World Health Organization;

PREFACE

Completing the Ph.D. degree is like climbing a mountain. This journey would never be possible without the support and help of a lot of people. I would like to express my most sincere gratitude to all of you.

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1.0 INTRODUCTION

1.1 HIV EPIDEMICS, SEXUAL TRANSMISSION AND PREVENTION

1.1.1 HIV epidemics and women

The human immunodeficiency virus (HIV) epidemic started in the mid- to late-1970s[1]. Since then, 76.1 million people have been infected with HIV and 35.0 million people have died from acquired immunodeficiency syndrome (AIDS)-related illness[2]. HIV continues to be a major health issue in the world. In 2016, the number of new HIV infections went to approximate 1.8 million, contributing to the estimated 36.7 million total HIV infection worldwide[2]. Among them, 25.5 million are located in sub-Saharan Africa[3].

Globally, women account for 51% of people with HIV infection[4]. This percentage varies largely with region: in western and central Africa, women make up nearly 60% of all HIV infected population[5]. Recent global statistics revealed that approximately 1 million women were newly infected with HIV-1 in 2016[4]. Women are disproportionally infected with HIV-1 especially in the younger age group: young women (aged 15-24 years) getting newly infected with HIV are 44% higher than young men[6]. Women also contribute to the majority of new HIV infections in children through mother-to-child transmission[7].With decades of effort to strengthen HIV prevention and treatment, the overall number of new infections has been static

since 2010[5]. However, AIDS-related illnesses is still the leading cause of death to women at the reproductive age in the world[4].

The fact that women are more susceptible to be infected by HIV than men can be attributed to a multitude of factors including societal factors, behavioral factors as well as physiological factors[3]. Due to gender inequality, women have limited access to health services and higher education, less ability of controlling decisions around sex, and are vulnerable to gender-based violence[8]. In addition, economic dependence, gaps in knowledge, limited perception of the risk of HIV acquisition, and age-disparate relationships all contribute to the disempowerment of women to protect themselves from HIV[9]. Furthermore, women are more vulnerable to HIV because the exposure area of the vagina to HIV is larger than that of the penis. Moreover, there are more immune cells in the female reproductive tract than the male that are susceptible to HIV infection[10].

1.1.2 Sexual transmission of HIV

Heterosexual transmission is the major mode of HIV-1 infection among women, particularly in sub-Saharan Africa[11]. Sexual transmission of HIV-1 occurs in the female vaginal or colorectal tissue by the exposure to cell-associated and cell-free virus. In this process, HIV-1 needs to cross the mucosal epithelium to reach the immune cells for further infection. Since the epithelium of colorectal tissue is single layer, while vaginal tissue is lined with multiple layered stratified epithelium, the incidence of HIV-1 transmission from male to female via rectal route (estimated 1/20-1/300 per sexual act) is higher than that via vaginal route (estimated 1/200-1/2000 per sexual act)[12-14]. Understanding the mechanism of HIV-1 sexual transmission is key to the design of vaccine or prevention strategies aimed at blocking HIV-1 access to the genital mucosal

tissues. Several mechanisms have been proposed to explain the process of HIV-1 transmission *in vivo*.

The transmission of HIV-1 through sexual intercourse may encounter several physiological barriers. First of all, the mucosal tissue provides a significant barrier to the permeation of virus since the mucosal tissue has limited permeability to particles with diameter larger than 30 nm, while the diameter of the HIV-1 virus is 80-100 nm[15]. Moreover, the stratified vaginal epithelial cells are not vulnerable to HIV-1 infection and not able to transfer the virus[16]. However, epithelial micro-abrasion and tears occur in 60% of women following sexual intercourse[17]. The micro-abrasion, alone with ulcerative sexually transmitted diseases, physical trauma, inflammation, varied hormonal status, and micronutrient levels could all diminish the protection by the stratified epithelium[18], thereby to allow the infiltration of HIV particles into the subepithelial layer and infect the local immune cells including T cells, macrophages, and dendritic cells[17].

Regarding the mechanism of HIV transmission across an intact epithelium, multiple studies have suggested that HIV-1 might interact with Langerhans cells (LCs), which are abundant in human cervical and vaginal epithelium. Moreover, LCs contain dendritic cytoplasmic processes that extend through the vaginal or ectocervical epithelial layer to the lumen. The exposure of dendritic branches in vaginal lumen could interact with HIV-1[19]. Although vaginal LCs do not express CD4 or CCR5, they do provide human leukocyte antigen - antigen D related (HLA-DR), CD1a, and a number of mannose dependent C-type lectin receptors that can allow efficient attachment of HIV-1[20-22]. Once captured by LCs, the virus is transported into the underlying lamina propria where abundant immune cells reside [23, 24].

A recent study from Bomsel's group has demonstrated the mechanism of cell-associated HIV-1 mucosal transmission in genital tissues by live imaging of a reconstructed *in vitro* urethral mucosal model[25]. In this model, it was found that in the first hour, cell-mediated HIV-1 contacted the epithelial cells and then transferred to the mucosal epithelium mediated by virological synapses. In the following 2-3 h, HIV-1 particles crossed the epithelium via transcytosis and then infected the macrophages in the next 2-3 days [26-29]. In turn, the infected macrophages displayed HIV-1 DNA and produced new viral particles for local expansion and systemic dissemination in the following weeks[18].

1.1.3 Female-controlled HIV prevention strategies

To reduce the HIV infection incidence, more effort is needed to put on the implementation of effective prevention tools. Women who are at high risk of HIV infection usually have difficulty negotiating condom use with their male counterparts. Therefore, female-controlled HIV preventative tools are essential to alleviate the disproportional burden of HIV on women. The currently available female-controlled HIV prevention strategies include female condoms and pre-exposure prophylaxis (PrEP) (oral PrEP and topical microbicides).

The female condom is a physical barrier for contraception that is used in the vagina during sexual intercourse. It is an efficacious dual functional product which can avert unintended pregnancy and risk of sexually transmitted infections including HIV-1[30]. There are numerous female condoms available globally, including FC2, VA w.o.w® Feminine Condom, The Woman's Condom, The Phoenurse, Cupid, Panty, and Velvet. FC2 is the only one approved by the Food and Drug Administration (FDA) to be used in the United States. Cupid is prequalified by world health organization (WHO) for distribution in Europe, and the others are undergoing

clinical trials[31]. Although female condoms show comparable efficacy as male condoms, their distribution and uptake remain significantly lower than expected, contributing to only 0.19% of global condom procurement[32]. The uptake of female condom could be impeded by high cost (cost about 2-5 times as much as male condoms), deeply rooted biases on its contraceptive efficacy, concerns about insertion, appearance and feel of the device (too noisy, messy and unattractive), as well as reluctance from male partners[33]. Efforts have been made to facilitate the use of the female condom including not only education and behavior intervention, but also product modification such as optimizing the material to reduce its cost, and enclosing the female condom into a dissolving capsule to aid insertion[34].

Oral PrEP is a self-directed and discreet HIV prevention strategy for people who are at risk of getting HIV infection. Truvada®, a tablet containing two nucleoside reverse transcriptase inhibitors (tenofovir disoproxil fumarate and emtricitabine), is an FDA approved medication for HIV prevention. The efficacy of tenofovir based PrEP has been studied in multiple clinical trials: iPrEx trial (cohort of gay men, other men who have sex with men (MSM) and transgender women), TDF2 Study (cohort of heterosexual men and women), Partners PrEP Study (cohort of serodiscordant heterosexual couples), IPERGAY (cohort of MSM), Proud (cohort of MSM), CDC 4370 (cohort of people who inject drugs), FEM-PrEP and VOICE (cohort of women)[35]. Overall, tenofovir based PrEPs showed over 90% efficacy in HIV prevention if participants were compliant with dosing regimens. Among MSM, continuous high efficacy of PrEP in preventing HIV infection has been observed across different clinical trials (44-92%)[36, 37]. However, lower efficacies were observed among women who got HIV infection through vaginal transmission (6-75%)[38-40]. To date, the access to oral PrEP is expanding, and more countries have approved the use of Truvada® for HIV prevention. Despite the effectiveness of oral PrEP,

its daily dosing frequency causes low user adherence which leads to low or no protection against HIV-1 infection[35]. Furthermore, in multiple clinical studies, oral PrEP containing antiretrovirals showed some modest side effects, such as emesis, stool softening, and diarrhea, in 1.0-18.5 % of participants[41]. Mildly decreased bone mineral density was also observed primarily in spine[42]. Therefore, the tolerability and long-term toxicity of oral PrEP need to be evaluated.

Topical microbicides are products that are applied vaginally or rectally for the prevention of sexually transmitted HIV. They have been developed into various dosage forms including gels, tablets, films, capsules, vaginal rings, enemas and suppositories. The first-generation topical microbicides were non-specific with a broad spectrum of antimicrobial activity. This class consists of surfactants such as nonoxynol-9 and C31G (SAVVY), polyanions such as Carrageenan, cellulose sulfate and naphthalene sulfonate, and acid buffering agent such as BufferGel[43, 44]. More than 10 phase II/III clinical trials have been conducted to evaluate 6 first generation topical microbicides in HIV prevention. However, it has been demonstrated that those first-generation topical microbicides had either no protection or even increased the risk of HIV acquisition due to the impairment of the epithelial integrity[45-47].

The second-generation topical microbicides are products containing different classes of antiretroviral drugs targeting various HIV life cycles during sexual transmission. Tenofovir was selected as a microbicide drug candidate due to its extensive clinical experience and the excellent safety in HIV treatment. 1% tenofovir vaginal gel has shown a 39% reduction of HIV acquisition overall after 18 months of use in a phase IIa CAPRISA004 clinical study among women in South Africa[48]. However, this finding was not confirmed in two other large clinical trials: FACTS 001 and VOICE. The inconsistent results were mainly caused by the stringent requirement of

daily or on-demand dosing that were hard to follow[49]. Alternatively, the dapivirine intravaginal ring (IVR) was designed for monthly use and consequently was shown to have improved the user acceptability and adherence over the tenofovir gel product. In ASPIRE and The Ring Study clinical trials, an encouraging and consistent effectiveness in HIV prevention was found, but user adherence dependent effectiveness was also prominent in these two studies[50-52]. Apart from the single entity loaded topical microbicides, products containing combination of antiretroviral drugs for different targets are also under development. Thus far, two IVRs (containing dapivirine &maraviroc and vicriviroc & MK-2048, respectively) have been developed and evaluated with safety in clinical trials (MTN-013/IPM 026 and MTN-027) [53, 54].

The quest for safe and effective topical microbicides is still on-going. More topical microbicides such as tenofovir and dapivirine vaginal films are under development. The excellent safety and acceptability of the dapivirine vaginal film have been demonstrated in Phase I clinical trial[55]. Additionally, the vaginal film showed a favorable pharmacokinetic profile as compared to that of gel and oral formulations, which was illustrated in FAME-05 and FAME 02B clinical trials[56, 57]. The efficacy of the dapivirine vaginal film in HIV inhibition was further confirmed in the *ex vivo* tissue explant model using the vaginal biopsies collected from the clinical studies[55].

1.1.4 Impact of user acceptability and adherence on product effectiveness in HIV prevention

One important lesson learnt from reviewing the currently available female-controlled HIV prevention tools is the critical role of the user acceptability and adherence in product efficacy.

Acceptability and adherence are the two major terms employed in the clinical trials to evaluate the preference of topical microbicides among women. Acceptability describes the willingness of people to use a product. It is determined by not only the product itself but also users' experiences, social and culture norms. Adherence, also termed as compliance, is used to describe whether people follow the product use regimens and instructions in clinical trials[58]. Composite approaches (such as the combination of self-report and biological markers) are usually applied in the clinical studies and proven to be useful in the measurement of user adherence to a product[59]. User adherence is usually assumed to be positively influenced by acceptability but there is no evidence demonstrating their relationship[60].

Unsatisfied user acceptability and adherence to medication pose significant obstacles to improve the outcome of prophylaxis in HIV prevention. This has been observed in multiple clinical trials evaluating the effectiveness of oral PrEP and topical microbicides in HIV prevention. For example, the iPrEx trial showed that Truvada® with 51% of user adherence (as indicated by the detection of tenofovir in plasma) exhibited 44% efficacy in HIV prevention[36]. However, the FEM-PrEP and VOICE clinical trials, which evaluated the same PrEP Truvada® but was conducted on African women, demonstrated no efficacy with low user adherence at 21–37% and 28–29%, respectively[39, 40]. Similarly, in the CAPRISA 004 trial, the users with adherence larger than 80% showed 54% reduction in the HIV incidences, while those with adherence between 50-80% and lower than 50% showed 38% and 28% reduction in HIV incidence, respectively[61]. In the VOICE and FACTS 001 trials, no protection from HIV infection was observed which was possibly due to the low user adherence[39, 62]. More than likely, the daily and coitally dependent dosing frequency was responsible for the low user compliance observed.

As an alternative, the IVR that requires less dosing frequencies was evaluated in Phase III ASPIRE trial and the Ring Study[52]. The dapivirine IVR showed consistent effectiveness in reducing HIV incidence among Africa women. Specifically, in the ASPIRE trial, 56% of protection from HIV infection was achieved among women over age of 21 years, while no protection was observed for those younger than 21 years. The failure of dapivirine IVR in women aged 18-21 was due to the low adherence of this product. Major reasons for non-adherence to IVRs reported by participants were health related-side effects from ring use, concerns of potential long-term effects, rumor/beliefs-related concerns, and opposition from partners particularly during sex. Other reasons were also reported such as menses-related concern, unknown efficacy, hygiene concerns, external-influence from peers and family members, and sole interest in study benefits.

Results from these clinical trials indicate that a myriad of factors, such as dosing frequency, age, culture, sex or vaginal product use experience and pros and cons of products, could affect the user adherence to microbicides. Therefore, no one product fits all and women need more choices when selecting HIV prevention tools to meet their own needs.

1.2 PHYSIOLOGICAL BARRIERS FOR THE DEVELOPMENT OF COITALLY-INDEPENDENT VAGINAL MICROBICIDES

Heterosexual transmission remains the major mode for HIV infection among women. Therefore, vaginal microbicides have great potential in reducing the acquisition of HIV through sexual transmission. To improve user acceptability and adherence and thereby efficacy, vaginal microbicide products that circumvent the need for frequent product administration are needed.

To develop a safe and effective coitally-independent vaginal microbicide, it is necessary to understand the vaginal anatomy and physiology which present barriers and opportunities for drug delivery.

1.2.1 Vaginal anatomy and physiology with respect to drug delivery

The vagina is a fibromuscular tube extending form the vestibule to the cervix. It is slightly S-shaped and sits at an angle of about 45° to the horizontal axis and is positioned between the rectum, bladder, and urethra[63]. The dimensions of the vagina range from 6.86 to 14.81 cm in length and 4.8 to 6.3 cm in diameter[64]. The surface area of the vagina ranges from 65.73 to 107.07 cm^2[65]. The true vaginal surface area could be underestimated since the vaginal surface has rugal folds and was unlikely to be fully distended during the measurement. The shape of the vagina varies considerably among women and can be categorized into 4 types: long parallel shape, conical shape, heart shape, and bulge or balloon shape[64]. Clearly, the varied shape, dimension and surface size of the vagina among women suggest the unlikelihood of "one product fits all" in the design of vaginal products.

The vagina is lined with a multilayered nonkeratinized, stratified squamous epithelium. The thickness of the epithelium changes with the hormone levels[66]. In the follicular phase, estrogen is abundant and prompts the maturation of epithelial cells which reduce the thickness of epithelium. In the luteal phase, progesterone is dominant and inhibits this maturation and facilitate the matured epithelial cells in turning to the superficial layer. The superficial layer is renewed approximately every three days[18]. The thickness change and renewal of the epithelium have crucial influences on permeation, targeting, and the pharmacokinetics (PK) of vaginally delivered drugs.

The vagina contains no secretory glands, but its surface is kept moist by vaginal fluid. Vaginal fluid consists of cervical mucus, microorganisms vaginal wall transudate, glycogen, sloughed epithelial cells, and exudates of white blood cells[67]. The vaginal fluid has acidic pH between 3.8 and 4.5 for healthy women of reproductive ages. The vaginal pH is maintained by *Lactobacillus species* such as *L. crispatus* and *L. jensenii*, which produce lactic acid from glycogen in the vaginal tract[68]. Lactobacilli also produce hydrogen peroxide that may help to prevent the overgrowth of harmful pathogens[69]. Multiple species of gram positive, gram negative, anaerobic and facultative *Lactobacillus* species, *Bacteroides species*, *Peptococcus* species, *Corynebacterium* species, *Staphylococccus epidermis*, *Peptostreptococcus* species, and *Eubacterium* species dominate in the vaginal flora and form a dynamic ecosystem[70]. Disruption of the vaginal flora is associated with bacterial vaginosis which is characterized by an overgrowth of anaerobic gram-negative rods and cocci. Another organism often associate with vaginal dysbiosis is *Trichomonas vaginalis*. The vaginal pH in bacterial vaginosis increases to 4.5 or even greater than 6[71]. The vaginal pH can also be increased by the introduction of the alkaline semen. The change of the vaginal pH could be a potential target for the design of pH-sensitive delivery systems. Besides of the pH change, vaginal infection as well as hormone levels also could alter the expression of transporters and metabolizing enzymes in the vaginal tract (fluid and/or epithelium), which can potentially affect the transport and biological stability of delivered drugs[72-76]. The volume of vaginal fluids is varied in the menstrual cycle from 1.96g/8 h during the mid-menstrual cycle to 1.37g/8 h during nadirs[63, 77]. The fluid volume is also influenced by the sexual intercourse. All those variations in the vaginal tract make it more complicated to develop a coitally-independent vaginal microbicide[78].

1.2.2 Mucus in female reproductive tract: a potential target for coitally-independent vaginal microbicides

To develop a coitally-independent vaginal microbicide, the prolongation of residence time for the delivered drug or vehicle in the vaginal cavity is desired. In addition to the large surface area, vaginal mucosa provides mucoadhesive targets which can be exploited for drug/delivery vehicle attachment. Cervical mucus is the major contributor to the adhesive property of vaginal mucosa.

Cervical mucus is a glycoprotein gel secreted from the goblet cells within the columnar epithelium from the endocervix and Bartholin's glands[79]. The presence of mucus in the female reproductive tract provides a barrier to prevent sperms, pathogens, and pharmaceutical agents from entering into the uterus and vaginal epithelium. Cervical mucus is continuously secreted, and the secretion rate is at 20-60 mg/day[80, 81]. It is a mixture of water (90-99%), low molecular weight components (salts, amino acids, sugar), and high molecular weight components (bactericidal proteins, plasma proteins and mucin (0.5-5%)). The viscoelastic gel property of mucus is dependent on mucins. Mucins are high molecular weight glycoproteins, which present negative charges due to the carboxyl and sulfate groups[82].

Mucins in the female reproductive tract can be classified into two categories based on the sequence of mucin genes: secreted mucins and membrane-associated mucins. Both types of the mucin share a structure feature which consists of adjacent amino acid repeats and abundant O-glycosylation sites[83]. Secreted mucins can be furthered divided into gel-forming mucins and soluble mucins[84]. Gel-forming mucins, including MUCs2, 5AC,5B, 6 and19, are ubiquitously expressed in almost all mucosal tissues and are the largest known glycoproteins. They have abundant cysteine-rich domains which link monomers to form homomultimers via disulfide bonds formation[85]. The bound oligosaccharides in the mucin glycoproteins greatly increase the

molecular weight of mucins and contribute to their increased viscosity. The high viscosity of mucins maintain an unstirred layer and adhere the epithelium firmly even under vaginal intercourse[86]. In addition, the glycoproteins in mucins present negative charge and strong proton acceptor and donor functions to mediate hydrogen bonding[87]. The unstirred mucus layer and proton acceptor and donor functionalities allow the mucosal layer as a potential target for the coitally-independent delivery system. On the other hand, soluble mucins including MUCs7 and 9 are the smallest known mucins[88]. The membrane-associated mucins (MUCs 1, 4 and 16) are expressed in oviduct, uterus, endocervix, ectocervix, and vagina[89, 90]. The transmembrane domain in these mucins tethers the mucin in the apical side[91]. They do not contain cysteine-rich domains and exist as monomers. Growing evidences suggest that MUCs 1, 4 and 16 are usually easy to be shed from the epithelial cells over time[92, 93].

Cervical mucin is dynamic and can be influenced by multiple factors. The secretion of the mucin changes cyclically in consequence of hormonal changes[94]. MUC 4 and 5B are the two predominant mucin proteins expressed in the endocervix during the menstrual cycle. MUC 5B displays the highest expression in the midcycle and drops remarkably in the luteal phase[95]. The pH, glycosylation, and activities of enzyme involved in mucins also changes with menstrual cycle[83]. The ovulation stimulates more neutral oligosaccharides than acidic ones (sulphated and sialidated), thereby to decrease the glycosylation of mucins which contributes the change of pH of mucins[96]. Additionally, the sialidase (an enzyme involves in the breakdown of mucins) activity reached to the maximum in the ovulatory phase[97]. Taken together, the changes in the secretion, pH, glycosylation, and enzyme activities of mucins could play a vital role in modifying the rheologic and biochemical properties of cervical mucus.

Cervical mucus is a semipermeable barrier that protects the female reproductive tract. It allows the exchange of nutrients, water, and hormones while being impermeable to most bacteria and pathogens. The spacing between the mucin mashes ranges from approximately 100 nm to 1000 nm, and this wide range in the size of mesh spacing is most likely due to the dynamic properties of mucins[98]. The mesh spacing of the cervical mucin was measured around 100 nm using Yudin's glutaraldehyde fixation procedure (without changing the mucin structure) [99]. Interestingly, the PEGylated nanoparticles with size of 500 nm were too large to diffuse through the fixed mucin but were able to penetrate through the cervical mucins without fixation procedure[98]. This is because mucins aggregate together to form cables with larger spacing size than individual mucin as illustrated in multiple methods. In addition, the viscosity and mesh spacing of mucins are influenced by hormone levels. During ovulation, the mucin concentration increases but the viscosity is reduced due to the decreased glycosylation and increased enzymes to breakdown mucins[100].

Mucin plays an important role in fertility as it accepts, filters, prepares, stores, and release sperm for transport to the oviduct for fertilization[101]. The glycoprotein composition, glycosylation, and mesh spacing of the mucus have critical influence on the survival and migration of sperm cells[102]. Although cervical mucus enables the permeation of sperm cells during the ovulation, it still presents a strong capacity in prevention of infections. The ionic groups on oligosaccharides and sulphated groups from mucins impede the adherence of bacteria to target cells[103, 104]. Moreover, characteristics of mucus are also informative during the diagnosis and therapy of infertility[105]. The expression and activity of MUCs have been utilized as indicators for conditions of mucus secretions preclinically and clinically. Therefore, it

is important that vaginal delivery vehicles and/or drugs do not interfere with the expression and activity of mucin proteins.

In summary, the vaginal route offers several advantages for drug delivery such as the large surface area, abundance blood supply and avoidance of hepatic first-pass effect[106, 107]. The therapeutic efficacy of vaginal delivered drugs is hampered due to the short retention time. The vaginal mucus exhibits high viscosity and multiple binding sites, which provides a potential target for extend the residence of the vaginal delivery system. Therefore, the unique anatomical and physiological properties of the vaginal route for drug delivery provide rationale for its use as an option for vaginal microbicides product.

1.3 CURRENTLY AVAILABLE COITALLY-INDEPENDENT DRUG DELIVERY SYSTEM FOR VAGINAL MICROBICIDES

Adherence issues associated with daily or coitally-dependent product use have motivated the development of coitally-independent microbicides. Coitally-independent microbicide can be achieved from the discovery of long-acting antiretroviral drugs and the design of dosage forms without the requirement of frequent dosing regimen. To date, only two antiretroviral reagents (protease inhibitor GSK744 and non-nucleoside reverse transcriptase inhibitor rilpivirine) have been proven to have long-term protection for HIV infection[108-110]. Meanwhile, a number of coitally-independent dosage forms have been explored in vaginal microbicides including IVRs, nanosystems and other modified traditional vaginal dosage forms.

1.3.1 Intravaginal rings

IVR is a polymeric device that can be positioned in the upper third of vagina for extended residence to release active pharmaceutical ingredients (APIs) in a sustained or controlled fashion. This property allows it to deliver drugs in coitally-independent manners for one month or even longer. Six types of IVRs have been designed to date: over-molded metal spring, matrix, reservoir/core, multiple partial core, insertable core and sandwich or shell ring[111]. The most common IVRs are in matrix and reservoir types. In matrix IVR, APIs are dispersed throughout the whole ring. The release rate of APIs is controlled by the diffusion and permeation through polymers in the matrix. In the later time frame, the release rate of APIs in matrix ring decreases over time due to the less concentration gradient, which diminishes the therapeutic effect[112]. The reservoir ring contains single or multiple cores that encapsulate the APIs in a nonmedicated polymeric sheath. This ring can be applied to deliver single or multiple APIs at independent release rates. For this ring, zero-order release kinetics of APIs can be achieved by adjusting the membrane material and the thickness of the polymeric membrane[113]. The polymer selection for fabricating IVRs is restricted by biocompatibility, flexibility, and drug permeability. To date, IVRs have been manufactured using silicone elastomers (polydimethylsiloxane), poly(ethylene vinyl acetate) (EVA), and thermoplastic polyurethane materials.

To date, IVRs have primarily been applied for delivery of hormones. They are also utilized in microbicides. The dapivirine silicone matrix IVR is the most advanced one that has been evaluated in clinical trials. In both The Ring Study, led by International Partnership for Microbicides (IPM), and ASPIRE, led by Microbicide Trials Network (MTN), the dapivirine IVR has demonstrated promising effectiveness in HIV prevention and long-term safety in more than 4,500 women in Malawi, South Africa, Uganda and Zimbabwe[51, 52]. Other antiretroviral

drugs have also been loaded into the IVR for clinical evaluations. The safety and pharmacokinetics of vicriviroc (MK-4176) and/or MK-2048 EVA core IVR is being evaluated in the clinical trial[54]. Vaginal rings with different functions such as more constant drug release rate or delivery of multiple APIs with different properties are also actively under investigation. Furthermore, evaluation of a 3-month dapivirine and levonorgestrel IVR has been initiated in the clinic [114]. Despite its merits, some weaknesses still exist for IVRs. In the ASPIRE study, no protection of dapivirine IVR from HIV infection was observed for women at younger age which was more than likely due to the low user adherence [52]. It was found that the low adherence to IVR was because the presence of the IVR caused discomfort in the vagina and some women preferred to remove the ring during the sexual intercourse. Therefore, more vaginal dosage forms need to be provided for women to improve the product efficacy.

1.3.2 Nanosystems in vaginal microbicides

Nanoparticles

Nanoparticle drug delivery systems not only provide the sustained or controlled delivery of APIs but also improve the drug solubility, protect drug payloads as well as enhance mucosal drug permeability[115]. With these advantages, nanoparticle delivery systems have been explored in the design of vaginal microbicides[116, 117]. Nanoparticles have been designed as not only active reagents for HIV prevention but also as carriers for the delivery of antiretroviral drugs.

An example of a bioactive nanoparticle is the dendrimer G2-S16. This dendrimer contains carbosilane surface groups and has been demonstrated the *in vitro* potent HIV inhibition at nanomolar range due to its ability to bind to gp120 and CD4[118-120]. This promising

microbicide candidate based vaginal gel needs to be assessed for safety in clinical trials. Another bioactive nanoparticle is SPL7013 dendrimer (VivaGel®), which inhibits HIV attachment via interacting with gp120 and coreceptors[121]. This dendrimer is derived from poly-L-lysine and shows potent anti-HIV activity *in vitro* and further evaluated in the clinic. However, the clinical trial of the SPL7013 containing gel has demonstrated mild side effects to the vagina, which halted further clinical development of this product[122].

As drug carriers, nanoparticles can be divided into polymeric or non-polymeric types. Polymeric nanoparticles can provide controlled or sustained release profiles for the payloads. They can be designed for site-specific delivery via the ligand modification on polymeric particle surface[123, 124]. Poly(ethylene oxide) (PEO) modified poly(caprolactone) (PCL) nanoparticles have been developed for the delivery of dapivirine as an alternative vaginal microbicide[125]. In the mouse model, it was found that this nanoparticle system was able to enhance the mucosal penetration of dapivirine even though the nanoparticles were rapidly cleared. In addition, after encapsulation into the nanoparticles, the local PK of dapivirine was improved as it exhibited more sustained dapivirine levels in vaginal lavages and tissues as compared with unformulated dapivirine[126]. Rilpivirine is a promising candidate for coitally-independent microbicide. Nanoparticles made from PEO modified poly(lactic-co-glycolic acid) (PLGA) were applied to deliver rilpivirine and further incorporated into the poloxamer based thermosensitive gel for intravaginal administration. The use of the gel enhanced the vaginal retention of nanoparticles and extended the drug levels in the vaginal tissue[127]. In addition to the polymeric nanoparticle delivery system, the commercialized liposome (Novasome®) has been utilized to deliver antiretroviral drug -2 RANTES intravaginally[128, 129]. The macaque study demonstrated that Novasome formulated -2 RANTES showed significantly higher protection from virus infection

as compared to the -2 RANTES in phosphate buffered saline (PBS). Interestingly, the placebo Novasome also provided antiviral efficacy in the macaques with viral challenges. This was because liposomes were able to disperse throughout the mucosal tissue and formed a barrier to impede the entry of virus[130]. In addition, Eszterhas et al. and Boyapalle et al have evaluated the ability of siRNA loaded chitosan-lipid nanocomplexes and liposomes as vaginal microbicides[131, 132]. Although the delivery of siRNA is very challenging with respect to the molecular stability and off-target effects, significant protection against viral challenges were observed both in *ex vivo* human tissue models and macaque models [131, 132].

Nanofibers

The application of nanofibers as drug delivery system is also under active exploration. The nanofiber has a mesh-like structure with variable pore size and fiber diameter[133]. It can be manufactured using multiple methods such as electrospinning, phase separation, and self-assembly. Nanofibers have been explored in the microbicide field due to their unique features. First, the nanofiber mesh can provide an extra barrier to impede the permeation of virus. Additionally, it has a large surface area, which provides more interaction between fibers and the vaginal mucous. Moreover, the nanofiber has the capacity to incorporate multiple APIs for HIV prevention[133]. Unlike nanoparticles that can internalize in cells and release drugs, nanofibers can only release the drug in a controlled manner through diffusion and erosion. Immediate- or sustained-release kinetics of drugs from nanofiber can be obtained by tuning polymer formulations and manufacturing parameters[134, 135].

Woodrow, K.A. et al has developed various electrospun nanofiber formulations using PEO, polyvinyl pyrrolidone, PLGA, poly-lactide (PLA), and PCL to load numbers of

antiretroviral reagents such as tenofovir, maraviroc, raltegravir, and zidovudine[136-140]. In addition, their group has also incorporated mucus-penetrating nanoparticles into the mucoadhesive nanofibers to achieve longer retention time in the vagina and sustained drug release rate[141]. Those nanofibers have been shown to have excellent *in vitro* safety profiles for vaginal administration. Moreover, the feasibility of scaling up the tenofovir loaded PVA nanofibers has also been illustrated [142]. Therefore, this dosage form is promising to be further investigated as coitally-independent vaginal microbicides.

The application of nanotechnology in vaginal microbicides product development is still at the pre-clinical stages. More efforts need to be put into the evaluation of the nanoparticle systems regarding the scale-up, selection of the appropriate vaginal loading platforms, and *in vivo* studies on the pharmacokinetics and safety. This information is necessary to advance such nanotechnology-based systems to the clinic. In addition, although improved local pharmacokinetic properties of microbicides have been achieved by nanosystems as compared to free drugs, maintaining sufficient drug levels for long period (as long as over one week) is still challenging.

1.3.3 Other sustained-release vaginal dosage forms

Traditionally, vaginal dosage forms such as vaginal tablets and gels are mostly applied for daily or coitally dependent microbicides. The most recent research is exploring the feasibility of manufacturing sustained-release vaginal tablets or gels by modifying their polymer formulations. A vaginal gel made of modified silicon elastomer achieved an enhanced sustained release profile over 24 h[143]. Another example is the IQP-058 containing osmotic tablet. It has been shown the capability of maintaining high levels of drugs in the vaginal mucosa for 10 days[144].

Controlled-release vaginal tablets are under investigation which utilize mucoadhesive polymers to prolong the retention time of microbicides at the site of action[145].

1.4 VAGINAL MUCOSAL DRUG DELIVERY SYSTEM: PROMISING APPROACH IN THE DEVELOPMENT OF COITALLY-INDEPENDENT VAGINAL MICROBICIDES

To design a coitally-independent vaginal microbicide, rather than the mechanical fixation of delivery systems such as the IVR, mucoadhesion is another promising approach to extend the retention time of the delivery vehicle inside of the vaginal cavity. Vaginal mucosa provides a large surface area for the intimate contact of the drug delivery system with the mucosal tissue. Furthermore, the biochemical structure of mucus in the vaginal secretions motivates the design of mucoadhesive formulations to prolong the residence of drug delivery systems in the vaginal tract.

1.4.1 Mechanisms of mucoadhesion

Mucoadhesion can be defined as the binding of natural or synthetic polymers to mucosal membranes[146]. The process of mucoadhesion is complex. In general, three stages are involved: mucoadhesive polymers get wetted or swelled first, and then the chains of mucoadhesive polymers interpenetrate into mucus networks followed by the formation of covalent/non-covalent bonds between entangled polymers and mucins[147]. Various theories

including wettability, electrostatic, fracture, adsorption, and diffusion-interpenetration have been proposed to explain the mechanisms involved in the mucoadhesion process[148].

The wettability theory mainly explains the mucoadhesion process of liquid and low viscous mucoadhesive delivery systems. The contact angle of polymers on a mucosal surface is an experimental measure of the wettability or spreadability of the delivery system. The lower the contact angle, the greater the wettability/spreadability of the mucoadhesive system[149]. However, polymers with extreme hydrophilicity are not favored since they present lower contact angle on mucus membrane than that of the mucus itself resulting in no adherence[150].

The electronic theory explains the mucoadhesion resulting from of the electronic interaction between polymers and the mucus. Mucin presents negative charges from the carboxyl and sulfate groups in oligosaccharide chains[151]. Therefore, polymers with positive ions can be applied to achieve mucoadhesion to the mucus due to the formation of electrostatic layers at their interface[152].

The fracture theory defines the force of detachment of the bonding between polymers and the mucus. The higher the fracture strength, the more adhesive the polymer is. The polymer with longer network strands and lower degree of cross-link shows greater work fracture[153]. The fracture strength can be determined by the Young's modulus, the fracture energy, and the critical crack length of the polymer[154].

The absorption theory describes mucoadhesion occurring via a variety of surface interactions between the polymers and mucus substrate. The surface interactions include chemisorption attributing to ionic, covalent, and metallic bonding as well as noncovalent bonding of can der Waals forces, hydrophobic interactions, and hydrogen bonding[148, 155]. Chemisorption is considered to be stronger than noncovalent bonding.

The diffusion-interpenetration theory explains the process of diffusion and interpenetration of polymer chains into the glycoprotein chains of the mucus. The interpenetration is dependent on the diffusion coefficient of both mucoadhesive polymer and mucus, which is influenced by the molecular weight, cross-link density, chain mobility/flexibility, and expansion capability[156]. Polymers with molecular weight over 100,000 Da, long chains, and less cross-link density are often considered to have high interpenetration and molecular entanglement[157]. In addition, mucoadhesive polymers presenting similar structure and functional groups to mucins often exhibit high miscibility, contributing to the increased mucoadhesion[158]. Overall, multiple mechanisms are generally involved in the process of mucoadhesion. Mucoadhesion is dependent on the nature of mucins, polymer properties, the surrounding environment, and physiological conditions.

1.4.2 Mucoadhesive polymers

Mucoadhesive polymers have gained considerable interests due to their ability to prolong the residence time of the delivered payloads at targeted mucosal sites. These polymers applied in the mucoadhesive drug delivery platform should be sufficiently flexible to penetrate into the mucus chains, biocompatible, non-toxic, and low cost. Based on the mucoadhesion mechanism, the mucoadhesive polymers can be categorized into two types: non-covalent bonding polymers and covalent bonding polymers.

Non-covalent bonding polymers

The non-covalent bonding mucoadhesive polymers can interact with mucin via hydrophilic-hydrophobic interaction, hydrogen bonding, Vander Waals and electrostatic interaction. This class of polymers includes polyacrylates, cellulose derivatives, chitosan, hyaluronic acid, and pectin.

Poly (acrylic acid) (PAA) is a homopolymer of acrylic acid or crosslinked with polyalkenyl ethers or divinylglycol. PAA exhibits mucoadhesion through the strong hydrogen bonds formed between its carboxylic groups and the oligosaccharide chains of mucin as well as the physical entanglement between the polymer and mucus layer [159]. Due to its excellent mucoadhesion, PAA has been modified into products with various cross-link densities, such as polycarbophil (Noveon®) and carbomer (Carbopol®) for swelling, pH-sensitive or sustained-release drug delivery systems[160, 161].

Cellulose derivatives include hydroxyethyl celluloses (HEC), hydroxypropylmethyl celluloses (HPMC), hydroxypropyl celluloses (HPC), and carboxymethyl celluloses (CMC). They are widely applied in mucoadhesive delivery system due to the hydrogen bonding between their carboxyl and/or hydroxyl groups and mucin[162]. The cellulose derivatives have comparable mucoadhesion to PAA and have been widely used for vaginal drug delivery due to their sustained mucoadhesion and safety profiles after vaginal administration over days[163].

Chitosan is a cationic polysaccharide which is derived from the deacetylation of chitin. It is a natural polymer with good biocompatibility and biodegradability *in vivo* [164]. The mucoadhesion of chitosan is mainly due to the electrostatic interaction between the positively charged amines on chitosan and the negatively charged sialic acid residues on the glycoprotein of mucin[165]. In addition, the hydroxy and amine groups on chitosan can form hydrogen bonding with mucin[166]. As a pharmaceutical excipient, chitosan has been applied in numbers of

formulations such as tablets, emulsions, and gels. Chitosan is also utilized for controlled drug release system due to its pH dependent solubility[167]. Moreover, it shows antimicrobial and anti-inflammatory activity[168, 169].

Hyaluronic acid (HA) is an anionic glycosaminoglycan containing carboxyl and hydroxyl groups that are able to form hydrogen bonding with mucin to produce mucoadhesion[170]. It was shown that HA with lower molecular weight exhibited higher mucoadhesion. Due to the intimate contact between polymer chains and mucosal tissue, HA displays penetration enhancement properties[171]. Thus, HA microsphere as a vaginal drug delivery system was utilized for systemic delivery of calcitonin [172]. Given its prolonged mucoadhesion, HA has also been applied in combination with HEC for the treatment of vaginal dryness. It has been also applied to deliver *Doederlein's bacillus* for restoring the normal bacteria flora in vagina[163].

Pectin is a water-soluble heterogeneous polysaccharide with a linear structure. Pectin exhibits mucoadhesion via the interpenetration and absorption into the mucin network. Pectin presents negative charge which facilitate the entanglement and hydrogen bonding between polymer chains and mucins[173]. Pectin has been applied in vaginal douches for better spread and hold of drugs to the vaginal wall[174]. It was also used in combination with Carbopol in mucoadhesive tablets to achieve better adhesion and swelling for vaginal administration[175].

Covalent bonding polymers

Thiomers refer to an array of hydrophilic polymers generated by the immobilization of sulphydryl bearing reagents on the backbone of the well-established mucoadhesive polymers mentioned above[176]. In contrast to common mucoadhesive polymers which exhibit mucoadhesion via non-covalent bonding, thiomer exhibits improved mucoadhesion due to their

ability to form disulfide bonds with the cysteine domain of mucin[177]. Thiomers are classified into cationic thiomers and anionic thiomers. Chitosan is the major cationic mucoadhesive polymer that can be thiolated at the primary amino group of its glucosamine subunits [178]. The anionic mucoadhesive polymers, such as PAA, CMC, and alginate, present carboxylic acid groups that facilitate sulfhydryl moiety attachment via the formation of amide bonds[179-186].

Thiomers can be synthesized through amide, amidine, amine bond formation and conversion of hydroxyl groups into thiol groups. Thiolation via amid bond formation is mostly mediated by carbodiimides using thiol bearing reagents such as cysteine, cysteamine, thioglycolic acid, or N-acetylcysteine[187]. For amidine bond formation, the thiol coupling molecules are isopropyl-S-acetylthioacetimidate and iminothiolane[188]. Amine bond formation can be achieved through a two-step reaction of cleavage on vicinal diol moiety with sodium periodate first and followed by coupling with cysteamine[189]. For the polymers lacking the carboxylic acid, primary amino or vicinal diol groups, thiolation can be achieved through the direct conversion of hydroxyl groups by bromine[190]. Furthermore, since the free thiol group is readily oxidized to a disulfide bond at pH levels greater than 5, pre-activated or S-protected thiomers have also been developed to maintain mucoadhesiveness[191].

In addition to their mucoadhesion property, thiomers present *in situ* gelation ability due to the inter- and intra-molecular disulfide bond formation via oxidation [192]. This property of thiomer has been widely applied in liquid or semisolid vaginal, nasal, and ocular formulations. Thiomer is also able to enhance the drug permeability in tissue or cell through reversible opening of tight junctions[193] and inhibition of efflux transporters such as P-glycoprotein (P-gp) and multidrug-resistance protein (MRP)[194].

Thiolated chitosan is ranked as the most mucoadhesive polymers as compared to other thiomers and exhibits about 10-fold higher adhesive retention time than most of non-covalent bonding mucoadhesive polymers[176]. This polymer has been evaluated in two clinical trials and revealed excellent tolerability. In addition, an eye drop containing chitosan-N-acetylcysteine (Lacrimera®; Croma-Pharama) for treatment of dry eye syndrome has been available in the European and Canadian markets[195].

1.4.3 Vaginal mucoadhesive dosage forms

The advances in mucoadhesive polymers have enabled their applications in the development of mucoadhesive dosage forms for various mucosal tissues. This dissertation focuses on the design of various vaginal dosage forms using mucoadhesive polymers. Traditional vaginal formulations include tablets, suppositories, and semi-solid dosage forms. However, the retention of these dosage forms is problematic due to the clearance of vaginal fluids. In recent years, vaginal mucoadhesive formulations have been exploited to extend the residence time of drugs in the vaginal lumen. In the design of vaginal microbicides, mucoadhesive gels, tablets, and films are currently under investigation to improve user compliance.

Vaginal gels

Vaginal gel is a semisolid dosage form with high content of water which is the most widely used mucoadhesive vaginal drug delivery system. It is a well-accepted product and has been applied for moisturizing, lubrication, adjusting vaginal pH, and the delivery of various APIs. The gel dosage form offers multiple advantages in the vaginal drug delivery. It is able to spread on the

mucosal surface to achieve an intimate contact with vaginal mucosa[196]. Because of the spreadability, gels can provide chemical or physical barrier to impede the entry of infectious virus including HIV[197]. Moreover, due to the high water content and rheological property, gels provide the functions of hydration and lubrication.

The employment of mucoadhesive polymers can improve the retention time of gels on the mucosal tissue to reduce the loss of drug and prolong the therapeutic effect [198]. Mucoadhesive polymers, such as polycarbophil, HEC, HPMC, or chitosan, have been utilized in gel formulations to eliminate the rapid clearance by vaginal fluids[199].One of the marketed vaginal gel containing 1-3% polycarbophil (Replens® gel) can retained on the vaginal mucosal tissue for 3-4 days [200]. The drug release rate from gel products can also be modified by changing the cross-linking density or molecular weight of mucoadhesive polymers[201].

There are numerous vaginal mucoadhesive gel products available on the market, such as Aci-Hel, Crinone Zidoval, Miphil, Replens, Conceptrol, Gynol-II and Advantage 24[202]. Thus, gel dosage form is a familiar vaginal dosage form to women. Given this and its other merits mentioned above, gels might be the most widely studied pharmaceutical formulations in the development of vaginal microbicides. To date, first-generation microbicides including nonoxynol-9, SAVVY, Carraguard, cellulose sulfate, PRO2000, and BufferGel, as well as second-generation microbicide 1% tenofovir vaginal gel have been evaluated in clinical trials for HIV prevention[61, 203]. No protection was observed for the first-generation microbicide gels in HIV prevention, whereas the 1% tenofovir gel showed encouraging effectiveness in HIV prevention.

However, there are some drawbacks associated with vaginal gels. The gel dosage form causes leakage and messiness after vaginal administration. It is difficult to control the accurate

amount of drugs being delivered due to the non-uniform distribution and leakage[204]. As compared to the solid dosage form, gel dilutes vaginal fluid which disrupts the vaginal innate immunity[55]. Also, the need of applicator increases the cost of this dosage form.

Vaginal tablets

Vaginal tablet is a solid dosage form which can be easily manufactured with low cost and scaled up to industrial levels. Tablets present advantages of precise dosing, discreet, potential for high drug loading, and stability compared to semisolid or liquid dosage forms. Vaginal tablets are usually designed in double convex or ovule shape for easy insertion[205]. Vaginal tablets have been used to deliver anti-infective agents, hormones, and plant extracts and commercially available. In addition to the active ingredients, tablets also contain excipients including diluent, binder or granulating agents, glidants, and lubricants. They can be manufactured by direct blending and dry granulation.

Tablets used for vaginal drug delivery can be classified into two categories based on the incorporation of different polymers: fast-disintegrating tablet and controlled-release tablet. The employment of mucoadhesive polymers is able to maintain the retention of tablets in the vaginal cavity after hydration by the vaginal fluid[206]. Mucoadhesive tablets can be prepared by compressing the mixture of mucoadhesive polymers and APIs or coating the mucoadhesive solution to the surface of tablets[207, 208]. Multiple mucoadhesive tablets have been developed for topical microbicides. Woolfson et al. developed freeze-dried dapivirine vaginal tablets through the incorporation of HPMC and carbopol to provide enhanced mucoadhesion[209]. Another example is the bioadhesive mini-tablets designed by Hiorth M. et al. The tablets employed various cellulose derivatives to not only improve the mucoadhesion to vaginal tissue but also exert pH dependent controlled release of drugs[210]. The most recent mucoadhesive

vaginal tablets for HIV prevention was developed by Notario-Péreza F. et al. This tablet containing tenofovir was formulated with HPMC, chitosan, guar gum and Eudragit® RS. This study showed that the designed tablet resided on the vaginal mucosa for 96 h and the drug was released in a sustained manner for 72 h[211].

Meanwhile, the tablet composition plays an important role in the user preference. The Product Acceptability Study (PAS II) investigated the acceptability of three vaginal dosage forms and showed that the vaginal tablet made by this particular formulation was the least preferred dosage form compared to the film and soft-gel capsule[212]. However, a microbicides Praneem polyherbal vaginal tablet developed by Panacea Biotec was shown with ease of use and no impact on sexual practice as reported by 95% of women[213]. This disparity in the user preference result demonstrates the importance of polymer selection in the design of vaginal tablets.

Tablets provide an alternative option for vaginal drug delivery. Nonetheless, some challenges remain in the development of effective microbicides. Tablets may not be able to dissolve fast enough to spread the vaginal cavity in the low volume of vaginal fluids[205]. Additionally, tablets have relatively large product volume that causes difficulty of insertion, dryness or irritation to the vagina[214]. As mentioned above, user preference is affected by tablet formulation which needs to be considered in the product development.

Vaginal films

Vaginal film is a solid dosage form, which has advantages including low product volume, discreet use, less leakage compared to gel products, and no need of applicator. The film has limited volume, which minimally interrupt innate immunity of vaginal mucosa[205]. Vaginal

films can be manufactured by solvent-casting and hot-melt extrusion methods based on the property of polymers and APIs[215, 216]. Solvent-cast method is preferred for water-soluble polymers and heat sensitive APIs. Hot-melt extrusion is more suitable for the hydrophobic polymers and APIs that are susceptible to hydrolytic degradation. From the product development stand point, film is a reproducible dosage form that has low unit dose cost [217]. It is manufactured as a thin strip which is generally in the square or rectangle shaped format and flexible to be folded for insertion.

The vaginal film has been utilized for delivery of the spermicide such as nonoxynol-9 as a contraceptive (VCF®). Other products loaded with deodorants and lubricants including (VCF® Dissolving Feminine Deodorant Film and VCF® Dissolving Vaginal Lubricant Film, Apothecus) are available on the market. More applications such as delivering topical microbicides or hormones are under investigation. The current vaginal films on the market/under investigations are fast-dissolving and the majority of them are made of hydrophilic mucoadhesive polymers[218]. By using mucoadhesive polymers such as PAA, polyethylene glycol (PEG), polyvinyl alcohol (PVA), and cellulose derivatives, films can disperse or dissolve in the vaginal fluid to form a bioadhesive and viscous gel which extends the residence of delivered APIs on the vaginal mucosa[216, 219-222]. In addition to the film forming polymer, plasticizers such as propylene glycol, glycerin, and PEG are also necessary to provide flexibility and ensure adequate tensile strength of vaginal film for packaging and handling[205]. Film properties such as disintegration, controlled release, and mucoadhesion can be modified by the amount and composition of excipients incorporated in film matrix. For example, to increase the disintegration of vaginal films in the vaginal fluid, super disintegrants such as veegum, croscarmellose sodium, and cross-linked polyvinyl pyrrolidone are commonly added to the film

formulation[223]. In the selection of excipients in vaginal films, both the compatibility among excipients and tolerability of excipients in the vagina need to be taken into consideration. The film dosage form is highly versatile and well-controllable. In order to control the drug release from film dosage forms, a double layered film format was developed using PVA and pectin to control the release of two antiretroviral drugs[224]. Apart from the multilayered film, the single layered thin film can be used to deliver multiple drugs simultaneously[225, 226]. In addition, films allow for the combination of different drug delivery strategies. For example, the nanoparticle loaded vaginal film products have also been developed [227-229].

Issues that may affect the women's acceptability for vaginal dosage forms need to be taken into consideration during the development of vaginal products. Since only a few vaginal film products are available on the market, the unfamiliarity of the vaginal film to women might limit the acceptability and trust by women to use this product. However, as mentioned before, PAS II showed that more women preferred to use films (61%) rather than soft-gel capsules (53%) and tablets (49%) than because of their fast dissolving and easier insertion[212]. Another report from M El-Sahn et al. illustrated that as compared to vaginal rings, films were chosen more often by women from Uganda, Nigeria and South Africa[230]. For some women, it was noticed that the issue of difficulties in the film insertion due to the stickiness of film on fingers was reported[55]. Additionally, the currently marketed and clinically evaluated vaginal films are fast-dissolving films which require frequent dosing regimen. This might cause lower user adherence to some women. Therefore, more efforts are required to improve the design of vaginal film dosage forms.

In summary, short retention time of drug delivery systems due to the rapid mucosal clearance present a major challenge in the design of coitally-independent vaginal drug delivery

systems. Vaginal mucosa provides a promising site for the delivery system to reside. To improve the mucoadhesion, numerous mucoadhesive polymers have been applied in different vaginal dosage forms. The mucoadhesive formulation provides a promising strategy to decelerate the loss of delivery systems and prolong the effect of APIs.

1.5 HYPOTHESIS AND SPECIFIC AIMS

As discussed above, several clinical studies evaluating IVR and gel products have shown the positive relationship between the effectiveness of topical microbicides in HIV prevention and product adherence. Multiple studies, encompassing 2500 women between ages of 15-40 years worldwide, have revealed that the polymeric vaginal film is a safe and highly acceptable dosage form[55, 212]. However, the current available vaginal films on the market or under clinical evaluation are daily or coitally-dependent, which requires high dosing frequency and may potentially lead to lower user adherence. One of the promising strategies to improve user adherence is to develop a product which is easy to administer and has reduced dosing frequencies. Therefore, we took the advantage of the film dosage form and aimed to design it as a coitally-independent dosage form to improve the user adherence of vaginal microbicides to women.

Provide and maintain an extended delivery of drugs in the target tissue is the key to design an effective coitally-independent microbicide for vaginal administration. Formulation with prolonged tissue residence is one of the promising strategies. Mucoadhesive polymers have been widely applied to prolong the retention time Among them, thiomers offer advanced mucoadhesion via the formation of covalent bond between polymer and vaginal mucus.

Thiolated chitosan is ranked as the most mucoadhesive polymer as compared to other thiomers. In addition to mucoadhesion, sustained drug release rate is also important for coitally independent dosage forms. PLA based polymer is a biocompatible and biodegradable polymer that has been widely applied for sustained or controlled delivery of APIs. The degradability and mechanical property of PLA based polymers can be tuned by copolymerization with polyglycolide or PCL. In addition, the PLA based polymers can be fabricated into different shape and drug delivery systems such as thin film and nanoparticles based on the different manufacture methods.

Based on the above information,

We hypothesize that through the modification of vaginal film polymeric matrix or incorporation of nanoparticle systems, enhanced drug release and mucoadhesion can be achieved. This can potentially lead to new designs of vaginal products which require less dosing frequency.

To achieve this goal, three specific aims are proposed:

Aim 1 explores the mucoadhesion and feasibility of thiomers in the design of vaginal films for delivery of dapivirine and levonorgestrel simultaneously as a multipurpose prevention technology.

Aim 2 investigates the feasibility of poly(lactide-co-ε-caprolactone) in the design of a vaginal film for delivery of integrase inhibitor MK-2048.

Aim 3 develops and evaluates a mucoadhesive-nanoparticle loaded vaginal film to achieve the sustained release profile for MK-2048.

2.0 FABRICATION AND CHARACTERIZATION OF MULTIPURPOSE PREVENTION TECHNOLOGY (MPT) BIOADHESIVE FILM

2.1 INTRODUCTION

As discussed in Chapter-1, user adherence is paramount to the effectiveness of vaginal microbicides in HIV prevention. One of the promising strategies for improving user adherence is to design the microbicide as a multipurpose tool. Contraceptives are used by 64% of women worldwide. It is believed that design of products which combine HIV prevention agents with products that women already use, may lead to enhanced compliance. It is important to note that 40% of all the pregnancies are unintended or mistimed[231]. This percentage is even higher in some developing countries such as South Africa[232], where unfortunately, higher incidence of HIV infection is also noted mostly due to the unprotected sexual intercourse[233]. Given that, more than 90% of children infected with HIV result from the mother-to-child transmission[234]. Reducing HIV infection rates in mothers would also contribute to the reduction in children infected by this mode. Therefore, an effective multipurpose prevention product for HIV infection and unintended pregnancy is a promising tool for reducing the overall healthcare burden to women.

Male and female condoms, diaphragms and cervical caps are the currently available multipurpose prevention technology (MPT) barrier methods. These MPT products provide

efficient protection for unintended pregnancy and sexual transmitted infections including HIV-1 if they are used correctly and consistently[235-239]. However, in practical use, the failure rate is much higher than expected, which is primarily due to the unsatisfactory adherence of those products[240]. The major reason of low adherence is the disturbance on the sexual intercourse and women's inability to negotiate condom use with their male partners in many cases[241]. Women need a new generation of safe and self-initiated MPT products with better user compliance to protect themselves from unintended pregnancy and HIV infection.

A number of the current MPTs being developed use intravaginal rings for delivering anti-retroviral drugs and contraceptives. A 3-month vaginal ring containing dapivirine (DPV) and levonorgestrel (LNG) is under clinical evaluation (MTN-030/IPM 041) for safety and pharmacokinetics (PK)[242]. The DPV vaginal ring has shown adherence-dependent effectiveness in HIV prevention in the ASPIRE clinical trial. Women who used the vaginal ring reported some issues such as ring expulsion and detectability by their male partners, which may reduce the acceptability of ring to women and possibly contributed low adherence[52]. An alternative dosage form which has been shown to be acceptable to women is polymeric film. This dosage form dissolves in the vagina and has low volume which has less detectability during sexual intercourse. The favorable safety and PK profiles of films have been illustrated in two clinical trials[55, 212, 243]. The polymeric film represents a promising alternative MPT dosage form to improve the product effectiveness.

The advantages of polymeric films as a potential vaginal dosage form has been outlined in Chapter-1. However, to the best of our knowledge, a vaginal film based MPT approach has yet to be explored. Further exploration of the polymeric film as an advanced functional drug delivery platform featuring mucoadhesiveness and controlled release is also novel. A series of

mucoadhesive polymers, including poly(acrylic acid), cellulose derivatives, chitosan, hyaluronic acid, pectin and thiomers, have been discussed in Chapter 1. Thiomer was reported as an advanced mucoadhesive polymer due its covalent bonding with mucin [244]. The excellent tolerance and safety of thiomer containing dosage forms has been demonstrated in animals[245, 246] and clinical trials[247]. In addition, the features of thiomers such as in situ gelling[248], controlled release[249, 250], tissue permeation enhancement[251], and excellent mucoadhesion at vaginal pH of 3.8-4.5[252] makes it an attractive excipient option for intravaginal drug delivery.

As compared to the first-generation microbicides, antiretrovirals-based microbicides have a specific mechanism of action to inhibit HIV. DPV, a non-nucleoside reverse transcriptase inhibitor, has been evaluated in multiple clinical trials with different dosage forms such as gels, polymeric films and vaginal rings, for the prevention of sexually transmitted HIV. Among them, the DPV vaginal ring demonstrated effective protection against HIV-1 infection in two Phase III the clinical trials[52].

Hormonal contraceptives have been utilized to eliminating the risk of unintended pregnancies since 1960. LNG is a leading drug prescribed as oral and topical contraceptives, which has demonstrated excellent efficacy and safety in birth control[253]. Because of this, LNG is prioritized to be used as the contraceptive hormonal component in many of the currently undergoing MPT products. When developing an MPT product, it is critical to investigate potential drug-drug interactions and their impact on the PK and pharmacodynamic (PD) properties[254]. Although no diminished effectiveness on the contraception was found when the oral hormonal LNG was used in combination with DPV vaginal ring in the clinic [255], the co-delivery of these drugs in a vaginal film has not been evaluated regarding their local PK and PD.

The aim of this proof-of-concept study was to describe the development and characterization of an innovative bioadhesive film platform for the combined delivery of an anti-retroviral drug, DPV, and a progestin, LNG. This study not only presented the *in vitro* feasibility of bioadhesive film in simultaneously delivering DPV and LNG, but also demonstrated this ability using the pigtailed macaque model. In this study, the pigtailed macaque model was utilized since it has shown advantages over small animals in the evaluation of topical microbicides. The pigtailed macaque shows similar reproductive tract to women such as regular menstrual cycle, typical hormone, genital anatomy and major vaginal flora[256, 257]. Our *in vivo* data demonstrated that both agents can be delivered in a sustained fashion, and provided adequate local (vaginal lavages and tissue) and systemic drug concentrations required for therapeutic efficacy.

2.2 METHODS

2.2.1 Materials

DPV was provided by the International Partnership for Microbicides (IPM, Silver Spring, MD, USA). LNG was purchased from CHEMO. PEG 8000 was purchased from Spectrum (Gardena, CA, US). Hydroxypropyl methyl cellulose E5 (METHOCEL™ E5) was obtained from Dow Chemical Company (Midland, MI, US). Polyvinyl alcohol 4-88 was purchased from Millipore Sigma (Temecula, CA). Ultrapure water was prepared by passing distilled water through a Milli-Q® Reagent Water System (Millipore®). Blue dye and 2-immunothiolane were purchased from Fisher Scientific (Pittsburgh, PA). Porcine gastric mucin and sodium starch glycolate were

purchased from Sigma-Aldrich (Carlsbad, CA). All the other chemical reagents for film preparation, mobile phase solvents, were purchased from Fisher Scientific (Pittsburgh, PA).

2.2.2 Synthesis and characterization of thiolated chitosan

Thiolated chitosan was synthesized following the method reported by Dr. Andreas Bernkop-Schnürch's group with some modifications[178]. 1g of chitosan (medium molecular weight around 300 kDa) was dissolved in 100 mL of 1% (v/v) acetic acid. The pH of the chitosan solution was adjusted to 5, 6 or 7 with 5 M NaOH. Premeasured 2-iminothiolane HCl was added in chitosan solution under stirring. The reaction with continuous stirring was stopped after 24 h. The solution was dialyzed multiple times against 5 mM HCl, 5mM HCl containing 1% NaCl, and 1mM HCl using dialysis tubing with 10k Da molecular weight-cutoff (MWCO). The final solution was lyophilized and stored under 4 °C for future use.

The degree of the thiolated groups to chitosan was determined using Ellman's assay. Briefly, 5 mg of thiolated chitosan was dissolved in 2.5 mL Milli-Q water. The Ellman's reagent was prepared by dissolving 3 mg of 5,5'-dithiobis (2-nitrobenzoic acid) in 10 mL of 0.5 M phosphate buffer pH 8.0. Then 250 µL of 0.5 M phosphate buffer pH 8.0 and 500 µL of Ellman's reagent were added to the above thiolated chitosan solution and incubated for 2 h. Afterwards, the samples were centrifuged at 12,000 g for 5 min to remove precipitated polymers. 250 µL of the reacted supernatant was transferred to a microtitration plate to measure the absorbance immediately at a wavelength of 450 nm. The standard curve was prepared by dissolving L-cysteine HCL in chitosan solution by series dilution (1-1000 µM). The absorbance was tested by the same method as described above. The amount of thiolated groups to the thiolated chitosan was calculated based on the standard curve.

2.2.3 High-performance liquid chromatography (HPLC) for simultaneous detection of DPV and LNG

A Waters HPLC system with 600 series pump, 2487 ultraviolet (UV) detector and Empower data acquisition system was used for analytical method development. The Waters Sunfire C8 HPLC column (2.5 µm, 4.6×50mm) was used to separate DPV and LNG. The mobile phase consisted of (A) 0.1% of trifluoroacetic acid in Milli-Q water and (B) 0.1% of trifluoroacetic acid in acetonitrile. It was using a gradient elution of 10% B initially, 10% B to 70% B from 0.7 to 4 min with linear change and then goes back to 10% B at 7 min with linear change and stays with 10% B at 7-30 min. The flow rate was 2 mL/min. Sample injection volume was 10 µL and both drugs were determined at wavelength of 254 nm. The autosampler was kept at 4 °C.

2.2.4 Liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) method development

Quantitation of DPV and LNG was conducted using LC-MS/MS. Specifically, a Thermo UltiMate3000 UPLC system coupled with a Thermo TSQ Quantum Access MAX mass spectrometer (Thermo Finnigan, San Jose, CA, USA) was used. The LC separation was carried out on a Phenomenex Hyperclone BSD C8 (3 µm, 4.6×150 mm) column. This method was developed for quantification of DPV and LNG in swab, vaginal and cervical tissues, and plasma samples. The mobile phase consisted of (A) 5 mM Ammonium Formate in 60% acetonitrile and (B) 5 mM Ammonium Formate in 80 % acetonitrile, using a gradient elution with mobile phase B starting from 0% of to 100% over 1.5 min and held at 100% for 2 min and then equilibrated back to 0% for the rest 2.5 min. The flow rate was 1.0 mL/min and injection volume was 40 µL.

Column was kept at 40 °C. The column outlet was directly coupled to the heated electrospray ionization sample inlet of the mass spectrometer using positive selective reaction monitoring scan. A spray voltage of 3000 V was applied to the ESI needle. The mass transitions were 330.2/158.0 for DPV, 334/145 for d4-DPV and 313.1/245.1 for LNG, using collision energies of 25V. The scan width (m/z) was set at 0.6, and the scan time was set at 0.5 sec. An XCalibur software package was used for acquiring data.

2.2.5 Film formulation development and physicochemical characterization

Bioadhesive film was manufactured using solvent-cast method as described before with some modifications (formulation is listed in **Table 1**)[226]. Briefly, thiolated chitosan was hydrated in Milli-Q. water first and stirred uniformly. Polyvinyl alcohol 40-88, polyethylene glycol 8000, and Methocel E5 were dissolved in Milli-Q. water and then mixed with thiolated chitosan solution until uniform. DPV and/or LNG were first dispersed in glycerin and propylene glycol, and then mixed with the polymer solution. The solution was casted on a polyester substrate by an automatic film applicator (Elcometer® 4340) using a 4 ″ doctor blade. The film sheet was allowed to dry for 15 mins at 72 °C before it was removed from the substrate. Once film sheets were obtained they were cut using a die press into 1 ″ × 1 ″ individual unit doses.

The quick-dissolving film was made as a control using the same manufacture method. The film formulation is listed in **Table 1**. This film consists of the same excipients but replacing thiolated chitosan with superdisintegrant sodium starch glycolate.

For both bioadhesive and quick-dissolving films, to aid in visualization for film retention in macaque studies, a water soluble blue colored dye (FD&C) was also added into films,

respectively. To prepare these films, the dye was first dissolved in aqueous phase and mixed with polymer solutions. The film was prepared by the same method as mentioned above.

Table 1. Film formulation of quick-dissolving film and bioadhesive film

Component	**Bioadhesive film (w/w %)**	**Quick-dissolving film (w/w %)**
Milli-Q water	86.10	83.6
Polyvinyl alcohol 40-88	7.02	7.02
Polyethylene glycol 8000	2.34	2.34
Methocel E5	1.75	1.75
Sodium starch glycolate	0	3.5
Thiolated chitosan	1	0
Glycerin	0.73	0.73
Propylene Glycol	0.73	0.73
Drug (DPV/LNG)	0.34	0.34
Total	100.00	100.00

The films were evaluated with physicochemical characterizations. Drug content: DPV/LNG films were dissolved in 50% of acetonitrile and heated up to 60 °C to ensure that the film matrix was completely dissolved. An aliquot was withdrawn and centrifuged at 9,000 rpm for 5 mins. The supernatant was further diluted accordingly and analyzed by HPLC as described above.

The water content, puncture strength and disintegration of all films manufactured in this study were tested using the published methods or method developed by our group[226, 258-260]. Briefly, 1). water content: The residual water content in the films was measured by the titration method using a Karl Fisher apparatus (Metrohm, 758 KFD Titrino). Films were weighed and sealed in a glass bottle. Oven temperature was set up at 120 °C for extracting the residual water in the film. Data was acquired and analyzed by Tiamo 2.2 software. 2). Puncture strength: puncture strength of TNP films was measured using a texture analyzer (TA. XT. Plus®, New

York, NY). Briefly, the film was fixed in the TA-108S5 fixture with five 15 mm openings, and the maximum force required to puncture the film using a rounded end ball probe (TA, 8A, 1/8") was recorded by the Exponent® software. 3). Disintegration: disintegration of TNP films was evaluated using a previously reported method that utilizes texture analyzer (TA. XT. Plus®, New York, NY)[261]. Briefly, the film was fixed in the TA-108S5 fixture. 15 µL of Milli-Q. water was added to wet the exposed films. A rounded end ball probe (TA, 8A, 1/8") with constant force of 5 g was loaded on the hydrated film spot. Process was ended once the film was punctured. The time (disintegration) from start to end of the trigger force was recorded by Exponent® software.

2.2.6 *In vitro* dissolution test

To compare the drug dissolution between DPV containing bioadhesive and quick-dissolving films, a USP IV apparatus (SOTAX CP7, Horsham, US) was used. Sink condition for DPV was maintained using 60 mL of 1% Cremophor EL in Milli-Q water as the dissolution medium. The drug release was monitored at 37 °C for 4 h to evaluate the release kinetics. The amount of drug released was determined by analyzing the samples online at predetermined time intervals using the connected UV-Vis system (Evolution 300, Thermofisher) at 254 nm.

To assess the drug dissolution of DPV/LNG single and combination bioadhesive films, a USP IV apparatus was used. LNG has poor solubility in 1% Cremophor EL solution. To maintain the sink condition for both DPV and LNG, 40% of acetonitrile was utilized as the dissolution medium. The drug release was monitored at 37°C for 60 mins. 0.5 mL samples were collected at predetermined time intervals and analyzed by HPLC as described above to quantify the amount of released drugs.

2.2.7 *Ex vivo* tissue mucoadhesion

The *ex vivo* mucoadhesion testing of films was carried out by texture analyzer (TA. XT. Plus®, New York, NY) using a 5 kg load cell. The film was attached to the head of a cylinder probe (1" radius, TA-57R) by double-sided adhesive tape. Porcine intestinal tissue was fixed in the tissue holder with an exposure area of 0.785 cm^2. Before the probe with the film contacted the tissue, 15 µL of the vaginal fluid simulant (VFS) was added to the surface of the tissue and left for equilibration for 15 secs. Following equilibration, the probe with the film was moved towards the tissue while constantly applying 150 g force for 60 secs. The probe was withdrawn to detach the tissue at speed of 0.5 mm/s. The change of the withdrawal force involved in detaching the film from tissue and the debonding distance were recorded by the Exponent software. The work of adhesion calculated from the area under the force versus distance curve was used for comparing the mucoadhesiveness of different film formulations.

2.2.8 Interaction of mucin and thiomers

The interaction between mucin and thiolated chitosan was evaluated by rheological property. 12% (w/v) of thiolated chitosan was hydrated in Milli-Q water and diluted with equal volume of 0.1 M phosphate buffer (pH 6.8) to obtain a concentration of thiolated chitosan at 6% (w/v). The prepared thiolated chitosan solution was mixed with equal volume of 8% (w/v) of porcine gastric mucin and stirred gently using a spatula. The solution pH was adjusted to 7 using 2 M NaOH and incubated for 20 min at room temperature. After incubation, 1% (w/v) of L-cysteine HCL was added under stirring. The mixture was incubated for 20 min at room temperature. Then the polymer-mucin incubates were transferred to viscometer and equilibrated on the plate for 3 min

at 25 ± 0.5 °C. Rheological profile was determined using the CP51 spindle on a cone/plate Brookfield Model HADVIII+ viscometer (Brookfield Eng. Lab., Inc., Middleboro, MA). Data was collected using Rheocalc software (Brookfield Eng. Lab., Inc.). Plastic viscosity and yield stress was calculated by Rheocalc Software using the Casson equation, which gave the best fit. Three controls, thiomer solution without cysteine and chitosan solution with/without cysteine, were prepared using the same method.

2.2.9 Tissue toxicity of thiomers

Human ectocervical tissue was obtained from Magee-Womens Hospital Tissue Procurement Center (University of Pittsburgh Medical Center, Pittsburgh, PA USA), under the protocols approved by the Institutional Review Board (PRO09110431). All the tissues used in this manuscript were collected from premenopausal women (age 30-55) undergoing hysterectomy with benign health conditions. The excised tissues were transferred to the lab within Dulbecco's Modification of Eagles Medium (DMEM) and then snap frozen with dry ice and methanol, and further stored at -80 °C for later use.

The tissue toxicity of thiomer was evaluated using MTT assay. The frozen tissue was thawed in 37°C water bath for 5 mins. The stromal layer was removed from the excised ectocervical tissue using a Thomas-Stadie-Riggs tissue slicer (Thomas Scientific, Swedesboro, NJ). Thickness of the tissue was measured using a digital micrometer. 6 mm biopsies of the ectocervical tissue were obtained using biopsy punch (INTEGRA YORK PA INC). Biopsies were incubated with 1 mL of DMEM (negative control), 1% w/v thiomers in DMEM, and 0.6% w/v of formulated Nonoxynol-9 (N-9, GYNOL II®) (positive control), respectively, and shaken in 37 °C water bath for 6 h. After 6 h, 200 µL of 0.5mg/mL MTT solution was added into the

treated tissues and incubated in 37 °C water bath for 3 h. Then MTT solution in tissue samples were replaced with 1 mL methanol and incubate overnight in the dark at room temperature. Tissues were removed out of the methanol and dried with paper towel for measuring the tissue weight. The methanol solutions were collected for absorbance measurement at 595 nm. The percentage of viability of tissues was presented as (abs-blank)/mg of tissue as compared to that of DMEM.

2.2.10 *In vivo* evaluations in macaques

Film retention and in vivo drug release

All the macaque studies were collaborated with Dr. Patton's group from the School of Medicine at the University of Washington. Five sexually mature female pigtailed macaques (Macaca nemestrina) with weighing 6-10 kg were used in this study. All five animals were of breeding age and housed at the Washington National Primate Research Center (WaNPRC). All the procedures pertaining the use of these animals have been approved by the Institutional Animal Care and Use Committee of University of Washington, and experiments were performed in accordance with the National Institutes of Health's laboratory animal use guidelines. Two types of 1" × 1" blue dye containing films were evaluated in this study: DPV bioadhesive films and DPV quick-dissolving film. Films were intravaginally inserted at time 0. Colpophotography was utilized to visualize the presence of the film before, after film insertion as well as each time point. A Dacron polyester-tipped swab was used for vaginal swab sample collection at each time point. DPV was extracted from swabs using acetonitrile. After drying under nitrogen, final samples were reconstituted in 500 µL of 60% of acetonitrile. Drug content was quantified by LC-MS/MS method as described above.

Assessment of DPV/LNG combination bioadhesive film in macaques

DPV/LNG single and combination bioadhesive films were studied in three arms. Five female pigtailed macaques were tested in each arm for PK evaluation. The study time-line and biological sample collection points are shown in **Figure 1**. Briefly, after vaginally insertion of each film, drug in vaginal fluid was collected using a Dacron polyester-tipped swab at time points of 6 h, 24 h, 2-day, 4-day and 7-day. Drug extraction method for DPV and/or LNG from the swab was same as described before. For vaginal and cervical biopsies collection: at 6h, biopsies were collected from three macaques; at 24 h, biopsies were collected from the other two macaques; at day-4, biopsies were collected from two macaques that were biopsied at 6 h; at day-7, biopsies were collected from one macaque that was biopsied at 6 h and two macaques that were biopsied at 24 h. This collection timeline was chosen to ensure sufficient recovery of macaque cervicovaginal tissue after each biopsy. The extraction solvent for DPV and/or LNG from tissue biopsies was a mixture of methanol, acetonitrile, and methyl tert-butyl ether (MTBE). After drying under nitrogen, final samples were reconstituted in 500 µL of 60% of acetonitrile. Drug content was quantified by LC-MS/MS method as described above. Plasma samples were collected at each time point for five macaques. Drug extraction from plasma samples was conducted by protein precipitation and supernatant was collected for drug analysis by the LC-MS/MS method described above.

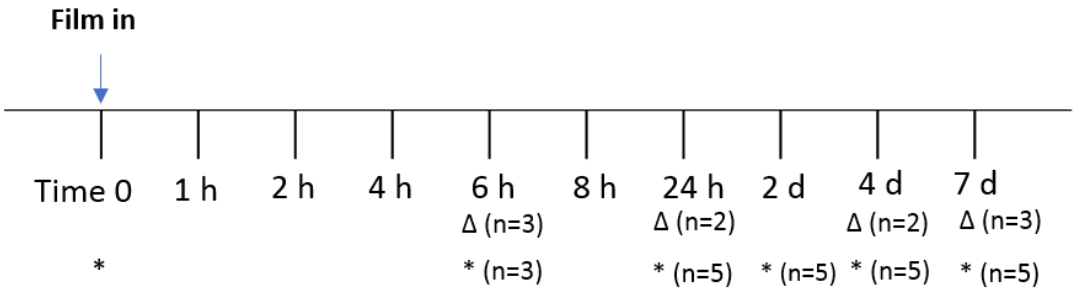


Figure 1. **Study design of *in vivo* assessment of DPV/LNG bioadhesive film**

Blood was collected at each time point
(*) indicates the time points for vaginal swab samples collection
(Δ) indicates the time points for vaginal and cervical biopsy collection
Films tested: DPV bioadhesive film, LNG bioadhesive film, DPV/LNG combination film

2.2.11 Statistical analysis

All data was presented as mean ± standard deviation (SD). Statistical analyses were conducted using the GraphPad Prism software version 7. The *t*-test was used for between two groups, with $p \leq 0.05$ as statistical significance and $p \leq 0.001$ for highly significant.

2.3 RESULTS

2.3.1 Thiolation degree determination in the synthesized thiolated chitosan

Thiolated chitosan was synthesized using chitosan with various molecular weights (**Table 2**). From the thiol degree characterization, a large variability was observed for chitosan with wide molecular weight range (50-700 kDa). To develop reproducible thiomers from a product development standpoint, the chitosan with intermediate molecular weight range (190-310 kDa)

was selected. More thiol groups on thiomers provide more mucoadhesiveness. The thiolation was conducted under different pH values of 5, 6, and 7 to access the effect of pH on the degree of thiolation in chitosan. With an increase in pH, a reduction in thiolation degree and solubility were observed. Additionally, since film manufacture requires preparation of aqueous solutions of thiomers and other excipients, the aqueous solubility of thiomer is crucial. The results showed that thiomer synthesized under at pH 5 displayed the highest thiolation degree and the best water solubility. Thus, thiomer synthesized by medium molecular weight range and at pH of 5 condition was chosen for further studies.

Table 2. Characterizations of thiomers

Chitosan (MWR, kDa)	pH	Thiol Degree (μmol/g)	Solubility in water
50-700	5	183.45±130.29	+++
190 -310	5	182.94±31.44	+++
190 -310	6	61.83±13.31	+
190 -310	7	71.26±27.11	+

MWR: the molecular weight range
+++: dissolve in water very fast (less than 1h)
+: dissolve in water slow (more than 8h)

2.3.2 Film physicochemical characterizations

DPV quick-dissolving film and DPV/LNG single entity and combination bioadhesive films were manufactured using the solvent casting method. These films were characterized for mass, thickness, water content, drug content, puncture strength and disintegration, and the results are shown in **Table 3**. Compared to the quick-dissolving film, all the bioadhesive films showed significantly increased puncture strength and disintegration time ($p < 0.05$). In addition, compared to DPV film and combination film, the mass and thickness of LNG film were higher whereas the water content was lower. LNG loaded film showed higher puncture strength and

disintegration as compared to DPV film, which might be due to the lower water solubility of LNG. Combination film showed longer disintegration than single entity films.

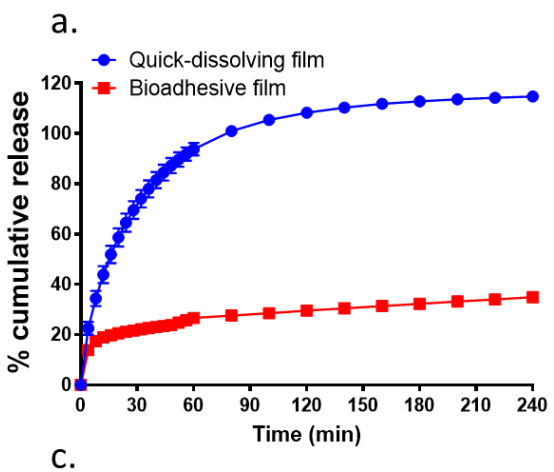
Table 3. Physicochemical characteristics of quick-dissolving film and bioadhesive MPT films

	DPV Quick-dissolving Film	**DPV Bioadhesive Film**	**LNG Bioadhesive Film**	**DPV/LNG MPT Bioadhesive Film**
Appearance	White, transparent, smooth and soft	White, transparent, smooth and soft	White, transparent, smooth and soft	White, transparent, smooth and soft
Weight (mg)	69.20 ± 1.76	69.35±5.9	79.14 ± 8.8	65.85 ± 3.93
Thickness (µm)	155.90 ± 9.75	110.75 ± 12.06	127.5 ± 11.8	105.28 ± 6.96
Water Content %(w/w)	4.87 ± 0.20	6.68± 0.43	4.95 ± 0.17	6.36 ± 0.50
Drug content (mg/film)	1.41 ± 0.13	1.68 ± 0.15	1.80 ± 0.09	1.71 ± 0.15/DPV 1.50 ± 0.13/LNG
Puncture Strength (kg/mm)	3.77 ± 0.58	12.41 ± 0.44	14.18 ± 2.28	13.03 ± 0.50
Disintegration (sec)	56.36 ± 6.49	153.12 ± 33.81	211.33 ± 70.52	228.44 ± 77.46

2.3.3 *In vitro* drug dissolution

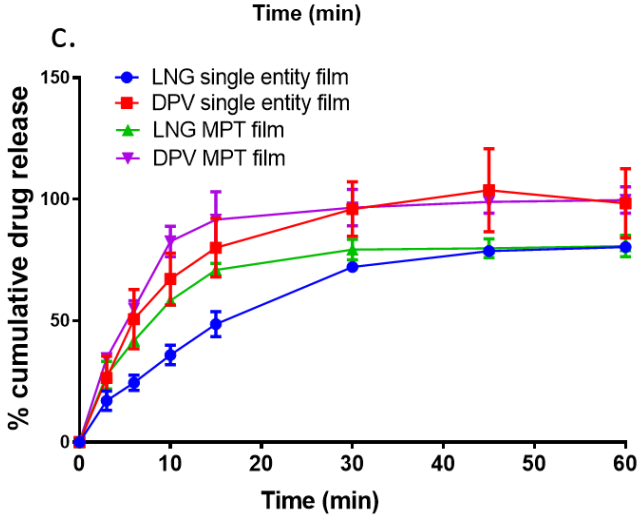
The release kinetics of DPV bioadhesive film was compared to the quick-dissolving film as shown in **Figure 2 a**. 100% of DPV was released out within 60 mins from the quick-dissolving film whereas bioadhesive film only released 27.61 ± 0.26 % of DPV. The drug release rate for DPV in the first 60 mins form both films was analyzed and compared. **Figure 2 b.** demonstrated that thiomer incorporation resulted in significantly slower drug release rate as compared to film without thiomer modification ($p < 0.001$).

Since the bioadhesive film formulation significantly reduced the drug release rate, this film platform was further evaluated for the co-delivery of DPV and LNG. For product quality control, the USP IV apparatus dissolution method using 40% of acetonitrile as the dissolution medium was applied. Both drugs can be released out from either the single entity bioadhesive film or the MPT bioadhesive film (**Figure 2 c.**). The drug release rate for the first 15 mins was analyzed and multiple comparison was conducted. As shown in **Figure 2 d**, either for single entity bioadhesive film or MPT bioadhesive film, DPV showed significantly faster drug release rate than LNG ($p < 0.001$). Meanwhile, both DPV and LNG showed faster drug release rate in the MPT bioadhesive film than in the single entity bioadhesive film (DPV: $p = 0.02$, LNG: $p < 0.001$).



b.

	Drug release rate (60 min)	Significance
Quick-dissolving film	1.36 ±0.07	-
Bioadhesive film	0.27 ±0.03	***



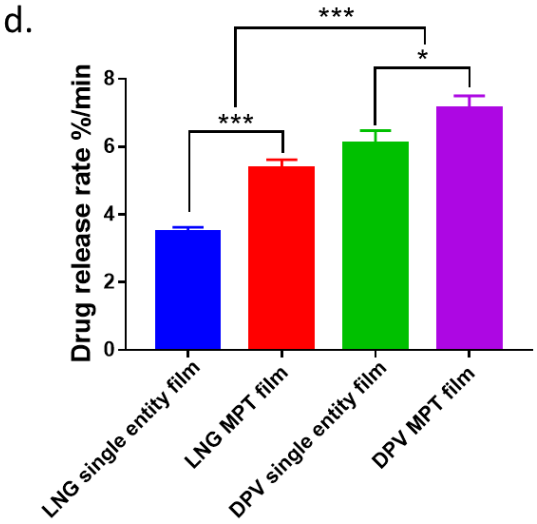


Figure 2. *In vitro* drug dissolution of films

a. Cumulative DPV release from quick-dissolving film and bioadhesive film in 1% Cremophor EL in Milli-Q water. **b.** The drug release rate for DPV in the first 60 mins form the quick-dissolving film and the bioadhesive film. **c.** Cumulative DPV/LNG release from single entity and combination bioadhesive films in 40% of acetonitrile in Milli-Q water. **d.** The drug release rate of DPV and LNG in the first 15 mins for the single entity bioadhesive films and the MPT bioadhesive films. (n=3, *** $p < 0.001$, * $p = 0.02$).

2.3.4 *Ex vivo* tissue mucoadhesiveness

Film tissue mucoadhesion was evaluated by Texture Analyzer using porcine intestinal tissue. The results of the tensile force and work of adhesion for quick-dissolving film and bioadhesive film are shown in **Figure 3 a and b**, respectively. The peak force for thiomer film detaching from mucus tissue was 9.7-fold higher than the quick-dissolving film ($p < 0.05$). Also, the work of adhesion increased 4.8-fold after incorporation of thiomers in the film matrix ($p < 0.001$). Both results indicated that the bioadhesive film showed improved tissue mucoadhesion as compared to the quick-dissolving film.

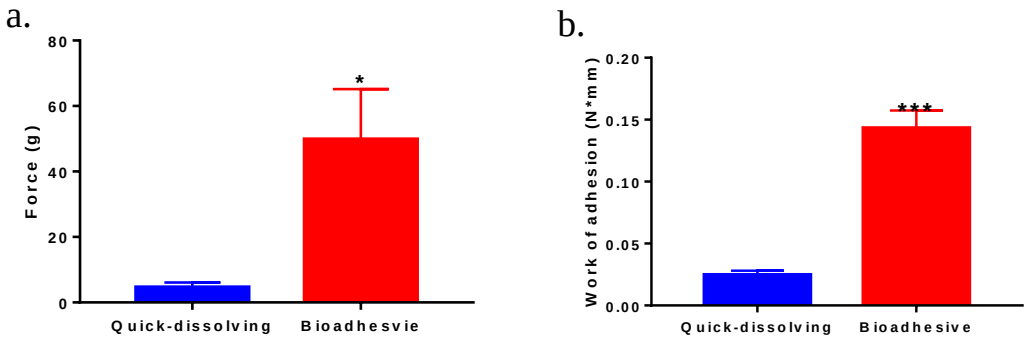


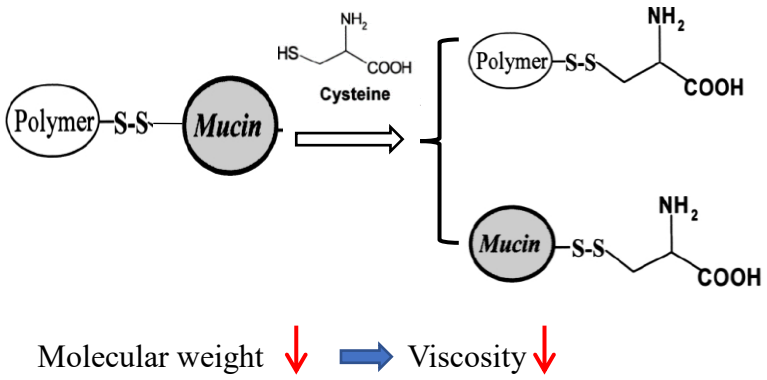
Figure 3. *Ex vivo* mucoadhesiveness of quick-dissolving and bioadhesive films on porcine intestinal mucosal tissues

a. Tensile force for departing quick-dissolving and bioadhesive films from mucosal tissues. **b.** Work of adhesion (area under the curve of tensile force vs. distance) as measurement of mucoadhesiveness of quick-dissolving and bioadhesive films ($n = 4$, * $p < 0.05$, *** $p < 0.001$)

2.3.5 Mechanism of polymer and mucin interaction

The interaction between mucin and thiomers was studied through rheological measurement. As illustrated in **Figure 4 a**, when polymer interacts with mucin via disulfide bond, the molecular weight of polymer-mucin conjugates increases which results in higher viscous solution. Since L-cysteine competitively interact with mucin via the same mechanism, the addition of cysteine in the polymer-cysteine conjugates solution breaks the conjugates into polymer-cysteine and mucin-cysteine which results in lower viscous solution. From **Figure 4 b**, it was observed that the viscosity of thiomers and mucin mixture was significantly reduced with the addition of cysteine as compared to the group without the addition of cysteine ($p < 0.001$). On the contrary, chitosan, a control polymer which has no thiol group, did not show the same trend after adding cysteine in the mixture of chitosan and mucin. These results indicate that in addition to the electrostatic interactions between mucin and chitosan, thiomer formed disulfide bond with mucin which further strengthens the mucoadhesiveness.

a.



b.

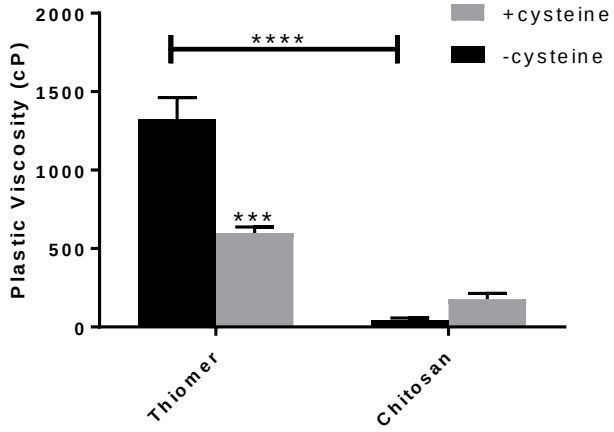


Figure 4. Mechanism of mucoadhesiveness for thiomers

a. Scheme for interaction mechanism between thiomers and mucins. **b.** Plastic viscosity evaluation for a mixture of thiomer and mucin with/without adding cysteine and a mixture of chitosan and mucin with/without adding cysteine. Viscosity comparison were performed between groups of with (black) and without (grey) cysteine using Student's T-test. Values indicate mean ± SD with triplicates. (n = 3, **** $p < 0.0001$, *** $p < 0.001$).

2.3.6 Tissue toxicity of thiomers

The tissue toxicity of thiomers was assessed in human ectocervical tissues using MTT assay. The percentage of tissue viability was determined and showed no significant difference as compared

to DMEM, the tissue culture medium (negative control). Whereas, the positive control, N-9, showed significant reduction ($p<0.05$) in tissue viability 17.18 ± 9.21 after 6 h incubation (**Figure 5**). Thus, thiomers showed excellent safety profile to the human ectocervical tissue.

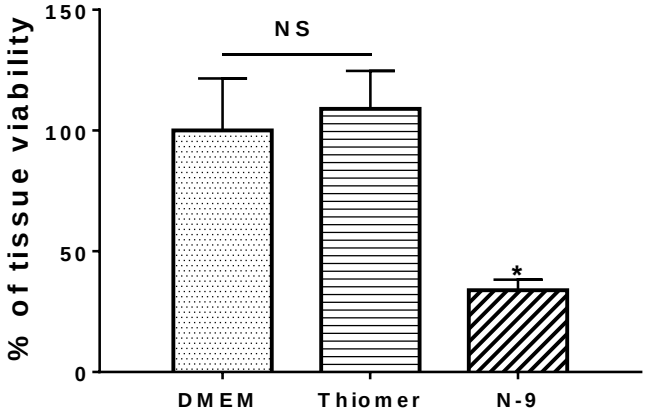


Figure 5. *Ex vivo* tissue toxicity of thiomers.
No significant difference was observed between DMEM and thiomers. Nonoxynol-9 (N-9) was chosen as a positive control. Values indicate mean ± SD with triplicates. (n = 3, * $p < 0.05$)

2.3.7 *In vivo* retention and safety of bioadhesive films in macaque genital tract

The comparison of *in vivo* retention of quick-dissolving film and bioadhesive film was conducted by loading water-soluble blue dye in films for visualization using colposcopy. Colposcopy images were taken at day 1, 2, 3, 4 and day 7 after the film insertion. The representative colposcopy images were shown in **Figure 6**, where bioadhesive film was retained in vaginal compartment for up to 7 days, while the quick-dissolving film can only stay for 3 days. No visual changes for each colposocpic check were observed in the vagina after the film insertion, suggesting that the bioadhesive film was safe. In addition, vaginal swabs were taken for evaluating the *in vivo* drug release from bioadhesive film and quick-dissolving film in vaginal

fluid. We observed that DPV was still detectable in the vaginal fluid for bioadhesive film at day-7, whereas no DPV was detectable for the quick dissolving film at day-7 (**Table 4**).

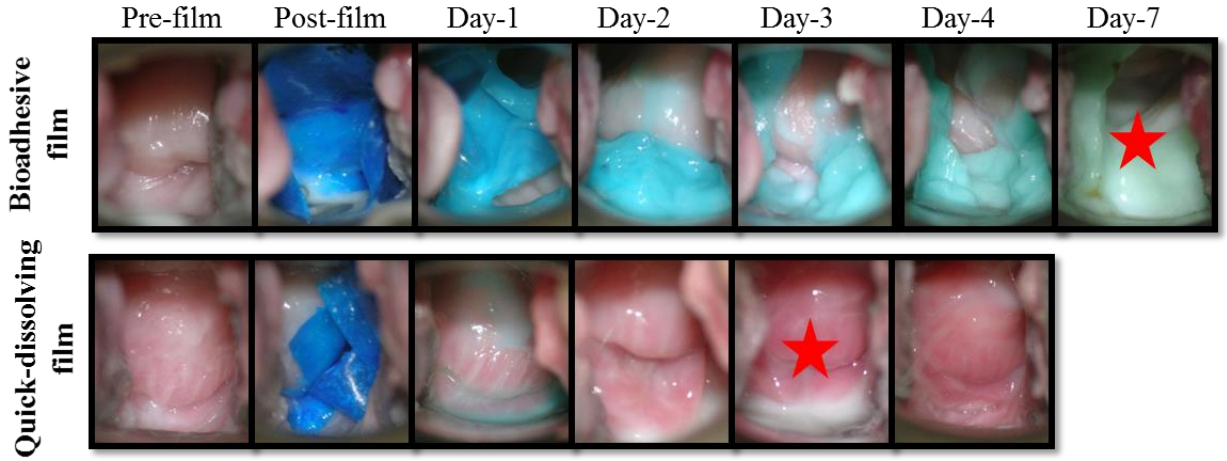


Figure 6. Tissue retention and distribution of bioadhesive film and quick-dissolving film in macaque model.
Representative colposcope image for blue dye loaded bioadhesive film and quick-dissolving film in macaque vaginal compartment (red star marks the last presence of film in vaginal cavity).

Table 4. *In vivo* release of DPV from bioadhesive film and quick-dissolving film formulations

	Bioadhesive film			Quick-dissolving film		
	# of samples	% above LLOQ	Median (range)	# of samples	% above LLOQ	Median (range)
1-day	5	100	12.3 (2.87-36.71)	5	100	18.56 (10.18-69.92)
2-day	5	100	2.62 (0.91-24.63)	5	100	1.68 (1.51-2.85)
3-day	5	100	0.49 (0.21-11.32)	5	100	0.49 (0.4-1.62)
4-day	5	100	0.40 (0.11-20.03)	5	100	0.21 (0.11-0.52)
7-day	5	40	33.01 (0.1-64.41)	ND	ND	ND

LLOQ: lower limit of quantification DPV = 0.2 ng/mL; ND: not detectable

2.3.8 Local and systemic PK of DPV/LNG bioadhesive film

After proving that the bioadhesive film was able to be retained on the vaginal mucosa for up to 7 days *in vivo*, DPV and LNG were loaded into the bioadhesive film for evaluation of the local and systemic PK profile. Vaginal swab was utilized to collect the drug released in vaginal fluid for

DPV/LNG single and combination films. The amount of the drug in the vaginal swab over time was shown in **Figure 7**. There was no significant difference for DPV release profile in vaginal fluid between DPV single and DPV/LNG combination bioadhesive films. The maximum DPV level was obtained for both films at 6 h after film insertion. The DPV concentration in vaginal fluid at day-7 for both films was detectable (lower limit of quantification (LLOQ) = 0.2 ng/mL) and at least 3-fold higher than its cellular IC_{50} (7.9 ng/mL) (**Figure 7 a**). LNG showed significantly higher concentration in vaginal fluid for the LNG single entity film than that of DPV/LNG combination film ($p < 0.05$). The maximum concentration at 24 h or 48 h for both single entity and combination LNG films was obtained after vaginal administration. However, LNG release from either the single entity or combination LNG films was not time-dependent (**Figure 7 b**). LNG single film showed detectable LNG levels (LLOQ = 0.2 ng/mL) at day-7 and DPV/LNG combination film showed detectable LNG levels at day-4 and one macaque showed detectable LNG at day-7. It was also observed that more of the LNG was detectable in the vaginal fluid than DPV for single films, which indicates that the vaginal mucosal clearance of DPV could be faster than that of LNG.

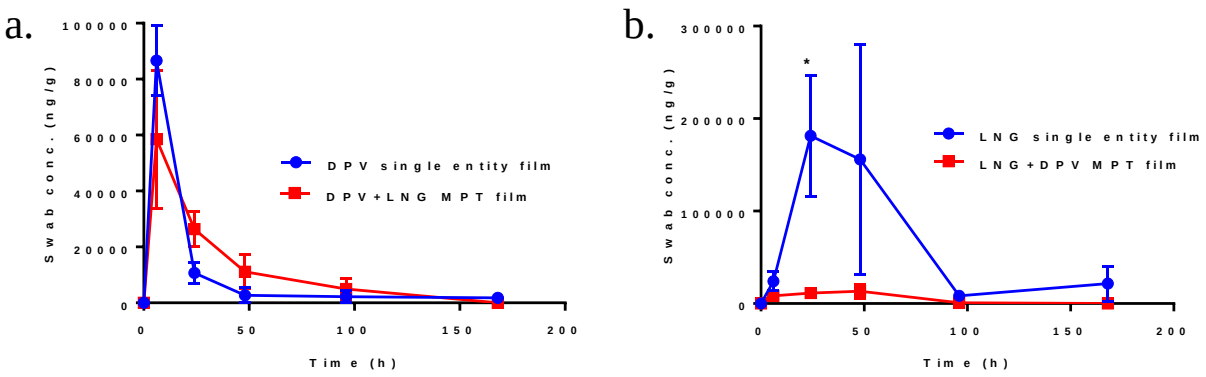


Figure 7. *In vivo* assessment of DPV/LNG single entity film and combination film in pigtailed -macaque models.

a. DPV drug level in vaginal swab over time for single entity and combination bioadhesive film. **b.** LNG drug level in vaginal swab over time for single entity and combination bioadhesive film ($n = 5$, * $p < 0.05$)

The vaginal and cervical tissue drug levels were also evaluated and listed in **Table 5 and 6**. It was observed that the highest DPV concentration in vaginal tissue was observed at 6 h for DPV single entity and DPV/LNG combination films. The maximum DPV level in cervical tissue was found at 6 h for single entity DPV film. The combination film showed comparable cervical tissue DPV level at 6 h, 24 h and day-4, and reduced at day-7. At day-7, DPV levels for single entity and combination films were 1-2 log higher than its IC_{50} in both vaginal and cervical tissues. The highest LNG concentration in vaginal tissues was observed at 24 h for LNG single film and at 6 h for DPV/LNG combination film, respectively. The LNG single entity film showed comparable LNG level in cervical tissue at 6 h, 24 h and day-4, and decreased at day-7. Both drugs can be detectable at day-7 in vaginal and cervical tissues for either single or combination films, which indicates that the bioadhesive film is able to deliver DPV or LNG individually or simultaneously to tissues for at least 7 days.

Table 5. DPV concentration in vaginal swab, vaginal and cervical tissue for DPV single entity and DPV/LNG combination bioadhesive films

DPV	6h			1-day			4-day			7-day		
	# of samples	% above LLOQ	Median (range)	# of samples	% above LLOQ	Median (range)	# of samples	% above LLOQ	Median (range)	# of samples	% above LLOQ	Median (range)
Vaginal swab												
Single film	3	100	86745 (64937-108127)	5	100	11328 (665-19246)	5	80	83 (0.1-8656)	5	60	1.5 (0.1-5339)
Combination film	3	100	49154 (21220-105229)	5	100	27023 (4574-41361)	5	100	2039 (82-20155)	5	80	26 (0.1-34)
Vaginal tissue												
Single film	3	100	6425 (2571-13071)	2	100	926 (107-1744)	2	100	1768 (361-3175)	3	100	215 (101-735)
Combination film	3	66.7	21238 (0.1-213096)	2	100	10332 (7452-13211)	2	100	2851.5 (1759-3944)	3	66.7	495 (0.1-605)
Cervical tissue												
Single film	3	100	2424 (359-4138)	2	100	298 (264-331)	2	100	478 (141-815)	3	100	342 (201-626)
Combination film	3	100	5390 (2959-10194)	2	100	6698 (2206-11190)	2	100	5581 (3258-7904)	3	100	94 (33-1943)

LLOQ: lower limit of quantification DPV = 0.2 ng/mL
Single entity film: DPV containing film; Combination film: DPV and LNG containing film
Values were presented as ng/g for vaginal swab, ng/g for vaginal and cervical tissue

Table 6. LNG concentration in vaginal swab, vaginal and cervical tissue for LNG single entity and DPV/LNG combination bioadhesive films

LNG	6h			1-day			4-day			7-day		
	# of samples	% above LLOQ	Median (range)	# of samples	% above LLOQ	Median (range)	# of samples	% above LLOQ	Median (range)	# of samples	% above LLOQ	Median (range)
Vaginal swab												
Single film	3	100	16820 (10602-45274)	5	100	12624 (43223-411887)	5	100	2888 (50-29221)	5	100	4664(85-97195)
Combination film	3	100	6533 (4390-14107)	5	100	11318 (210-16382)	5	100	13 (3-3704)	5	20	0.1 (0.1-2.8)
Vaginal tissue												
Single film	3	100	1631 (444-19086)	2	100	21344 (13300-29388)	2	100	3902 (1062-6741)	3	100	188 (103-226)
Combination film	3	100	5548 (473-132699)	2	100	1785 (1168-2402)	2	100	303 (102-504)	3	66.7	264 (0.1-679)
Cervical tissue												
Single film	3	100	7258.5 (3059-11458)	2	100	7582.5 (6759-8406)	2	100	9335.5 (4620-14051)	3	100	154 (118-166)
Combination film	3	100	2771.9 (2273-3450)	2	100	1987.5 (873-3102)	2	100	901.42 (554-1248.84)	3	66.7	13.9 (0.1-222)

LLOQ: lower limit of quantification LNG = 0.2 ng/mL
Single film: LNG containing film; Combination film: DPV and LNG containing film
Values were presented as ng/g for vaginal swab, ng/g for vaginal and cervical tissue

In addition to the local PK for DPV and LNG, we also evaluated their plasma levels. Minimum systemic exposure of DPV was observed for DPV single entity and DPV/LNG combination films. LNG plasma levels were observed within 1-2 h post film dosing and achieved 1-log higher than the minimum LNG plasma concentration for effective contraception (0.3 ng/mL)[262]. The peak plasma concentration for LNG was obtained at 8 h for both films (Combination: 2-logs higher than 0.3 ng/mL). As compared to the single LNG film, LNG containing combination bioadhesive film showed significantly ($p < 0.05$) higher plasma exposure at all the time points (**Figure 8**). Furthermore, when co-delivering with DPV, the plasma level of LNG persisted up to 7 days.

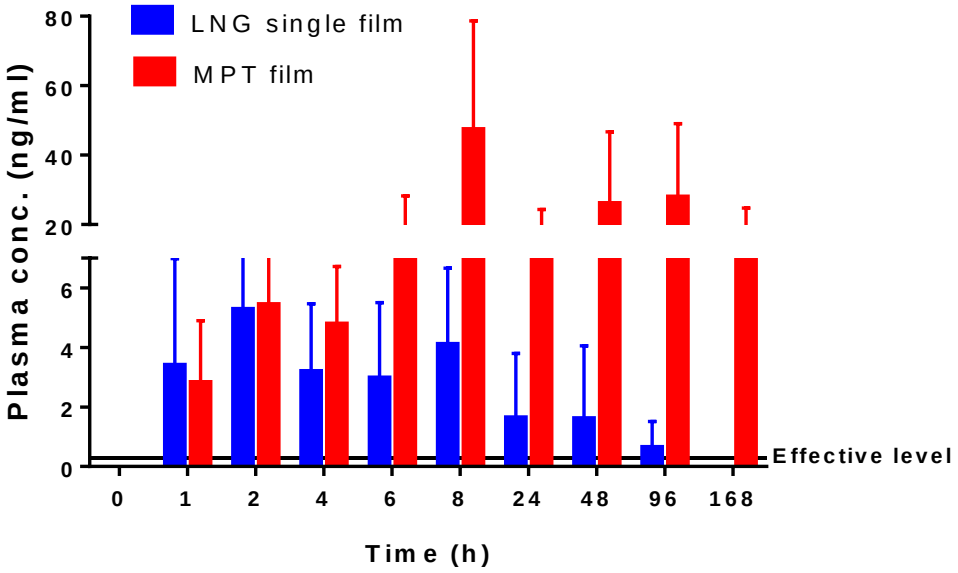


Figure 8. Plasma levels of LNG for single and combination bioadhesive films.
The co-delivery of LNG with DPV in bioadhesive films exhibited up to 7-day persist plasma level of LNG which was higher than the minimum LNG plasma concentration for effective contraception. (n=5)

2.4 DISCUSSION AND CONCLUSION

MPTs offer advantage of simultaneously meeting multiple sexual and reproductive health needs such as unintended pregnancy and sexually transmitted infections (STIs) including HIV[263]. Polymeric film is a well-accepted dosage form and has been developed for delivering combinations of antiretroviral drugs[264, 265]. User adherence for current available films is associated to frequency of dosage administration. Thus, dosage forms with less dosing frequency is promising to increase the user compliance [266]. The primary objective of this study was to evaluate the feasibility of bioadhesive films for co-delivering antiretroviral drug (DPV) and progestin (LNG) as a novel weekly administered MPT product.

The polyvinyl alcohol based quick-dissolving film has been evaluated in the clinical studies and showed favorable acceptability and safety. The feasibility of formulating DPV in this film was also confirmed. Thus, it is reasonable to utilize this film platform to explore more applications such as bioadhesion. The DPV containing bioadhesive film was assessed with physical properties including appearance, weight and thickness, as well as chemical properties including water content, drug content, puncture strength, and disintegration. Comparing to the quick-dissolving film, the incorporation of thiomer significantly increased the puncture strength and disintegration to the film (**Table 3**). Thiolated chitosan synthesized in this study has high molecular weight. Thus, the incorporation of thiolated chitosan could slow the hydration of the polymeric film. In addition to the DPV film, LNG and the combination of LNG/DPV were also formulated in the bioadhesive films. With the physicochemical assessment, it was found that there was no significant difference between the single entity film and combination film (**Table 3**).

Thiomer is reported as an advanced bioadhesive polymer due to its covalent bond formation with mucin[244].This was confirmed when it was incorporated in our polymeric film matrix using an *ex vivo* quantitative tensile method. The mucoadhesion of the bioadhesive film was increased approximately 5-fold after incorporating thiomers (**Figure 3**). The *in vivo* film retention in vaginal tissue was also evaluated in pigtailed macaques using colposcopy imaging. This is the first time that the *in vivo* residence time of thiomer containing formulations were investigated in vaginal compartment. The result showed that the bioadhesive film was able to retain in the vaginal compartment for much longer time than the quick-dissolving film (**Figure 6**), which demonstrated that thiomer incorporation significantly improved the tissue retention both *in vitro* and *in vivo*.

In addition to the film persistence observed *in vivo*, the DPV release from the bioadhesive film was sustained *in vivo* as compared to the quick-dissolving film. The *in vivo* release patterns for DPV from bioadhesive film and quick-dissolving film were consistent with the *in vitro* disintegration data. The sustained drug release rate can be attributed to the addition of thiomers, which increased the disintegration of the film significantly (**Table 3**). The observed longer disintegration time can be explained by the high content of cross-linked disulfide bonds within the polymeric network[267]. It indicates that water takes more time to penetrate into the bioadhesive film matrix that slows the erosion process, thereby to achieve a lower release rate[268]. Collectively, from the *in vitro* and *in vivo* studies, the DPV bioadhesive film with 7-day release profile demonstrates the potential of such film platform to be developed into a weekly MPT product.

After confirming that this film platform can be used to deliver drug for one week, DPV and LNG were co-formulated into the bioadhesive film as a potential MPT product and further evaluated in the nonhuman primate model. The investigation of local and systemic PK of anti-retroviral and progestin is regarded as a very important step in the development of MPT product[269]. The mechanism of action for DPV demands sufficient concentration in the target mucosal tissue, which is the major site for sexually transmitted HIV infection. The study of the LNG intrauterine device not only showed the systemic effectiveness in inhibition of ovulation via binding to the progesterone and estrogen receptors, but also demonstrated the local effectiveness in thickening cervical mucus to inhibit sperm motility[253]. Thus, for intravaginal delivery of LNG, tissue concentration and systematic circulation are both desirable.

Our data showed sufficient DPV concentration in vaginal fluid, vaginal and cervical tissues even with co-delivery of LNG. Minimum systemic exposure of DPV drug concentration

was detected at each time point. We also observed slightly higher but not statistically significant tissue DPV level for DPV/LNG film than that of DPV single film (**Table 5**). This increase might be due to the lower vaginal mucosal clearance of DPV for DPV/LNG combination film. This is also reported by Akil A. *et al.* where the co-delivery of DPV with tenofovir increased the tissue accumulation of DPV in the *ex vivo* human ectocervical tissue explant model[265]. Their results indicate that co-delivery could have impact on the local PK of drug. In our study, DPV and LNG showed different PK profiles for their co-delivery. LNG displayed lower drug concentration in the vaginal fluid for the combination film as compared to single entity film ($p < 0.05$), which could result in the slightly lower vaginal and cervical tissue concentration observed for the combination film (not statistical significant due to small sample number) (**Figure 7**). However, LNG combination film showed significantly higher drug concentration in the plasma compared to the single entity film (**Figure 8**). These results indicate that drug-drug interaction could also occur during the co-delivery of antiretroviral drug and contraception.

One of the possible explanations for the drug-drug interaction during the co-delivery of DPV and LNG is the involvement of cytochrome P-450 (CYP450) enzymes. Multiple studies have shown that CYP3A4 was expressed in the vaginal and cervical tissue[270-272]. LNG is the substrate of cytochrome P-450 isoenzyme 3A4 (CYP3A4)[273], and DPV can stimulate the increase of CYP3A4 expression at mRNA levels[270]. Thus, when co-delivery with DPV, the metabolism of LNG could be increased in the vaginal and cervical tissues. This could result in the decreased vaginal and cervical tissue levels of LNG that were observed in our study. On the other hand, LNG is the inhibitor of CYP2C19 and CYP3A4[254], while DPV is the substrate of both enzymes and they are also expressed in vaginal tissues. Although LNG's inhibitory activity for these enzymes is weaker than other progestin[274], the inhibition of CYP3A4 and CYP2C19

could increase DPV levels in vaginal and cervical tissues. Moreover, both of DPV and LNG have high protein bonding property. The increased plasma drug level especially for LNG after co-delivery could be due to the competitive protein binding, but this needs to be studied. Overall, these results indicate that the drug-drug interaction needs further exploration given that DPV and LNG combination products are being pursued in a vaginal ring platform.

In conclusion, our study designed a novel MPT bioadhesive films for delivering antiretroviral drug and progestin simultaneously and evaluated the PK profile of the co-delivery of DPV and LNG using bioadhesive film in the nonhuman primate model. In this study, we presented the *in vivo* one-week residence of the bioadhesive film in the vaginal compartment. It also demonstrated the ability of film platform to deliver LNG intravaginally with or without combination of DPV. The co-delivery of DPV and LNG showed different PK profiles as compared to individual entity delivery, which indicated potential drug-drug interactions occurred locally or systemically. These results support further investigation of DPV and LNG film as the MPT product for HIV prevention and unintended pregnancy.

2.5 ACKNOWLEDGEMENT

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3.0 DEVELOPMENT AND CHARACTERIZATION OF MK-2048 EXTENDED RELEASE FILM

3.1 INTRODUCTION

Chapter-2 has demonstrated the feasibility of using thiomers in vaginal film to enhance the tissue mucoadhesiveness *in vitro* and *in vivo* which had achieved one-week retention time on the vaginal lumen. Although thiomers have been explored for the sustained release in oral drug delivery, the extended release property of thiomers for vaginal delivery might be diminished because thiomers are more soluble in low pH such as that of vaginal fluids. As shown in Chapter-2, this thiomer containing bioadhesive film was not able to achieve sufficient dapivirine (DPV) levels in the vaginal compartment after 2 days. Therefore, the polymer formulation of film matrix needs to be further modified to achieve the desired sustained release profile for APIs.

To achieve a one-week extended drug release profile from the vaginal film, the selected polymer needs to meet the following criteria: 1) appropriate biodegradation rate to maintain one-week drug release; 2) biocompatible and safe in the vaginal compartment; 3) manufacturable as film and compatible with other film components. Poly(lactide-co-Ɛ-caprolactone) (PLACL) stands out as one of the most promising polymers for the design of an extended release vaginal film. It is a synthetic biodegradable polymer. This polymer can obtain tunable degradation rate and elasticity via adjusting the ratio of lactide to caprolactone[275]. It shows great

biocompatibility with cells and tissues and degrades over time without causing significant toxicity[276, 277]. Because of these features, PLACL has been widely applied in controlled drug release systems[278-283] such as electrospun nanofibers and nanoparticles. PLACL is also amenable to be manufactured as polymeric films using different manufacturing methods such as electrospinning, solvent casting or hot melt extruding[284, 285]. Overall, PLACL is an appropriate polymer candidate for designing an extended-release vaginal film in coitally-independent applications.

Along with a robust extended-release film as drug delivery system, a potent and efficacious API is also required. MK-2048, a second-generation integrase inhibitor, possesses great potency against integrase (IN) and the mutation of INR263K with the half maximal inhibitory concentration (IC_{50}) of 2.6 nM and 1.5 nM[286], respectively. In addition, MK-2048 has been shown to have a long tissue half-life[53]. Our preliminary study investigated the tissue levels of MK-2048 in a macaque model. Results revealed that MK-2048 retained significant drug levels in the macaque cervical tissues for four days after administration and tissue drug concentration remained at levels well above its IC_{50} after two separate coital events. Therefore, MK-2048 is a promising candidate to be incorporated into the coitally-independent dosage form.

The aim of this work is to improve the mucoadhesive film described in Chapter 2 with sustained release profile using biodegradable polymers to achieve a one-week dosing regimen. In this study, we presented the synthesis and characterization of PLACLs with different chemical compositions. The lead film formulation was selected based on the drug release profile, and further evaluated with respect to its *in vitro* and *ex vivo* characteristics including physicochemical properties, mucoadhesion, tissue toxicity, drug tissue permeability as well as *in*

vitro bioactivity in HIV inhibition. In this chapter, thiomer containing film was utilized as a control for evaluating the impact of PLACLs on the tissue mucoadhesion in the *ex vivo* model.

3.2 METHODS

3.2.1 Materials

L-lactide was kindly provided by Corbion. Ɛ-Caprolactone was purchased from Alfa Aesar (Haverhill, MA). Stannous octoate was purchased from Sigma Aldrich (Cleveland, OH). PEG 400 was purchased from Spectrum (Gardena, CA). Hydroxypropyl methyl cellulose E5 (HPMC E5) was obtained from Dow Chemical Company (Midland, MI). Polyvinyl alcohol 4-88 (PVA 4-88) was purchased from Millipore Sigma (Temecula, CA). Ultrapure water was prepared by passing distilled water through a Milli-Q® Reagent Water System (Millipore®). Cell culture reagents were obtained from GIBCO, Invitrogen by Life Sciences, Inc (Lenexa, Kansas). Alexa Fluor® 647 C2 Maleimide was purchased from ThermoFisher (Pittsburgh, PA). MK-2048 was provided by Merck Sharp & Dohme Corp. (NJ, USA). Phosphate buffered saline 10× molecular biology grade was purchased from Mediatech, Inc (Manassas, VA). All the other chemical reagents for film preparation were purchased from Fisher Scientific (Pittsburgh, PA).

3.2.2 Synthesis of PLACL

Three PLACLs with different molar ratios of L-lactide (LA) and Ɛ-caprolactone (CL) were synthesized using ring-opening polymerization based on the previously published method[275].

Briefly, the synthesis was carried out in a 50 mL flask placed in a temperature-controlled oil bath. Premeasured amounts of LA and CL were simultaneously added and melt. The flask was purged with nitrogen for 30 min and then 1/2000 stannous octoate of LA and CL in equimolar ratio was added into the flask under stirring at 100 rpm and reacted at 140 °C. The reaction was stopped after 24 h. The product was dissolved in chloroform and precipitated in the excess of methanol to remove the catalyst impurities and unreacted monomers. The product was dried at 35 °C under vacuum until it reached the constant weight. The copolymer was named according to the feeding ratios of LA to CL, as PLACL 70, 80 and 90, respectively.

3.2.3 Characterizations of PLACLs

Gel Permeation Chromatography (GPC): The molecular weight of PLACL samples was determined by GPC. PLACLs were dissolved in tetrahydrofuran to a concentration of 5 mg/mL. Once dissolved, the samples were filtered using a 0.45 µm nylon membrane. Molecular weights and dispersities were acquired on a Waters GPC (THF) system with Jordi 500 Å, 1000 Å and 10000 Å divinylbenzene columns and a refractive index detector (Waters) which was calibrated using polystyrene standards of 62300 Da, 30000 Da, 9000 Da and 2500 Da. The injection volume for each sample was 20 µL and the flow rate was 0.5 mL/min.

Proton nuclear magnetic resonance (^{1}H-NMR): The chemical composition of PLACL samples was analyzed using ^{1}H-NMR spectroscopy (Bruker 400). All samples were dissolved in chloroform and the spectra were recorded at 400MHz.

Fourier-transform infrared spectroscopy (FTIR): Chemical bond formation of the PLACL was studied using ATR-FTIR. The FTIR spectra were recorded in absorption mode with a resolution of 4 cm^{-1} using Bruker Vertex 70 FTIR spectrometer.

Differential scanning calorimetry (DSC) analysis: DSC (Mettler Toledo) was utilized to study the thermal properties of PLACL copolymers. Sample (approx. 5 mg) was sealed in an aluminum pan and first heated from room temperature to 250 °C (1st cycle), then cooled to -70 °C (2nd cycle), and finally reheated to 250 °C (3rd cycle) at a heating/cooling rate of 10 °C/min. All the processes were carried out under nitrogen atmosphere. Glass transition temperature (T_g) and melting temperature (T_m) were obtained from the heating cycle (-70 °C to 250 °C, 3rd cycle). DSC data was analyzed using STARe software.

3.2.4 Film formulation development

The polymeric film was manufactured using a modified solvent casting method [226]. Thiolate chitosan used in this study was synthesized and characterized using the method that described in Chapter-2. The film formulation was composed of PLACL, thiolated chitosan, polyvinyl alcohol (PVA 40-88), METHOCEL E5, propylene glycol, and glycerin. Briefly, thiomers were hydrated in Milli-Q water and PLACL was dissolved in methylene chloride. The other excipients were dissolved in Milli-Q water and then mix with thiolated chitosan solution and PLACL solution in the laboratory fume hood for evaporation of methylene chloride. MK-2048 was dispersed in plasticizers and mixed with film solution. Once the excipients and drug were mixed uniformly, the solution was cast onto a polyester substrate using a 4″ doctor blade and dried on an automatic film applicator (Elcometer® 4340) with temperature set at 60 °C for 30 mins. The film sheet was peeled from the substrate and cut into 1″ × 1″ individual unit doses. All films utilized in this study were manufactured using the same method with variation of formulation compositions. PVA film was composed of PVA 40-88, METHOCEL E5, propylene glycol, and glycerin. Thiomer film was composed of thiolated chitosan, PVA 40-88, METHOCEL E5, propylene

glycol, and glycerin. Thiomer PLACL film was composed of thiolated chitosan, PVA 40-88, METHOCEL E5, propylene glycol, glycerin, and PLACL 70, 80 or 90.

3.2.5 Mechanical characterization of films

The mechanical properties of films containing different polymer compositions were evaluated using uniaxial tensile testing with ADMET MTEST Quattro mechanical testing system (n = 4). The PVA based film and commercialized film product VCF® were used as control groups. The casted films were cut into rectangular shape (10 mm × 7 mm). Samples were stretched until failure at a constant jogging speed of 10 mm/min. The stress (MPa) was obtained by dividing the applied force (N) with cross-section area (mm^2) and % elongation (strain) was obtained from the displacement using $((L - L_0)/L_0 \nearrow 100)$, where L_0 was initial gauge length and L was instantaneous gauge length. Elastic modulus was calculated from the linear stress–strain curve between 3% and 15% strain.

3.2.6 High-performance liquid chromatography (HPLC) method for MK-2048

Dionex Ultimate 3000 HPLC system (Thermo fisher) with diode array detector (Pump DGP-3600A, Autosampler WPS-3000TSL, Photometer PDA-3000) and Chromeleon data acquisition system was used for analytical method development. The Xbridge C18 HPLC column (5 µm, 2.1 × 50mm, waters) with guard column was used to separate MK-2048. The mobile phase consisted of (A) 0.1% of formic acid in Milli-Q water and (B) acetonitrile using a gradient elution of 10% B at 0-2 min, 10% B at 2-6 min with linear change to 50% B at 7 min and then goes to 10% B at 8 min with linear change and stays with 10% B at 8-11 min. The flow rate was 1 mL/min.

Sample injection volume was 20 µL and MK-2048 was determined at wavelength of 334 nm. All samples were performed at 4 °C.

3.2.7 *In vitro* drug release

To determine the kinetics of drug release from MK-2048-loaded films in different media, an *in vitro* release study was conducted using a USP dissolution apparatus I (Distek dissolution system model 2100; North Brunswick, NJ). The dissolution was operated at 10 rpm and 37 °C. 0.5% w/v of sodium dodecyl sulfate (SDS) in acetate buffer (pH 4.2) or 0.5% w/v of SDS in Milli-Q water were used as the media for evaluating the drug release from different film formulations. The test was carried out for up to 7 days. At appropriate time intervals, 1 mL aliquots were withdrawn and replaced by 1 mL of fresh media. The MK-2048 in collected samples was analyzed using HPLC method described before.

3.2.8 Physicochemical characterization and stability of films

The thiomer PLACL80 films were characterized for mass using analytical balance (Mettler Toledo XS105), thickness using thickness gauge (Mitutoyo Corporation), moisture content using Karel Fisher apparatus (Metrohm, 758 KFD Titrino) in accordance with the titration method specified by the manufacturer, and puncture strength using texture analyzer (TA. XT. Plus®, New York, NY). The drug content in films were determined by extracting the drug using 40% acetonitrile and analyzing by HPLC method. Films were placed in the humidity chamber at 25 °C 60% relative humidity (RH) and 40 °C 75% RH, respectively. The drug content in films was monitored for 6 months.

3.2.9 Distribution of thiolated chitosan in the film matrix

Thiolated chitosan were labeled with Alexa Fluor® 647 C2 Maleimide by click-chemistry reaction. The SH- group in thiolated chitosan was conjugated with the fluorophore to indicate its distribution in the film matrix. The stable conjugates of thiolated chitosan-fluorescence were purified by dialysis tubing with molecular weight cut of 10,000 Da. The fluorescence labeled film was manufactured using the same method as mentioned in method 3.2.7 but replacing the thiolated chitosan with thiolated chitosan-fluorescence conjugates. Images were taken in two directions (film surface and cross-section) by inverted confocal laser scanning microscope (Olympus Fluoview™ 1000, Japan).

3.2.10 *Ex vivo* mucoadhesiveness

The ex vivo mucoadhesiveness for each film was evaluated using the method as described in Chapter 2 (section 2.2.7).

3.2.11 *Ex vivo* tissue epithelial integrity

The human ectocervical tissues used in this study were obtained through the University of Pittsburgh Health Sciences Tissue Bank as per approved IRB protocol PRO09110431. All tissues were de-identified and collected through an Honest Broker. The human ectocervical tissues were collected from three premenopausal women (30-55 years old) undergoing hysterectomy with benign health conditions. The excised tissues were transferred to the lab in Dulbecco's

Modification of Eagles Medium (DMEM). Tissues were snap frozen and then stored in -80 °C for later use.

The impact of the thiomer containing films on the integrity of human ectocervical tissue were evaluated with transepithelial electrical resistance (TEER) using Ussing Chamber system (Physiologic Instruments, Inc. San Diego, CA)[287]. The frozen tissue was thawed in 37°C water bath for 5 mins. The stromal tissue was removed from the excised ectocervical tissue using a Thomas-Stadie-Riggs tissue slicer (Thomas Scientific, Swedesboro, NJ). Thickness of the tissue was measured using a digital micrometer by mounting between two glass slides. The excised tissue was placed in the Ussing Chamber slider and equilibrated for 30 mins with exposure to PBS in apical and basal chambers. The tissue exposure area was 0.26 cm^2. TEER was monitored by Acquire and Analyze Rev II software, version 3.1 after adding thiomer film or thiomer PLACL80 film into the apical hemichamber. Both hemichambers were bubbled with continuous oxygen to keep the tissue alive. The data were presented as the percentage of initial TEER for 6 h. PBS was used in the apical hemichamber as the negative control to show whether tissue was alive over 6 h incubation. 0.6% (w/v) of formulated Nonoxynol (N-9, GYNOL II®) was used as a positive control for validation of this TEER assay. After the TEER study, tissues were collected for checking the epithelial morphology using Hematoxylin and Eosin (H&E) staining.

3.2.12 *Ex vivo* tissue permeability

The tissue permeability of thiomer containing films was evaluated using the In-Line Cell system (PermeGear, Hellertown, PA). Excised human ecto-cervical tissue was placed in between donor and receptor compartments. Before tissue placement, tissue procurement and processing were

conducted in the same way as in the TEER study. The tested articles introduced into the donor compartment were unformulated MK-2048, single 6mm biopsy punches of MK-2048 PVA base film, MK-2048 thiomer film, and MK-2048 thiomer PLACL80 film dispersed in 450 µL VFS. The receptor medium used was DMEM at a flow rate of 50 µL/min. Excised human ecto-cervical tissue was exposed to each treatment for 6 h and the cells were water jacketed to maintain a temperature of 37°C. 3 mL receptor sample was collected throughout the experiment every hour. After the exposure period, the tissue was collected and cut in half for morphology evaluation using H&E staining, and MK-2048 tissue concentration determination by HPLC method as described before. The apparent partition coefficients (P_{app}) were calculated using the following equation:

$$\mathbf{Papp} = \frac{dX}{dt} \times \frac{\mathbf{1}}{\boldsymbol{A} * \boldsymbol{Cd}}$$

Where *dX* is the cumulative amount of MK-2048 (ng) in the receptor chamber, *A* is the surface area of the cervicovaginal tissue (cm^2) and *Cd* is the initial concentration of MK-2048 in the donor compartment (ng/mL).

3.2.13 *In vitro* anti-HIV activity

Cellular toxicity and *in vitro* efficacy studies were studied using TZM-bl cell assay as previously described[288]. TZM-bl cells were seeded in a 96-well plate. 1″ × 1″ Films were dissolved in 1 mL saline solution with 10-10^6 dilution using cell culture medium. 100 µL of film solution was added into TZM-bl cells for incubation. For cytotoxicity study, film solutions were incubated with TZM-bl cells overnight and media was replaced with 100 µL of Celltiter-Glo. After 10 mins incubation, luminescence was determined by plate reader. The % viability of cells treated with

film solution was calculated by the luminescence of film treatment over that of cells without any treatment. For HIV inhibition efficacy, film dilutions were added to TZM-bl plates and followed by adding HIV-1_{BAL} into the cells. Cells were incubated for 48 h and then media was replaced with 100 µL of Bright-Glo. After 2 mins incubation, luminescence was determined by plate reader. The % of HIV inhibition was calculated by using the equation: (1-cells with film treatment/cells with HIV-1 infection only) * 100.

3.2.14 Compatibility with *Lactobacillus*

The standard microbicide safety test was used as previously described[289] to assess lactobacillus compatibility with 1″ × 1″ MK-2048 thiomer PLACL80 film. Briefly, bacterial suspensions were prepared in ACES buffer and the films were then dissolved and mixed in the suspensions. The suspensions were then incubated for 30 min at 37°C. Samples were taken at time zero and again after 30 min. Viability was determined by standard plate count. A sample was considered to be compatible with *Lactobacillus* if the reduction in viability was < $\log_{10}$.

3.2.15 Statistical analysis

All values are reported as mean ± standard deviation (SD). Statistical data analyses were performed using the Student's t-test between two groups and one-way ANOVA with Tukey's post hoc test for the analysis among three or more study groups, with $p < 0.05$ as the minimal level of significance, $p < 0.01$ for very significant and $p < 0.001$ for highly significant. Two-way ANOVA with Dunnet's multiple comparison test was used for the stability data. All tests were performed using the GraphPad Prism software version 7.

3.3 RESULTS

3.3.1 Characterizations of PLACL

A series of PLACL co-polymers were obtained by ring-opening polymerization using stannous octoate as an initiator[275] (**Figure 9**). The polymer composition was controlled by varying the feeding ratio of monomers. **Table 7** summarizes the characteristics for synthesized PLACLs. The number average molar mass (M_n) and mass average molar mass (M_w) of PLACL with different LA to CL ratios were confirmed by GPC. The molecular weight of all the synthesized PLACLs were in the similar range (12-17 kDa). The chemical composition of PLACLs was confirmed by ^{1}H-NMR (**Figure 10**). The peak between (5.3-5.0 ppm) represents the proton from the methine group in LA-A, and the highest peak is the proton from the methyl group in LA-B. The ratio of peak areas integrated from the two signals was 1 to 3 which corresponds to that in the lactide structure. The peak between 4.3-4.0 ppm was the proton from the methylene in CL-Ɛ, and the peak between 2.5-2.0 ppm was the proton from the methylene in CL-α (**Figure 10**). The actual monomer ratios calculated from the ^{1}H-NMR by comparing the methylene hydrogen with in LA-A and CL- Ɛ correlated well with the feed ratio, which confirms the synthesis process. The chemical bonds in PLACL were confirmed by FTIR. **Figure 11** highlights the areas, which are typically associated for C=O, C-H and C-O bonds. This corresponds to the bond that links the two monomers together. Additionally, no other bonds that do not belong to PLACL were observed, which indicates the success of the copolymerization. DSC was used to evaluate the thermal properties of PLACL with different monomer ratios. T_g was determined through the heating cycle (-70 °C to 250 °C). It was noticed that T_g of all these three copolymers was below

the body temperature (PLACL 90: 36.86 °C, PLACL 80: 27.75 °C, PLACL 70: 16.57 °C), and the lower T_g was observed with the increased percentage of CL in the copolymers (**Table 7**).

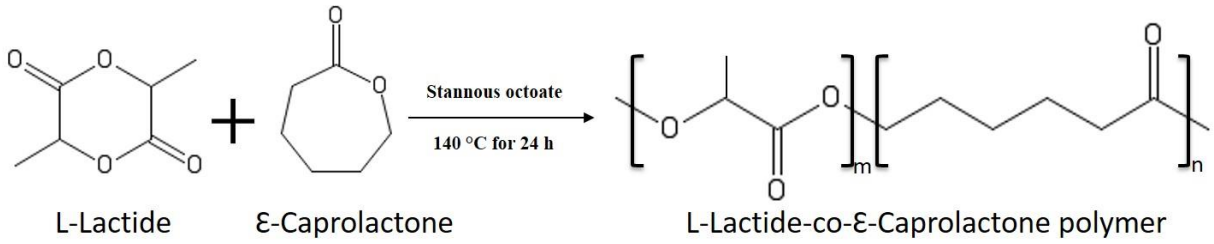
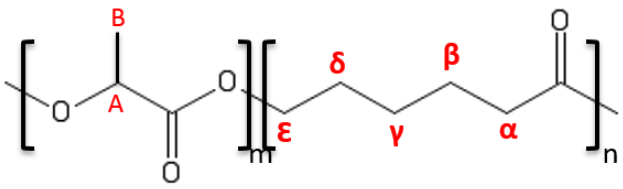


Figure 9. Poly (L-lactide-co-Ɛ-caprolactone) Copolymerization Scheme

Table 7. Characteristics of poly (L-lactide-co-Ɛ-caprolactone)

Polymer	Monomer feeding ratio (LA/CL)	M_n (kDa)	M_w (kDa)	M_w/M_n	Actual ratio of LA/CL	Tg (°C)
PLACL 90	90:10	13.8	22.9	1.66	90:10	36.86
PLACL 80	80:20	12.3	19.8	1.61	81:19	27.75
PLACL 70	70:30	16.8	26.7	1.59	65:35	16.57

M_n: number average molar mass, M_w: mass average molar mass
Actual ratio of LA/CL was calculated based on ^{1}H-NMR.



L-Lactide-co-Ɛ-Caprolactone polymer

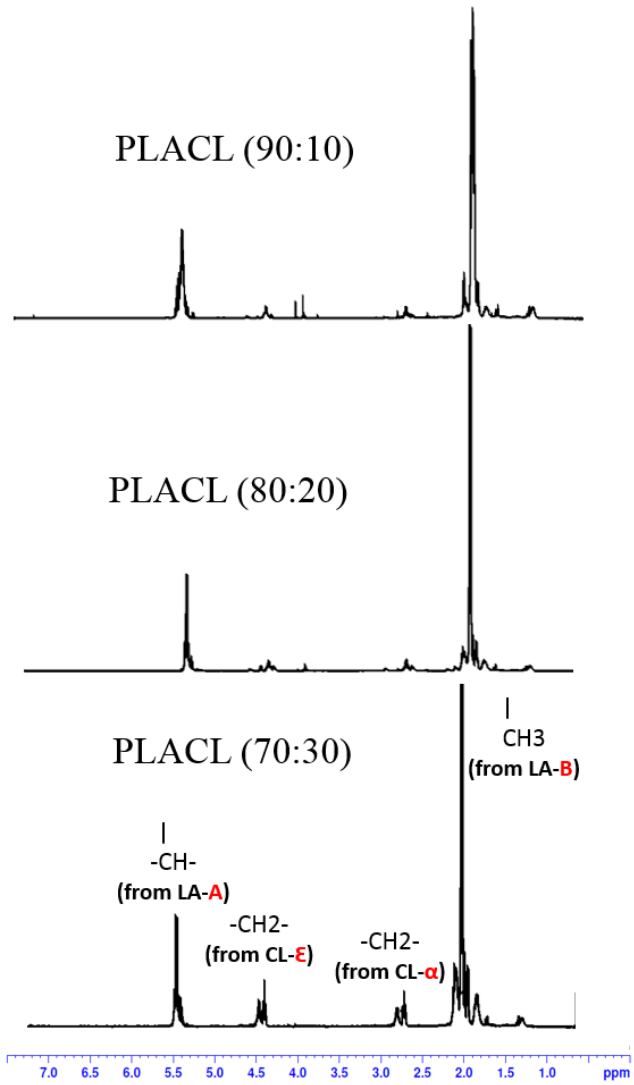


Figure 10. ^{1}H-NMR of PLACL 70, PLACL 80 and PLACL 90

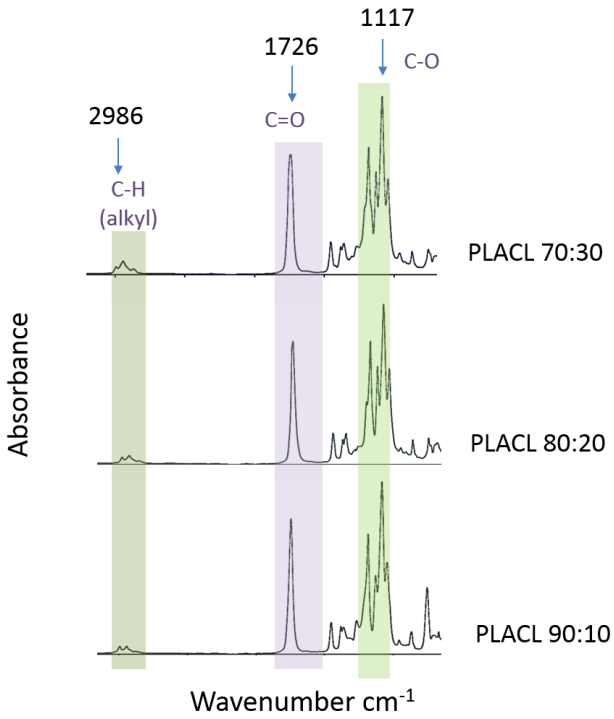


Figure 11. FTIR spectra of PLACL 70, PLACL 80 and PLACL 90

3.3.2 Effect of matrix composition on elasticity of films

A soft and flexible film is more desirable and acceptable by women[290]. The elasticity was used to evaluate the flexibility of films. In this study, the commercialized film VCF® and the PVA film evaluated in clinical trials[55] were utilized for comparing the elasticity of films modified with thiomer and/or PLACLs. Higher of the elastic modulus, lower of the elasticity of the film. The elastic modulus of VCF® and PVA film was 30.77 ± 6.17 MPa and 34.84 ± 3.20 MPa, respectively. The incorporation of thiomer significantly increased the elastic modulus of film to 79.57 ± 15.87 MPa. However, with the addition of PLACLs into the thiomer films, the elastic modulus of these films was significantly reduced and returned to the similar level of

VCF® and PVA films (24.91 MPa to 37.50 MPa) (**Figure 12**). It demonstrates the feasibility of using PLACL copolymers to modify the elastic properties of the polymeric films.

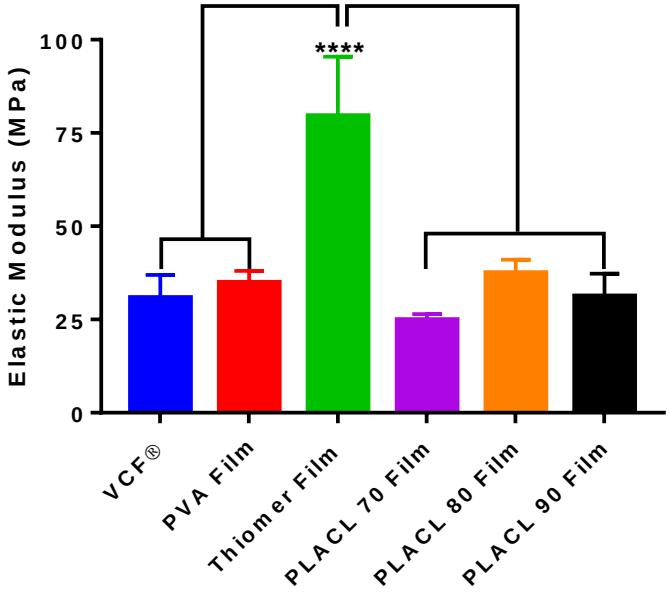


Figure 12. **Elasticity of films with polymer modifications.**
The elastic modulus of film was increased very significantly by incorporation of thiomers in PVA film matrix, but was compromised by the addition of any PLACL compositions. As compared to the commercialized VCF®, films with modification of PLACL with any composition can maintain the similar elasticity even co-formulated with thiomers. n = 4, significant difference was noted at **** $p<0.0001$. The data were analyzed by One-way ANOVA with Tukey's multiple comparisons test. PLACL 70 Film represents PVA film with addition of thiomer and PLACL 70; PLACL 80 Film represents PVA film with addition of thiomer and PLACL 80; PLACL 90 Film represents PVA film with addition of thiomer and PLACL 90.

3.3.3 *In vitro* drug release

To differentiate the drug release profile from different film platforms, a USP I dissolution method was applied using modified dissolution media and stirring speed. MK-2048 is a very hydrophobic antiretroviral drug. The aqueous solubility of MK-2048 was determined to be 10 µg/mL. Thus, solubilizers SDS and Cremorphor at different concentrations were evaluate for their ability to increase the solubility of MK-2048 for drug release study. It was observed that

addition of solubilizers significantly improved the solubility of MK-2048. The solubility of MK-2048 in 0.5% SDS, 1% SDS and 1% Cremophor EL was 537.31 µg/mL, 1355.98 µg/mL and 95.30 µg/mL, respectively (**Table 8**). 0.5% SDS was chosen as the dissolution medium to maintain the sink condition in practice. *In vitro* drug release was conducted in buffers with pH 7.0 and 4.2, respectively. The Higuchi model, which describes the drug release from the matrix system, was used to plot the release profile of formulated MK-2048. Biphasic MK-2048 release was observed for films containing PLACL. It was shown that 100% of MK-2048 was released in 8 h for thiomer film in both buffers. PLACL's incorporation in film matrix significantly slower the release rate of MK-2048 in both buffers as compared to the thiomer film. PLACL 70 film and PLACL 90 film showed similar release profile at first 8 h, whereas PLACL 90 film showed slower release rate than PLACL 70 film after 8 h (**Figure 13 a and b**). PLACL 80 film showed the slowest release profile over all the other films. In addition, the buffer pH had no significant effect on MK-2048 release rate for all the polymeric films (**Figure 13 c**). In the following study, PLACL80 film was selected as the leading film formulation for further investigation due to its comparative elasticity with commercialized VCF® and slowest drug release rate.

Table 8. Solubility of MK-2048 in different surfactant solutions

Surfactants	**Conc.(µg/mL)**
0.5% SDS	537.31 ± 2.3
1% SDS	1355.98 ± 5.64
1% Cremophor	95.30 ± 4.06

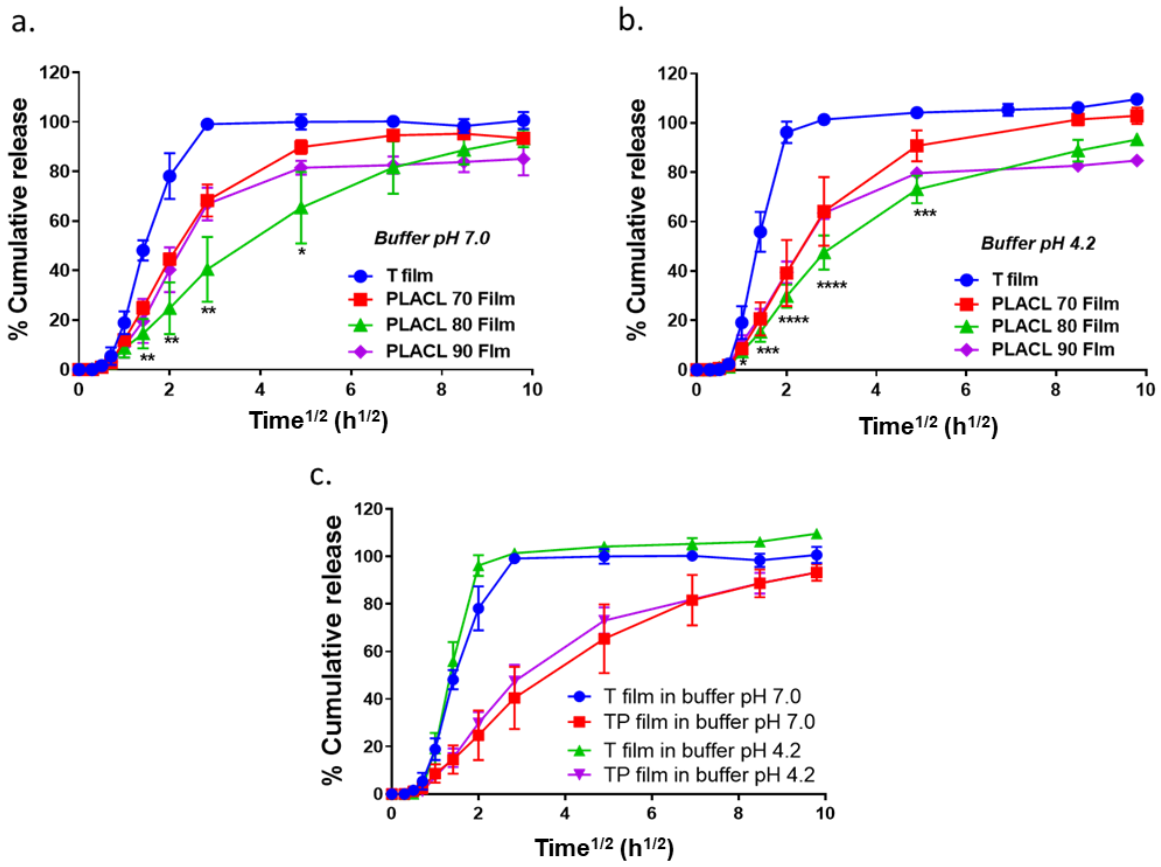


Figure 13. Effect of PLACL composition on *in vitro* drug release from films
Cumulative release of MK-2048 from thiomer film (T film), PLACL 70 Film, PLACL 80 Film and PLACL 90 Film in **a.** 0.5% SDS in Milli-Q water with pH 7.0 and **b.** 0.5% SDS in acetate buffer with pH 4.2. **c.** Effect of pH on *in vitro* drug release from thiomer film (T film) and PLACL80 film (TP film). The comparison was conducted between each formulation to thiomer film group. Data were analyzed using One-way ANOVA with Tukey's multiple comparisons test. n = 3, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3.3.4 Film physicochemical characterizations

The thiomer PLACL80 film was loaded with two dosing levels of MK-2048: 3.56 ± 0.18 mg/film and 6.89 ± 0.18 mg/film. There was no significant change in the water content and puncture strength between placebo and drug-loaded films, suggesting the drug incorporation had little influence on the physicochemical properties of vaginal polymeric films (**Table 9**). The

stability for drug content in films was monitored for both films in two storage conditions. No significant reduction ($p > 0.05$) of MK-2048 in either of the films was observed when they were maintained under 25°C/60% R.H. and 40°C/75% R.H. over a 6-month period (**Figure 14 and 15**).

Table 9. Physicochemical characterization for TP film

	Mass (mg)	Thickness (mm)	Puncture strength (kg/mm)	Water content%
Placebo TP film	93.15 ± 4.70	0.12 ± 0.01	6.20 ± 1.10	5.09 ± 0.55
MK-2048 LD TP film	72.53 ± 5.35	0.12 ± 0.01	5.10 ± 0.28	5.57 ± 0.13
MK-2048 HD TP film	77.2 ± 12.38	0.12 ± 0.005	5.41 ± 0.53	5.23 ± 0.36

TP film: thiomer PLACL80 film; LD: low drug loading of 3.56±0.18 mg/film; HD: high drug loading of 6.89±0.18 mg/film

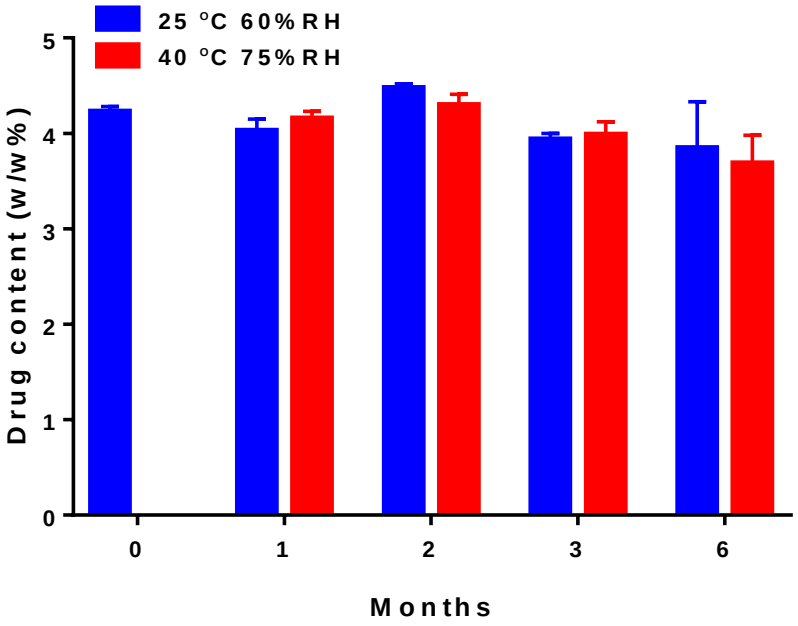


Figure 14. 6-month stability of low drug loading MK-2048 TP film

Two-way ANOVA with Dunnett's multiple comparison test was conducted. No significant reduction of MK-2048 in films was observed in both storage conditions ($n = 3$, $p = 0.1226$ for 6-month film stored in 25 °C 60% RH; $p = 0.2061$ for 6-month film stored in 40 °C 75% RH). TP: thiomer PLACL80 film; RH: relative humidity.

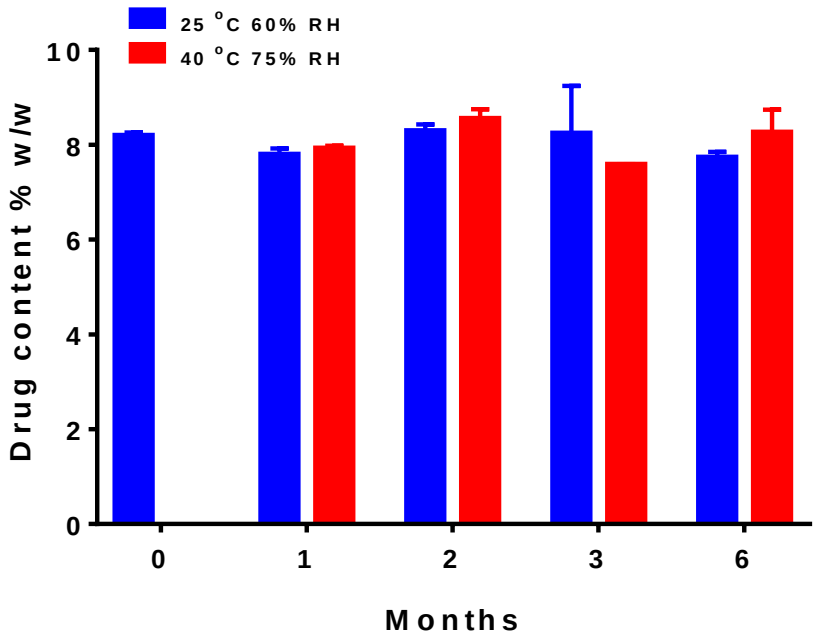


Figure 15. 6-month stability of high drug loading MK-2048 TP film

Two-way ANOVA with Dunnett's multiple comparison test was conducted. No significant reduction of MK-2048 in films was observed in both storage conditions (n = 3, p = 0.3697 for 6-month film stored in 25 °C 60% RH; p = 0.9976 for 6-month film stored in 40 °C 75% RH). TP: thiomer PLACL80 film; RH: relative humidity.

3.3.5 Thiomer distribution in film matrix and the enhanced tissue mucoadhesiveness

The distribution of thiomers was observed by conjugating the thiol groups with cy5 fluorescent dye. As shown in **Figure 16**, cy5 was visualized on not only the surface of the film but also the cross section, which demonstrates that thiomers were distributed throughout the bulk of the film. No segregated areas of high fluorescence intensity were observed indicating even distribution of dye-conjugated thiomers.

The *ex vivo* mucoadhesiveness of films was investigated by texture analyzer using porcine intestinal tissue. The quantitative work of adhesion was utilized to represent the tissue mucoadhesion of different films[291]. The effect of thiomers on tissue mucoadhesion was

studied using the PVA film as the comparison group. The group of PVA PLACL80 film was used as another controller to evaluate the effect of PLACL 80 on the mucoadhesion of films. By comparing the PVA film and PVA PLACL 80 film, there was no significant change in the tissue mucoadhesion observed by adding the PLACL 80 into the film matrix. Meanwhile, the addition of thiomers significantly improved the tissue mucoadhesiveness as compared to PVA film. We also observed that the thiomer PLACL80 film showed significantly improved tissue mucoadhesion compared to PVA film and PLACL 80 film. No significant difference was observed between thiomer film and thiomer PLACL80 film (**Figure 17**). This indicates that the tissue mucoadhesion of thiomers was not affected when co-formulated with PLACL 80 in film matrix.

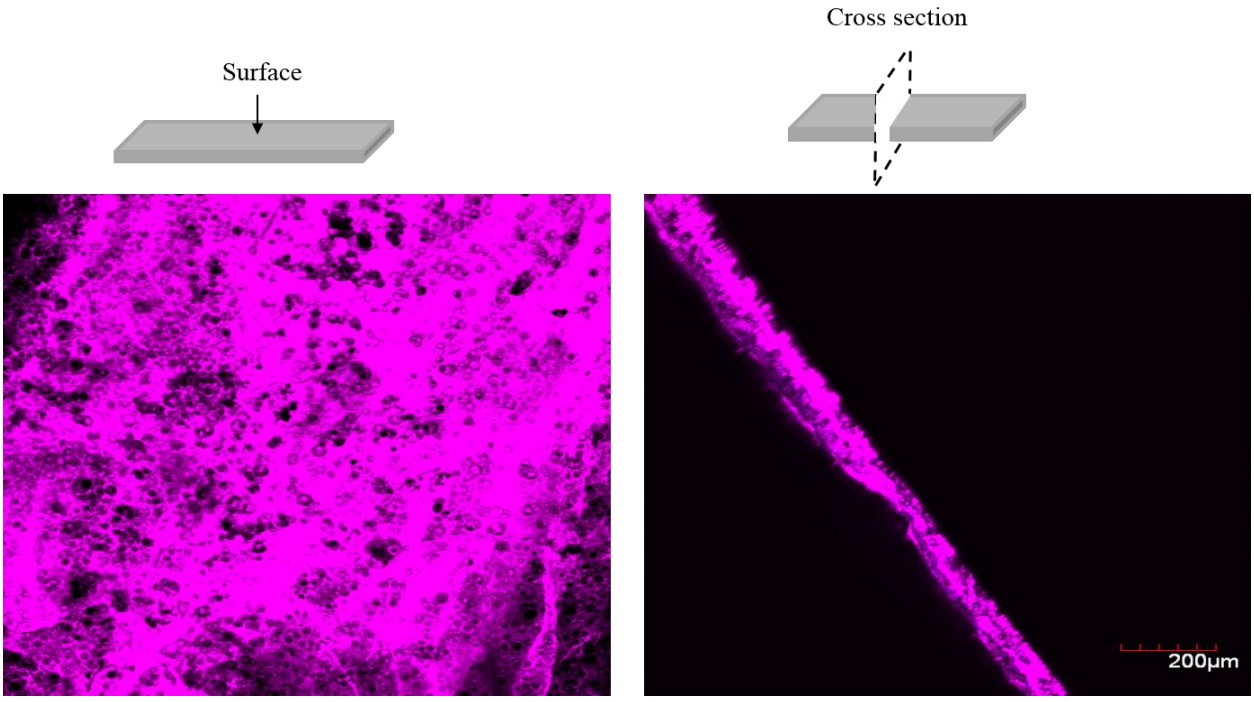


Figure 16. Confocal images of distribution of thiomers in film matrix from surface and cross-section visualizations

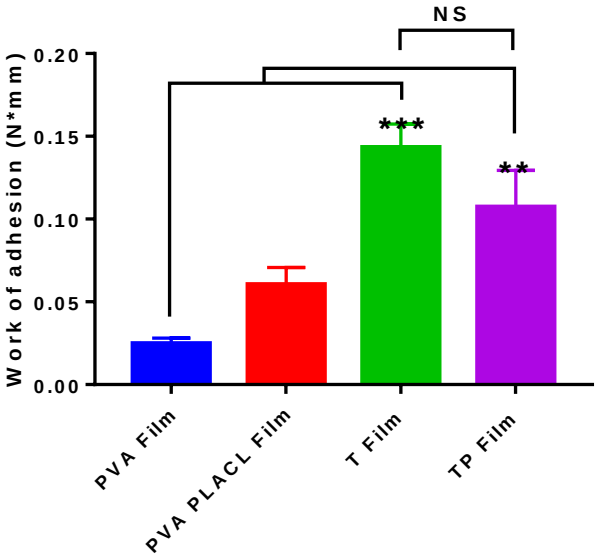


Figure 17. *Ex vivo* mucoadhesiveness of films.
The addition of thiomer into the PVA film matrix significantly enhanced the tissue mucoadhesion of films. Addition of PLACL80 into the thiomer film has no significant impact on the tissue mucoadhesion of films. Comparison was conducted between film formulations and PVA base film group. Data were analyzed using One-way ANOVA and Tukey's multiple comparisons test. Significant difference at ** $p < 0.01$ and *** $p < 0.001$. NS represents as not significant different ($n = 4$). PVA film is the clinical evaluated vaginal film. PVA PLACL film is the PVA film with addition of PLACL80. T film is PVA film with addition of thiomer. TP film is PVA film with addition of thiomer and PLACL80.

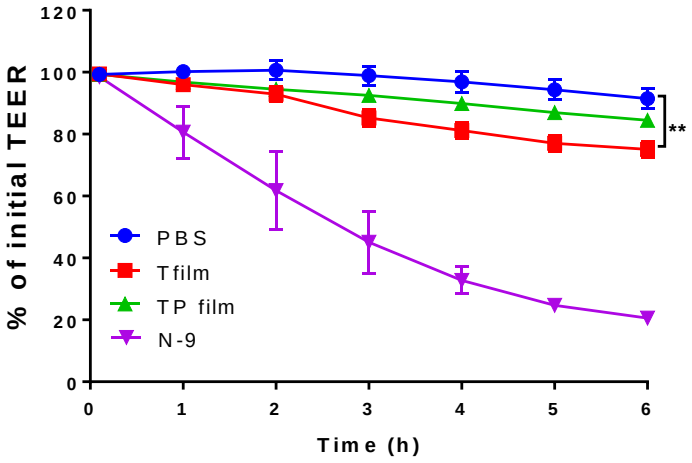
3.3.6 Impact of film formulation on epithelium integrity

The impact of thiomer containing films on tissue integrity was assessed by measuring the TEER of the excised human ectocervical tissue following a 6 h exposure to thiomer film or thiomer PLACL80 film (**Figure 18 a**). PBS was used as the negative control that showed only 8.5% reduction in TEER over 6 h incubation, while 0.6% of formulated N-9 (GYNOL II®, positive control) showed 79.4% decreased of TEER after 6 h incubation as compared to its initial TEER. The thiomer film had 24.9 ± 3.7% reduction in TEER as compared to its initial value. The reduction in TEER for thiomer film was significantly different from that for PBS ($p = 0.008$).

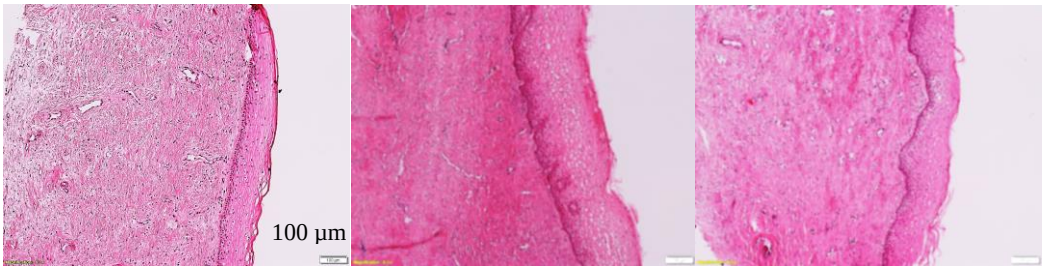
However, as demonstrated in Chapter-2, thiomer has no significant toxicity to the excised human ectocervical tissues. Thiomer PLACL80 film showed 15.5 ± 2.5 % reduction in TEER after 6 h exposure, but no significant difference was observed between groups of thiomer PLACL80 film and PBS ($p = 0.26$).

The post exposed tissue was further evaluated for changes in epithelial morphology using H&E staining. **Figure 18 b** shows that the exposure of N-9 to tissue resulted in disruption in the epithelial layer, whereas no significant changes in the tissue epithelial morphology were observed for PBS, thiomer film and thiomer PLACL80 film.

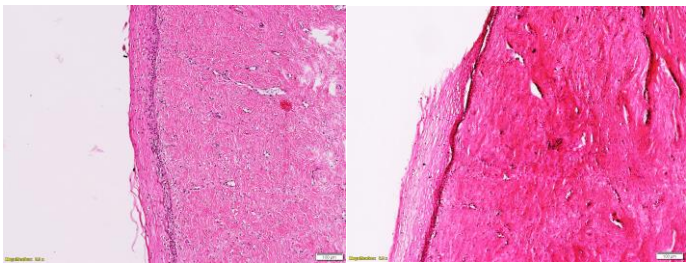
a.



b.



Pre-treatment T-film TP-film



PBS N-9

Figure 18. Impact of thiomer film on tissue epithelium integrity using Ussing Chamber system.

a. shows the change of the TEER for PBS (negative control), thiomer film (T film), thiomer PLACL80 film (TP film) and N-9 (GYNOL II®) (positive control). One-way ANOVA with Tukey's multiple comparison test was conducted. The reduction in TEER for thiomer film was significantly different from that for PBS (** $p = 0.008$). No significant difference was observed between PBS and TP film ($p = 0.26$) ($n = 3$); **b.** No significant changes on the morphology of human ectocervical tissue for pre-and post-treatment of PBS, T film and TP film using H&E staining.

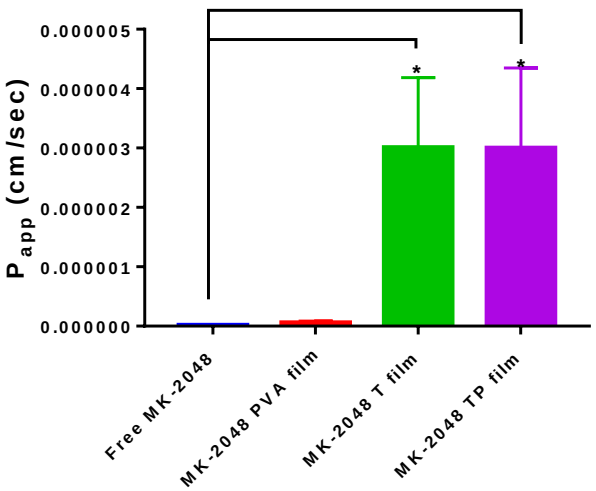
3.3.7 Enhanced tissue permeability of thiomer containing films

The permeation of MK-2048 formulated in thiomer containing film through human cervicovaginal tissue was evaluated using In-Line Cell system. The drug substance and all the films formulated MK-2048 was able to penetrate the epithelium of excised human ectocervical tissues under the tested experimental condition. No significant difference was observed between MK-2048 and MK-2048 PVA film. As compared to the drug substance, thiomer film and thiomer PLACL80 film showed significant increased P_{app} of MK-2048 (**Figure 19 a**). The % of tissue absorbed MK-2048 for free or formulated testing groups was investigated over 6 h and summarized in the **Figure 19 b**. It was observed that 12.15± 5.68 % of MK-2048 permeated into ectocervical tissue for thiomer film (**$p < 0.01$) and 24.70 ± 9.15% of MK-2048 for thiomer PLACL80 film (****$p < 0.0001$). Both films showed significantly higher permeated drug as

compared to free MK-2048 (0.24 ± 0.11 %) and PVA film formulated MK-2048 (1.25 ± 0.60 %).

To ascertain that the improved tissue permeability is not due to the morphology changes in the excised human ectocervical tissue, all the pre- and post-treatment tissues were stained for morphology observation. As compared to the tissue morphology prior to film treatment, no peeling or breach of the epithelial layers was observed in the tissues exposed to either of thiomer containing films, (**Figure 19 c**). These results demonstrated that the thiomer PLACL80 film formulated MK-2048 could be efficiently absorbed into the cervicovaginal tissue without affecting tissue morphology.

a.



b.

Tested articles	% in tissue	% Permeated (total)	Fold increased in % Permeated (total)
Free MK-2048	0.22 ± 0.09	0.24 ± 0.11	-
MK-2048 PVA film	1.09 ± 0.43	1.25 ± 0.60	5.2
MK-2048 T film	12.15± 5.68	16.00 ± 3.29	66.7 (**)
MK-2048 TP film	24.70 ± 9.15	29.82 ± 5.80	124.25 (****)

c.

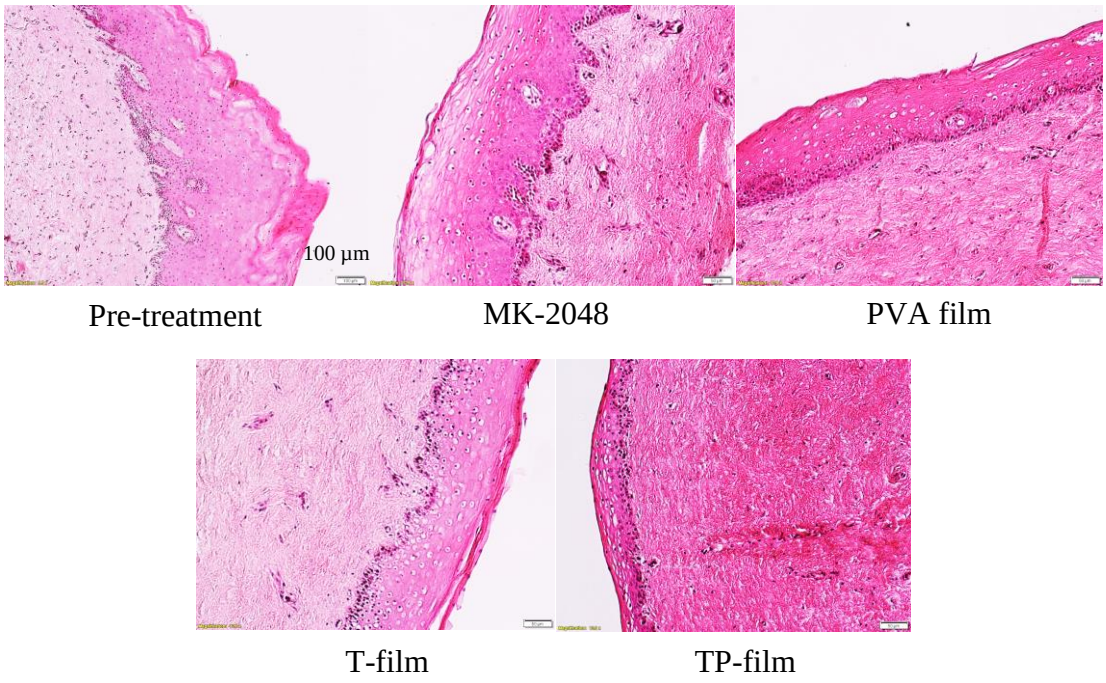
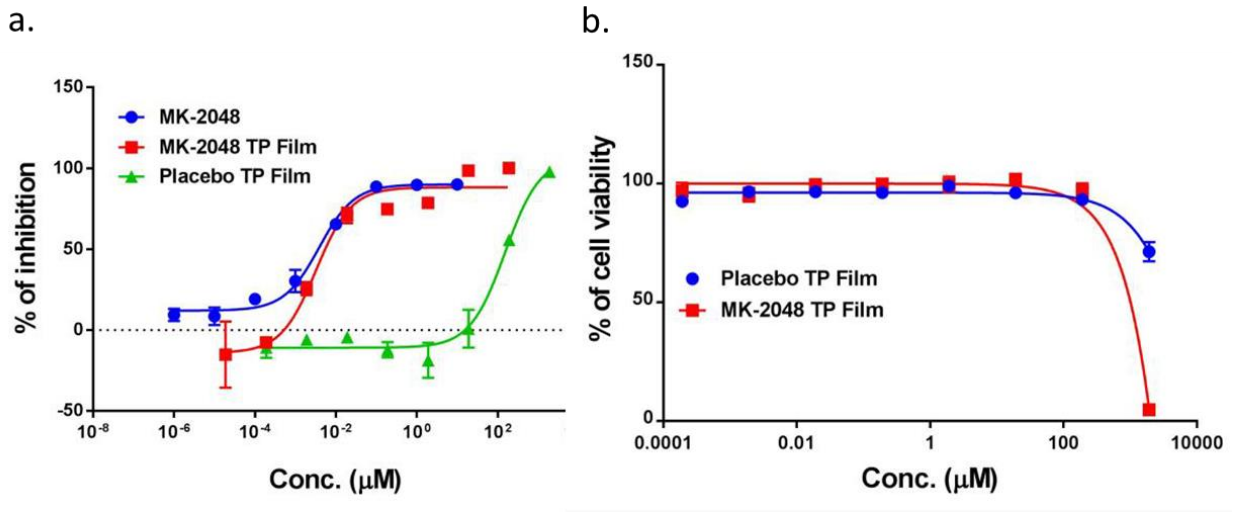


Figure 19. *Ex. vivo* tissue permeability using In-Line Cells system.
a. Shows the apparent partition coefficients (P_{app})for free MK-2048, PVA base film, thiomer film (T film) and thiomer PLACL80 film (TP film). Values indicate mean ± SD (n = 3). One-way ANOVA was used to analyze the data. (*p<0.05) **b.** Comparison of % permeated MK-2048 of free, PVA film, T film and TP film. Enhancement ratios result from the comparison of each tested film formulations with the free MK-2048 control. Indicated values represent the means ± SD (n = 3, **p < 0.01 and ****p < 0.0001 compared with free MK-2048). **c.** Comparison of morphology of human ectocervical tissues for pre-and post-permeability treatment

3.3.8 *In vitro* assessment of anti-HIV activity

The anti-HIV-1_{Bal} activity of free MK-2048, thiomer PLACL80 film formulated MK-2048 and placebo thiomer PLACL80 film was evaluated in the cell-based HIV assay with a single round of infection. The thiomer PLACL80 film formulated MK-2048 showed highly potent antiviral activity with 50% of effective concentration (EC_{50}) of 3.25 nM, which was similar to free MK-2048 (EC_{50} of 3.88 nM) in this assay (**Figure 20 a and c**). The placebo film also showed anti-HIV activity at high concentration. This activity observed in the placebo film is more than likely

due to the presence of the polymer excipients. **Figure 20 b** confirmed that the anti-HIV activity of the treatments was not due to the cytotoxicity.



c.

	MK-2048 TP Film	MK-2048 free drug
IC_{50} (nM)	3.25	3.88

Figure 20. Bioactivity of MK-2048 films

a. Anti-HIV activity of MK-2048 and thiomer PLACL80 (TP) film formulated MK-2048 using TZM-bl assay; **b.** cytotoxicity of MK-2048 and placebo TP film in TZM-bl cells. **c.** summarizes the IC_{50} of MK-2048 TP film and unformulated MK-2048. MK-2048 still maintained the potency in HIV inhibition in the TZM-bl cells after being formulated in the TP film (n = 3).

3.3.9 Compatibility of thiomer PLACL80 film with *Lactobacillus*

The compatibility of MK-2048 thiomer PLACL80 film with the normal vaginal flora *Lactobacillus* was evaluated against three Lactobacillus strains (*L. crisp ATCC 33197*, *L. jensenii ATCC 25258* and *L. jensenii LBP 25AB*). The Log (T_{30}min plate count –T_0 plate count) of less than 1 was considered as passed. MK-2048 thiomer PLACL80 film was safe against all tested *Lactobacillus* strains (**Table 10**).

Table 10. Compatibility of thiomer PLACL80 (TP) film with normal vaginal flora *Lactobacillus*

Lactobacillus strains	Log (T_{30}min plate count –T_0 plate count)
	TP-film
L. jensenii ATCC 25258	-0.040
L. jensenii LBP 25AB	-0.489
L.crisp ATCC 33197	-0.082

3.4 DISCUSSION AND CONCLUSION

User adherence plays a critical role in the successful application of antiretroviral microbicides for HIV prevention[49, 292-296]. The effectiveness of on-demand/coitally-dependent microbicides relies on self-motivated product use. It is imperative to develop coitally-independent product for HIV prevention. This can be addressed through the discovery of potent long-lasting anti-retroviral agents[297] or the development of suitable dosage forms. In this study, we have designed an extended-release polymeric vaginal film with enhanced mucoadhesive property, to deliver MK-2048, a potent second-generation integrase inhibitor, for HIV prevention.

Polymeric vaginal films studied to date were quick-dissolving and administrated in a coitally-dependent manner[226, 258, 264, 288]. PLACL is a biodegradable copolymer which is commonly utilized in tissue engineering or long-term sustained drug delivery system. Our target is to reduce the dosing frequency and develop a one-week administrated vaginal film. Ryoichi Wada et al. reported that the molecular weight of PLACL has impact on drug release: higher of the molecular weight, lower of the drug release[298]. As shown in Ryoichi's study, film made with PLACL 70 (molecular weight of 140 kDa) exhibited about 20% of Cisplatin release in one

week. Therefore, we incorporated PLACL 70, 80 or 90 with lower molecular weight between 12.3-16.8 kDa into the film to achieve a one-week drug release profile. In our study, film with incorporation of the PLACL showed much faster drug release rate as compared to the most of the reported PLACL containing drug delivery systems. In addition, the release rate of MK-2048 could be further adjusted by incorporation of PLACL with different LA/CL ratios. We observed that the overall drug release rate of PLACL 80 film was the lowest as compared to PLACL 70 and PLACL 90 films (**Figure 13**). This effect of the LA/CL ration in copolymers on the drug release rate showed in our study is different from the reported result from Ryoichi Wada et al, but consistent with the result from Jelonek, K et al.[299]. This difference is more than likely due to the API properties or the synthesized PLACL polymer properties.

In these studies, the incorporation of thiomers significantly increased the film elastic modulus (**Figure 12**). This is because the T_g of thiolated chitosan is approximately 100 °C (data not shown), which makes it very brittle at the body temperature or room temperature. Stiff vaginal films are not desirable since they may create discomfort during the insertion, which will lead to low user acceptability and adherence for women. However, the increased stiffness induced by thiomers can be circumvented through the incorporation of PLACL. DSC analysis revealed that the T_g of PLACL can be modified by tuning the ratio of LA to CL. All PLACLs synthesized in this work have T_g below the body temperature (**Table 7**). Therefore, PLACL works like a plasticizer that makes the thiomer film to be more elastic. The elasticity of these films was maintained at the similar level as that of the commercial film VCF® and clinical PVA film, which have already shown good acceptability for women. Considering that the PLACL 80 film displayed the lowest drug release rate, it was selected as the leading film candidate for further studies.

Thiolated chitosan was shown to possess more than 7-day tissue retention time *in vitro*, outperforming other thiolated polymers as reported by A. Bernkop-Schnu¨rch's group[178]. To the best of our knowledge, we are the first to formulate thiomers into film formulation and Chapter 2 has demonstrated that the thiomer containing film was able to be retained in the vaginal compartment for at least 7 days *in vivo* (**Figure 6**). Thus, the thiomer containing film was utilized as a comparator for evaluation of the mucoadhesion of the designed thiomer PLACLs film using the developed *ex vivo* mucoadhesion method. The result showed that the excellent tissue mucoadhesion of thiomer containing film was not compromised after co-formulation with PLACL 80 (**Figure 17**). This might be due to the tissue adhesion property of the low molecular weight PLACL[300]. In addition, for most mucoadhesive polymers, insufficient retention time is caused by the continuous daily mucus turnover, which clears the mucus-bound polymers. This can be circumvented by the incorporation of PLACL 80, which sustainably provides free thiol group for film matrix to interact with mucin, thereby extending the residence time for film in vaginal cavity. The increased residence of the film leads to continuously prolonged delivery of MK-2048 for HIV prevention.

It was observed that the tissue permeability of MK-2048 was significantly improved by formulating the drug into the thiomer containing film (**Figure 19**). It was also found that the human ectocervical tissue epithelial integrity was affected by the film containing thiomers as illustrated by the decreased TEER after 6 h-exposure (**Figure 18 a**). As reported by Gradauer et al.[301] and Song et al.[302], the enhanced permeability is usually related to reduced TEER indicating change in the barrier function of epithelial layer in tissue. However, the histology staining of the cervical tissues that were used for TEER and permeability study showed no significant morphology changes in the epithelium of excised human ectocervical tissues (**Figure**

18 b). Also, the tissue toxicity data support that thiomer is highly tolerable to human ectocervical tissue as demonstrated at Chapter 2 (**Figure 5**). We suspect that the improved tissue permeability is caused by thiomer via tight junction opening of epithelial layer[303]. Furthermore, thiomer is a P-gp inhibitor[301] and our previous work from Zhou et al. reported that the P-gp transporter is highly expressed in human cervicovaginal tissues[304]. Therefore, thiomer is very promising to serve as an efficient and safe absorption enhancer for vaginal delivery of antiretroviral P-gp substrates in HIV prevention.

We also studied the cytotoxicity of the thiomer PLACL80 film in the TZM-bl cell, which is an engineered cervical epithelial cell line. No cytotoxicity was observed for the placebo thiomer PLACL80 films (**Figure 20 b**). The MK-2048 thiomer PLACL80 film showed extremely high CC_{50} as compared to the drug level for HIV inhibition. In addition, the ability of MK-2048 and film formulated MK-2048 to inhibit HIV infection was investigated using TZM-bl assay. The EC_{50} of MK-2048 for the cellular HIV inhibition was comparable to its reported integrase inhibition activity[286]. The thiomer PLACL80 formulated MK-2048 showed similar EC_{50}, which indicates that the film excipients have no negative interference to the anti-HIV activity of API (**Figure 20**). Interestingly, we also found that the placebo thiomer PLACL80 film has a baseline *in vitro* inhibitory effect on HIV infection (**Figure 20 a**). Since the safety of the placebo film has been confirmed in the cytotoxicity study, one possible mechanism of the anti-HIV activity of the placebo film could be the interaction between the thiomers and the cysteine domains in HIV-1 Tat, which inhibits its activation of the transcription of HIV and promotion of more infections[305]. However, further investigation needs to be conducted to study the impact of excipients on HIV-1 inhibition.

In conclusion, MK-2048 can be successfully formulated into the thiomer PLACL80 polymeric films that show a sustained release profile, enhanced tissue permeability and potent *in vitro* anti-HIV activity. The designed sustained-release film platform significantly improved the tissue mucoadhesion for vaginal delivery and was biocompatible with human ectocervical tissue and epithelial cells. These results demonstrate that this novel film platform has potential to address the user adherence issue in HIV prevention.

3.5 ACKNOWLEDGEMENT

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4.0 DEVELOPMENT AND ASSESSMENT OF MK-2048 LOADED MUCOADHESIVE NANOPARTICLES-IN-FILM AS VAGINAL MICROBICIDES FOR HIV PREVENTION

4.1 INTRODUCTION

Nanoparticles are increasingly getting attention for potential delivery systems as vaginal microbicides due to their unique merits[115]. Some of the antiretroviral drugs such as MK-2048 need to enter the target cells to interrupt the viral cycle life. For a nanoparticle based vaginal microbicides to deliver such drugs to the targeted subepithelial site, the first hurdle is to penetrate the mucus layer which has a fast clearance rate. Dapivirine-loaded nanoparticles have been shown to be rapidly cleared from the mucosal layer within 24 h in a mouse model[126]. Therefore, the ideal nanoparticles should first be retained on mucus layer to avoid rapid vaginal clearance before migrating through mucus towards the epithelial layer.

To achieve this goal, two strategies have been studied. The first one utilizes nanoparticles that can diffuse through mucus layer at a faster rate than the clearance rate[306]. The other one utilizes mucoadhesive nanoparticles to prolong their retention time[307]. PEGylated nanoparticles have shown excellent *in vitro* mucus penetration ability in diluted cervical mucus and have been applied for mucosal drug delivery[308]. However, there is no significant improvement in the local PK for the polyethylene glycol-poly(lactic-co-glycolic acid) (PEG-

PLGA) nanoparticle loaded film as compared to the quick-dissolving film[229]. The PEGylated nanoparticles were also loaded in mucoadhesive fibers for achieving sustained drug delivery[141]. In a mice model, nanoparticle loaded fiber showed a 7-day retention in the vagina but only reached 3-day sustained drug release[141]. This result indicated that the nanoparticle itself needs to be retained in mucosal to provide sufficient drug release. Therefore, the nanoparticle based vaginal microbicides need to be further improved.

Thiomers are a new generation of mucoadhesive polymers which have improved mucoadhesion due to the disulfide bond formation with mucin[176]. Thiomer nanoparticles have been developed and applied for buccal, ocular, and intestinal drug delivery with enhanced tissue residence time[309]. In addition, thiomer is also reported as a permeation enhancer by opening tight junction and inhibiting efflux transporter [178, 301]. These features of thiomer enable it to simultaneously improve the mucoadhesion and permeation during the delivery of antiretrovirals to the mucosal tissue. However, since thiomer is a hydrophilic polymer, it has low encapsulation efficiency for hydrophobic drugs[310]. The antiretroviral drug utilized in this study, MK-2048, is a hydrophobic integrase inhibitor with potent anti-HIV activity[286]. The low drug encapsulation efficiency is undesirable for manufacturing nanoparticle loaded film due to the high amount of antiretroviral drugs needed in the carrier for monthly dosing regimen goal. In addition, the low drug encapsulation efficiency also makes it necessary to load more nanoparticle into polymeric film which could potentially impact the mechanical properties of films[311]. To overcome this problem, the PLGA nanoparticle (PNP) stands out, because it has shown high drug encapsulation efficiency for hydrophobic drug and offers sustained drug release[312]. We hypothesized that the surface coating of thiomer onto PNP could provide mucoadhesion, penetration enhancement, and high drug encapsulation efficiency, which are favorable for sustained-release vaginal drug

delivery. The nanoparticles were further incorporated into the polymeric film since film is a discrete and highly acceptable solid dosage form and can provide a more stable environment for the nanoparticles rather than the gel.

The objective of this study was to develop a vaginal film loaded with thiomer coated PLGA nanoparticles (TNP) for the delivery of MK-2048 in preventing sexually transmitted HIV. In this study, we optimized the manufacture process for TNP and evaluated the *in vitro* and *ex vivo* characteristics of TNP loaded film including the nanoparticle properties, physicochemical properties of TNP films, mucoadhesion, drug release, tissue permeability, cytotoxicity, lactobacillus compatibility as well as bioactivity in HIV inhibition. Our results indicate that this novel vaginal nanoparticle loaded film prolongs tissue mucoadhesion, enhances tissue permeability, imparts sustained release profile for MK-2048, and possess excellent safety profile. We envision that this promising coitally-independent topical microbicide tool can improve user adherence in HIV prevention.

4.2 MATERIALS AND METHODS

4.2.1 Materials

MK-2048 was provided by Merck Sharp & Dohme Corp. (NJ, USA). PLGA with molar ratios of 50:50 lactic acid: glycolic acid was purchased from Sigma-Aldrich (Resomer [503, Mw 24,000-38,000 D]; St. Louis, MO). PEG 8000 was purchased from Spectrum (Gardena, CA, US). Hydroxypropyl methyl cellulose E5 (METHOCEL^{TM} E5) was obtained from Dow Chemical Company (Midland, MI, US). Polyvinyl alcohol 4-88 (PVA) was purchased from Millipore

Sigma (Temecula, CA). Cell culture reagents were obtained from GIBCO, Invitrogen by Life Sciences, Inc (Lenexa, Kansas). Ultrapure water was prepared by passing distilled water through a Milli-Q® Reagent Water System (Millipore®). All the other chemical reagents for nanoparticle manufacture and film preparation were purchased from Fisher Scientific (Pittsburgh, PA).

4.2.2 Development of high-performance liquid chromatography (HPLC) method for MK-2048

The HPLC method for MK-2048 was described in Chapter 3 (section 3.2.6).

4.2.3 Development of thiomer coated PLGA nanoparticles (TNPs)

Thiomers were synthesized using the method as described in Chapter 2. **Table 11** shows the formulations and manufacturing parameters for MK-2048-loaded TNPs using a reported emulsion-solvent evaporating method with modifications[313]. Briefly, MK-2048 and PLGA were dissolved in methylene chloride (DCM) as the oil phase. 2mg/mL of thiomer solution and 4% (w/v) polyvinyl alcohol (PVA) solution were mixed well at equal volume as the aqueous phase. The oil phase and aqueous phase were homogenized using Vibra-Cell probe sonicator (Sonics and Materials, Newton, CT) for 300 s at 50 W and 50% intensity. The resulted oil-in-water emulsion was then diluted in Milli-Q water under magnetic stirring in an ice bath at 300 rpm. TNPs were then washed three times with Milli-Q water by centrifuging at 15,000 × g 4 °C for 20 mins (Sorvall Ultra 80, Waltham, MA). The concentrated TNPs were resuspended in 3 mL of Milli.Q water and then lyophilized overnight under vacuum at 0.120 mbar and at −50°C using a FreeZone 6 lyophilizer (Labconco, Kansas City, MO). The lyophilized nanoparticles were

stored at 4°C until use. The placebo/MK-2048 loaded PNPs and placebo TNPs were manufactured using a similar method as described above.

Table 11. Formulation and manufacture parameters for scale-up process

	PLGA (mg)	PVA % (w/v)	Thiomers % (w/v)	DCM (mL)	Aqueous phase (mL)	Excess water (mL)	Sonication probe[1]	Sonication time (s)	Stirring time (h)
#1-77.5 mg	20	2	0.1	1	2	10	S2	50	4
#2-2.3 g	1200	2	0.1	12	24	50	S6	300	Overnight

[1]S2-Sonication probe diameter is 2 mm; S6- Sonication probe diameter is 6 mm.

4.2.4 Characterization of TNPs

Drug encapsulation efficiency was determined by indirect method. The drug content in the supernatant of TNPs/PNPs was determined by HPLC method after washing with Milli-Q water three times. The % of encapsulation efficiency (%EE) was calculated by $\frac{Loading\ drug\ content - drug\ content\ in\ supernatant}{Loading\ drug\ content} \times 100$. All nanoparticles were dispersed in water and characterized for hydrodynamic diameter, polydispersion index (PdI), and zeta potential at 25 °C using a ZetaSizer Nano ZS90 (Malvern Instruments, Malvern, UK). The drug loading % was calculated by $\frac{Experimental\ drug\ content\ in\ particles}{(Polymer\ mass + Experimental\ drug\ content\ in\ particles)} \times 100$. Thiolated degree on TNPs was determined by Ellman's assay[314]. Thiomer coating efficiency% on TNPs was determined by $\frac{Thiol\ degree\ in\ particles}{Thiomers\ loading\ content} \times 100$. The morphology of lyophilized TNPs was evaluated by scanning electron microscopy (SEM) (JEOL JSM6510) using 0.5 kV electron beam and imaged at magnification of 30 k×.

4.2.5 Development and characterizations of TNP film

Films were manufactured by solvent casting method. Briefly, the film forming excipients (PVA40-88, METHOCEL E5 and polyethylene glycol 8000 (PEG-8000)) were dissolved in Milli-Q water. Then the MK-2048 TNPs/PNPs or placebo TNPs/PNPs were dispersed in mixture of plasticizers (glycerin and propylene glycerol) and added into the film solutions with overhead stirring. The uniformed mixed film solution was casted onto the polymeric substrate under 72 °C heating for 15 min using the film applicator (Elcometer® 4340). The obtained film sheet was cut into 1″ × 1″ size using dye compressor. PVA film (without TNPs) was manufactured based on the same method without dispersing nanoparticles in plasticizers.

Films were evaluated by the following features: 1). Drug content: MK-2048 TNP films were dissolved in 40% of acetonitrile and sonicated for drug extraction. An aliquot was withdrawn and centrifuged at 12,000 rpm for 15 min. The supernatant was further diluted accordingly and analyzed by HPLC as described above. 2). Water content, 3). Puncture strength, 4). Disintegration followed the methods described in Chapter 2 section 2.2.5. 5). Morphology and elemental analysis of TNP film: the SEM imaging of films with/without TNPs was performed at 1.0 kV for base film and 0.5 kV for TNP film. The elements in TNP area and film base were analyzed by energy dispersive X-ray spectroscopy (EDS) at 5.0 kV, Oxford INCA EDS system.

4.2.6 *Ex vivo* tissue mucoadhesiveness

Mucoadhesion studies of TNPs and PNPs were performed on porcine intestinal tissue using the experimental setup established by Ranga Rao & Buri with some modifications[315]. Briefly,

porcine intestinal mucosa was mounted on a tube-shaped platform and placed in a cupboard with an angle of 45°. A constant flow rate of 1 mL/min was provided by using a syringe pump (KD Scientific, Holliston, USA). To humidify the tissue, vaginal fluid simulant (VFS) was used to rinse the tissue for 5 mins before exposing to nanoparticles. After the equilibration period, a premeasured amount of nanoparticles were transferred to the mucosa and rinsed continuously with VFS. After 30 min, 1, 2, or 3 h, the collected particles and fluids in the reservoir were sampled and centrifuged at 12,000 rpm for 10 mins. The content of MK-2048 detected using HPLC was used to quantify the amount of washed nanoparticles.

4.2.7 *In vitro* drug release

To investigate the in vitro drug release from nanoparticles and nanoparticle films, PBS with 1% Cremophor® (pH 7.4), and VFS (pH 4.2) were used as dissolution media. MK-2048 loaded TNP and TNP films were dispersed in 5 mL of each dissolution medium, with continuous shaking, at a temperature of 37°C. At predetermined intervals, the TNP/TNP film were isolated via centrifugation at 12,000 rpm for 10 mins, and the supernatant was collected for HPLC analysis to determine the released drug content. The nanoparticles were then resuspended in fresh dissolution media and returned to the *in vitro* release set-up.

4.2.8 Cell culture

The vaginal epithelial (VK2/E6E7) and TZM-bl cell lines were purchased from American Type Culture Collection (ATCC). The VK2/E6E7 cells were cultured in keratinocyte-serum free medium (GIBCO-BRL 17005-042) with 0.1 ng/mL human recombinant EGF, 0.05 mg/mL

bovine pituitary extract, 44.1 mg/L calcium chloride (final concentration 0.4 mM) at 37 °C under 5% of carbon dioxide (CO_2). The TZM-bl cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 1% 200 mM L-glutamine at 37°C in 5% CO_2.

4.2.9 *In vitro* cellular uptake of TNPs

Before conducting cellular uptake studies for developed nanoparticles, the cytotoxicity of MK-2048, MK-2048-loaded TNPs and placebo TNPs were assessed in VK2/E6E7 cells. Briefly, cells were seeded in 96-well plates at 1×10^4 cell/cell followed by 24 h incubation in cell culture media. Cells were treated with different MK-2048 formulations of varying concentrations (at the equivalent concentrations of unformulated MK-2048). Placebo TNP was used as a control to treat cells at amounts equivalent to MK-2048 encapsulated TNPs. After the 24-h treatment of nanoparticles, cells were incubated with MTT solution for 4 h and washed with dimethyl sulfoxide (DMSO). Plates were read at a wavelength of 570 nm for calculating the cell viability.

The hydrophobic fluorescein isothiocyanate (FITC) was encapsulated into PNP and TNP via the same fabrication method as the MK-2048 PNP and MK-2048 TNP. VK2/E6E7 cells were seeded into the 12-well plates at a density of $10^{\wedge 4}$ cells/ well at 37°C for 48 h till 80% confluence was achieved. Fluorescent dye-loaded NPs were added to the cells at 100 µg/mL of the total 200 µL of cell culture media for 2 h at 37 °C. Then cells were washed with cold PBS three times, and fixed with 4% paraformaldehyde for 30 min. Afterwards, cells were stained with NucBlue™ (Fixed Cell ReadyProbes™ Reagent) for 10 mins. Subsequently, cells were washed with cold saline. Finally, the cellular uptake of FITC in various formulations was observed under

microscope for fluorescent imaging (OLYMPUS, Pittsburgh PA). Cell images were taken at 358/461 nm for 4',6-diamidino-2-phenylindole (DAPI), 495/519 nm for FITC.

Quantitative cellular uptake of PNP and TNP was assessed by flow cytometry. Briefly, VK2/E6E7 cells were seeded into the 12-well plates at a density of $3\times10^{\wedge5}$ cells/well. After overnight attachment, cells were treated with 100 µg/mL FITC loaded PNP and TNP, respectively. Cells without treatment were used as a control. Following incubation at 37 °C for 2 h, cells were washed with cold Dulbecco's phosphate-buffered saline (DPBS) and treated with trypsin-EDTA and incubated at 37 °C for 3-5 mins. Cell suspension was neutralized with DMEM: F12 with 10% FBS. After centrifugation, cell pellets were resuspended in 200 µL of cell culture media for the flow cytometry analysis using FlowJo software version 9 (Tree Star, Inc., Ashland, OR). Cell-associated FITC was excited at 495nm, and fluorescence was detected at 519 nm. A total of 10,000 events were collected for each sample.

4.2.10 *Ex vivo* tissue permeability

The *ex vivo* tissue permeability of TNP films were evaluated using the In-Line Cell system as described in Chapter 3 (section 3.2.12).

4.2.11 *In vitro* anti-HIV activity

The anti-HIV activity of placebo TNP film, MK-2048 loaded PNP film and TNP film were evaluated in TZM-bl model as described in Chapter 3 (section 3.2.13).

4.2.12 Compatibility with *Lactobacillus*

The compatibility of films with Lactobacillus was evaluated using the standard microbicide safety test as described in Chapter 3 (section 3.2.14)

4.2.13 Statistical analysis

All values are reported as means ± standard deviation (SD). Statistical data analyses were performed using the Student's t-test between two groups and one-way ANOVA with Tukey's post hoc test for the analysis of three or more study groups, with $p < 0.05$ as the minimal level of significance, $p < 0.01$ for very significant and $p < 0.001$ for highly significant. All tests were performed using the GraphPad Prism software version 7.

4.3 RESULTS

4.3.1 Characterizations of TNPs and TNP film

The batch size of TNPs was scaled up from 77.5 mg to 2.3 g with various modifications as described in **Table 11**. The characteristics of these nanoparticles are listed in **Table 12**. The particle size of scale 1 and 2 were both around 250 nm. The scaled-2 TNPs exerts more uniform particle size, lower zeta potential, higher drug encapsulation efficiency and two times of drug loading as compared to those of the scale-1.

Placebo PNPs, MK-2048 PNPs and placebo TNPs were manufactured using the scale-2 formulation and manufacture parameters. Their particle size, zeta potential, drug encapsulation efficiency, drug loading% and thiomer coating efficiency are listed in **Table 13**. The particle size of TNPs was increased by about 40 nm as compared to PNPs. Zeta potential of TNPs displayed positive confirming the particle surface coating of positively charged thiomers, whereas PNPs displayed negative charge. Using the optimized formulation and manufacture parameters, the high drug encapsulation efficiency was achieved with excellent reproducibility (multiple batches were manufactured and obtained the similar drug encapsulation efficiency). With MK-2048 encapsulation, the zeta potential was increased for both of TNPs and PNPs, which attributed to the positive charge of MK-2048 under neutral pH. Thiomer coating efficiency was determined as 68. 85 ± 1.57%. Particle size of three batches of TNPs was evaluated by DLS and showed in **Figure 21 a** with excellent reproducibility. The morphology of the lyophilized TNP was spherical as obtained through SEM (**Figure 21 b**).

To prepare nanoparticles-in-films, the lyophilized TNPs were dispersed in PVA based film matrix. The weight, thickness, puncture strength, water content and disintegration of PVA base film, low TNP loading film and high TNP loading film were characterized (**Table 14**). With TNP loading, film weight, thickness and disintegration were increased, whereas puncture strength and water content were decreased. This is because the addition of TNPs increased more hydrophobic polymers in the film matrix.

Table 12. MK-2048 TNP scale-up characterizations

Samples	Size (nm)	PDI	ZP (mV)	% EE	% DL
Scale 1-77.5 mg	257.6 ± 7.3	0.190 ± 0.007	17.9 ± 1.9	87.4 ± 3.04	12.9
Scale 2-2.3 g	242.8 ± 1.6	0.098 ± 0.06	11.3 ± 0.7	99.7 ± 0.01	26.04

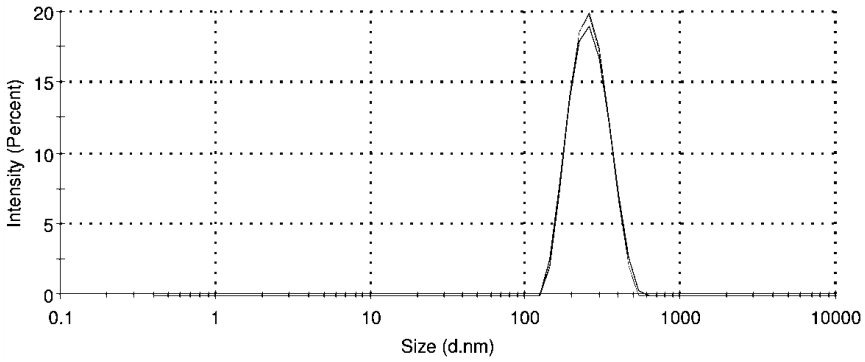
PDI: Polydispersity index; ZP: zeta potential; EE: encapsulation efficiency, DL: drug loading

Table 13. Biophysical characteristics of MK-2048 loaded and blank TNPs

Samples	Size (nm)	ZP (mV)	Drug EE%	DLC%	% of TCE
Placebo PLGA NP	212.4 ± 2.3	-24.8 ± 0.5	NA	NA	NA
MK-2048 PLGA NP	209.0 ± 0.5	-23.4 ± 0.1	99.7 ± 0.03	26.32	NA
Placebo Thiomer PLGA NP	253.7 ± 5.0	9.5 ± 0.2	NA	NA	NA
MK-2048 Thiomer PLGA NP	242.8 ± 1.6	11.3 ± 0.7	99.7 ± 0.01	26.04	68.85 ± 1.57

DLC: drug loading capacity; TCE: thiomer coating efficiency; NA: not available
Particle size of all the nanoparticles has PDI less than 0.2

a.



b.

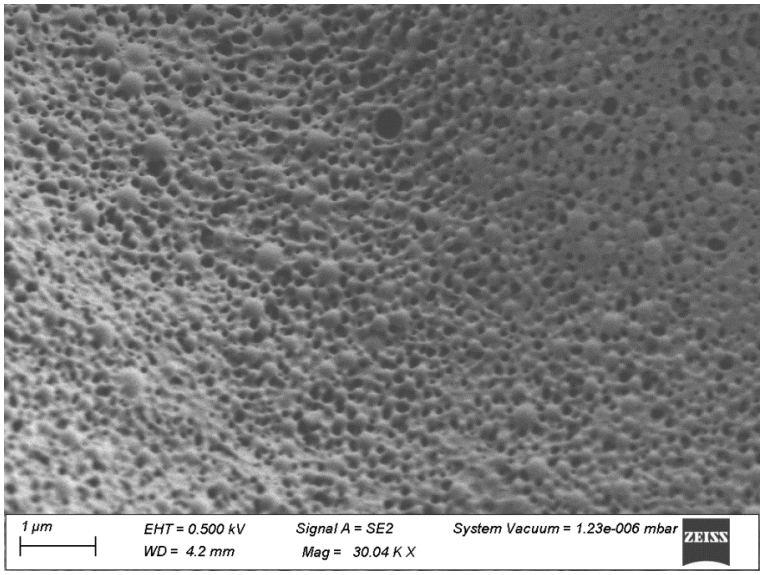


Figure 21. **Characterizations of TNPs**

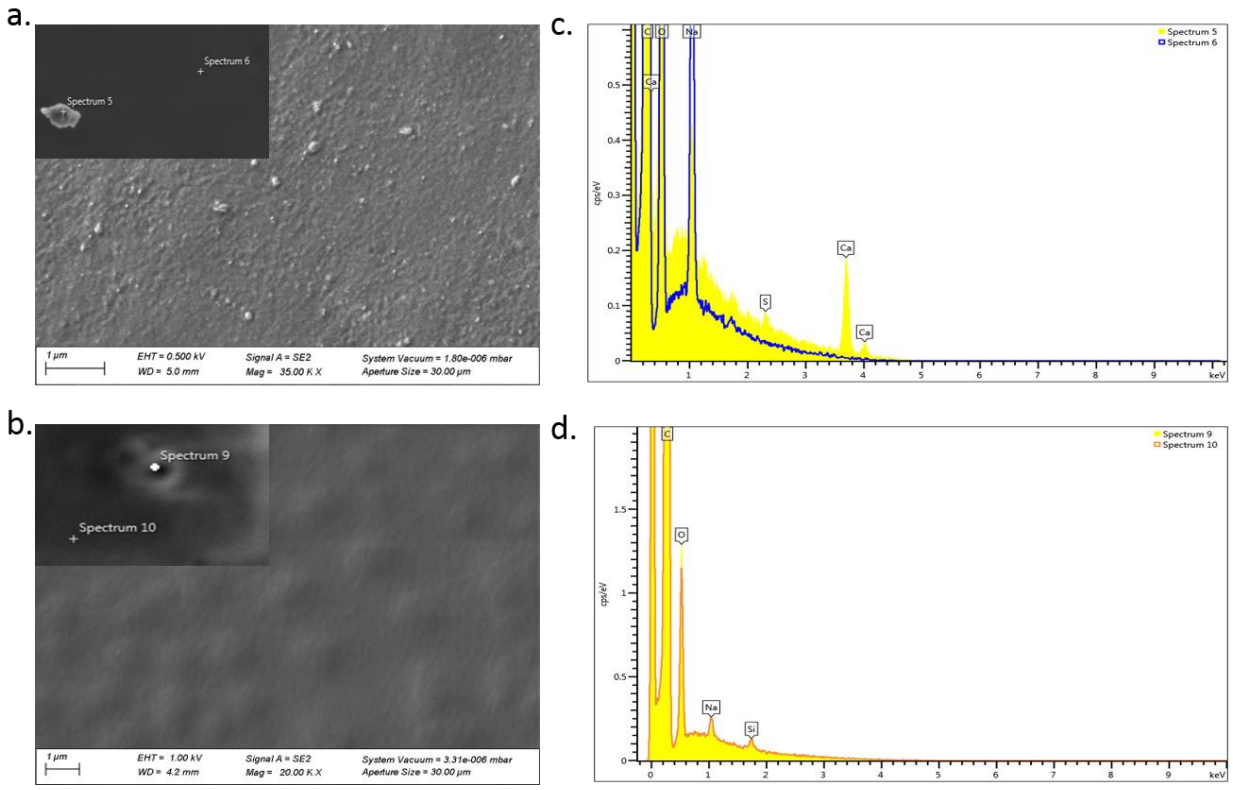
a. Size distribution and **b.** Morphology of MK-2048 TNPs were examined by dynamic light scattering and SEM, respectively.

Table 14. Physicochemical characteristics for TNP and PVA films

Characterizations	PVA film	TNP Film
NP loading %	0	26.45%
Weight (mg)	69.22 ± 11.54	92.75 ± 11.44
Thickness (mm)	0.10 ± 0.02	0.23 ± 0.02
Puncture strength (kg/mm)	13.06 ± 1.44	4.07 ± 0.21
Water content %	4.30 ± 0.18	2.84 ± 0.07
Disintegration (s)	95.6 ± 10.19	221.49 ± 33.77

4.3.2 Morphology and element analysis of TNP film

SEM was utilized to observe the morphology of TNP film and PVA base film. As shown in **Figure 22 a and b**, spots were uniformly distributed in TNP films while no spots were present in PVA base film. To indicate the spots in the TNP film, areas with and without spots in TNP film, and two random areas in PVA film were further analyzed for elements. The results showed that as compared to the film part without any spots, the spots in the TNP film contained two extra elements of sulfur and calcium which are from thiomers (**Figure 22 c, d and e**). This result indicates that TNPs were loaded in PVA film with intact morphology and the surface modification of thiomers was not affected by the film manufacture process.



e. Summary of the elements in TNP film and base film

	Common elements	**Different elements**
TNPs area	C, O, Na	S, Ca
Films without TNP	C, O, Na	-

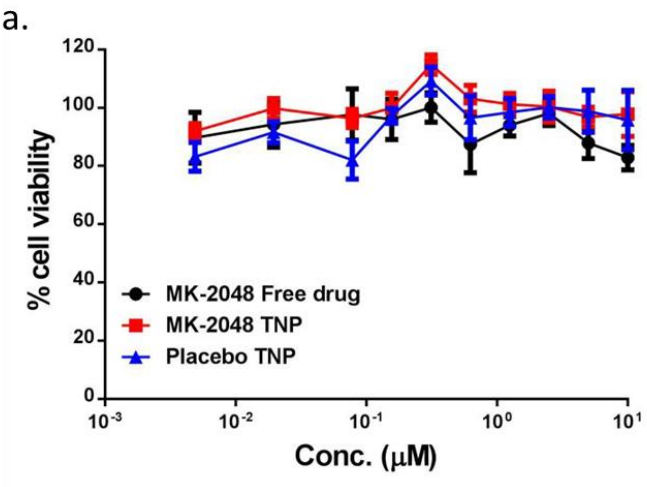
Figure 22. Characterizations of TNP films

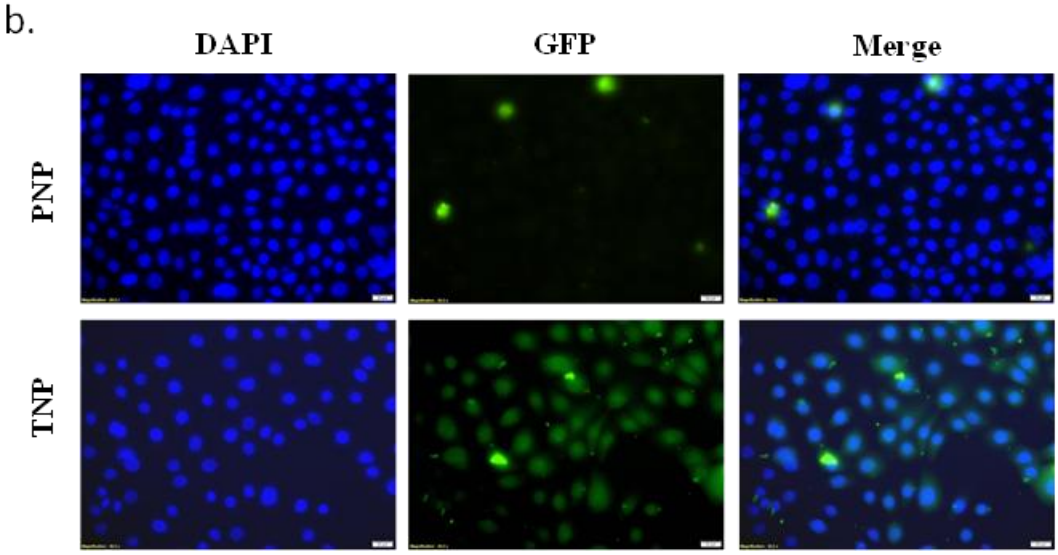
Morphology of **a.** TNP film and **b.** PVA film by SEM; and elements analysis of **c.** TNP film and **d.** PVA film by EDS. **e.** is the summary of the elements in films with/without TNP areas.

4.3.3 Cellular uptake of TNPs

Free MK-2048, MK-2048 TNPs and placebo TNPs showed no significant toxicity for VK2/E6E7 cells (**Figure 23 a**). More TNPs were taken up by VK2/E6E7 than PNPs at 2 h (**Figure 23 b**).

The cellular uptake of TNP and PNP was further analyzed by flow cytometry and the quantitative result confirmed that TNP has improved the VK2/E6E7 cell uptake as compared to PNP (**Figure 23 c**).





C.

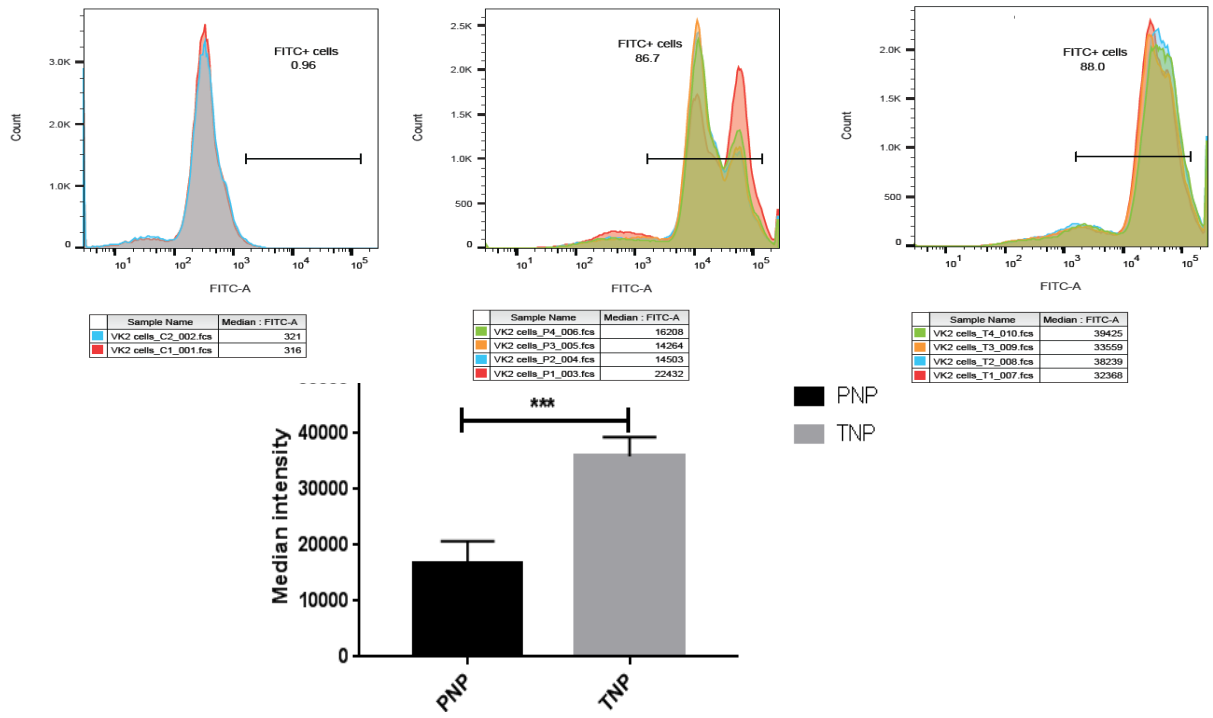


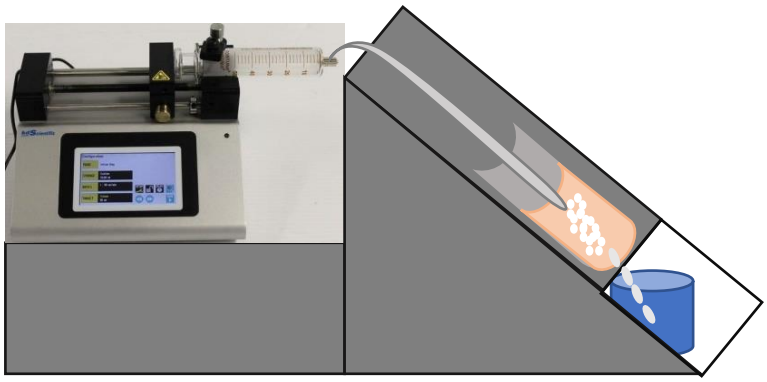
Figure 23. Cellular uptake of TNPs

a. Cytotoxicity of TNPs in VK2/E6E7 cells; **b.** Microscope image of FITC loaded PLGA nanoparticles (PNP) and thiomer coated PLGA nanoparticles (TNP) and **c.** flow cytometry of PNP and TNP for cellular uptake in VK2/E6E7 cell line.

4.3.4 Tissue mucoadhesion of TNPs

The tissue mucoadhesion was evaluated by the incline model using porcine intestinal mucus tissue (**Figure 24 a**). As shown in **Figure 24 b**, more PNPs were washed out from the tissue than TNPs ($p < 0.05$) at 30 mins, 1 h, 2 h, and 3 h. This indicates that TNPs are more likely to be retained on mucosal tissue than PNP. Coating of thiomer on nanoparticles significantly improved the tissue mucoadhesion.

a.



b.

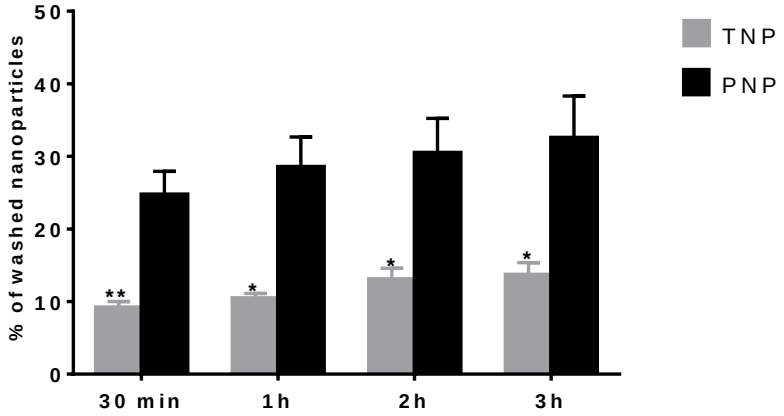


Figure 24. *Ex vivo* mucoadhesiveness of TNPs.
a. Incline model of mucoadhesion. **b.** % of washed nanoparticles for TNP and PNP for different time periods (n = 3, ** $p < 0.01$ and * $p < 0.05$).

4.3.5 *In vitro* drug release of TNPs and TNP film

The *in vitro* drug release profile of MK-2048 TNPs and MK-2048 TNP film was evaluated over 30 days in buffers of PBS with 1% Cremophor® EL pH 7.4 and VFS. The *in vitro* release of MK-2048 from TNPs and TNP films in both buffers followed a biphasic release profile (**Figure 25 a and b**). In VFS, there was 10.13 ± 1.05% and 26.67 ± 1.67% of MK-2048 released from TNPs and TNP film respectively, with initial burst release within first 24 h. A sustained release of MK-2048 from TNP and TNP film in VFS was obtained over 30 days. The total amount of

MK-2048 released from TNPs and TNP film over 30 days was 24.7 ± 2.6% and 42.2 ± 3.1%, respectively. In addition, the TNP film showed faster release rate of MK-2048 than that of TNP in both buffers. In PBS with 1% Cremophor® EL pH 7.4, 32.3 ± 7.0% and 49.3 ± 11.2% of MK-2048 were released from TNPs and TNP film for the first 24 h. MK-2048 was released continuously from TNPs and TNP film over the next 30 days. The total amount of released MK-2048 from TNP and TNP film was 73.6 ± 13.7% and 96.1 ± 1.8%, respectively.

In order to reveal the mechanism of drug release from fabricated TNPs and TNP film, various kinetic mathematical modeling was used to analyze the *in vitro* release profiles of MK-2048. Three mathematical models used were the Higuchi model, the Peppas–Sahlin model, and the Weibull model. Higher correlation was observed in the Peppas-Sahlin model (**Figure 25 c and d**). Therefore, our results indicate that release of MK-2048 from TNPs and TNP film is not predominantly driven by a solo mechanism, but a combined mechanism of Fickian (pure diffusion phenomenon) and non-Fickian release (due to the relaxation of the polymer chains between the networks).

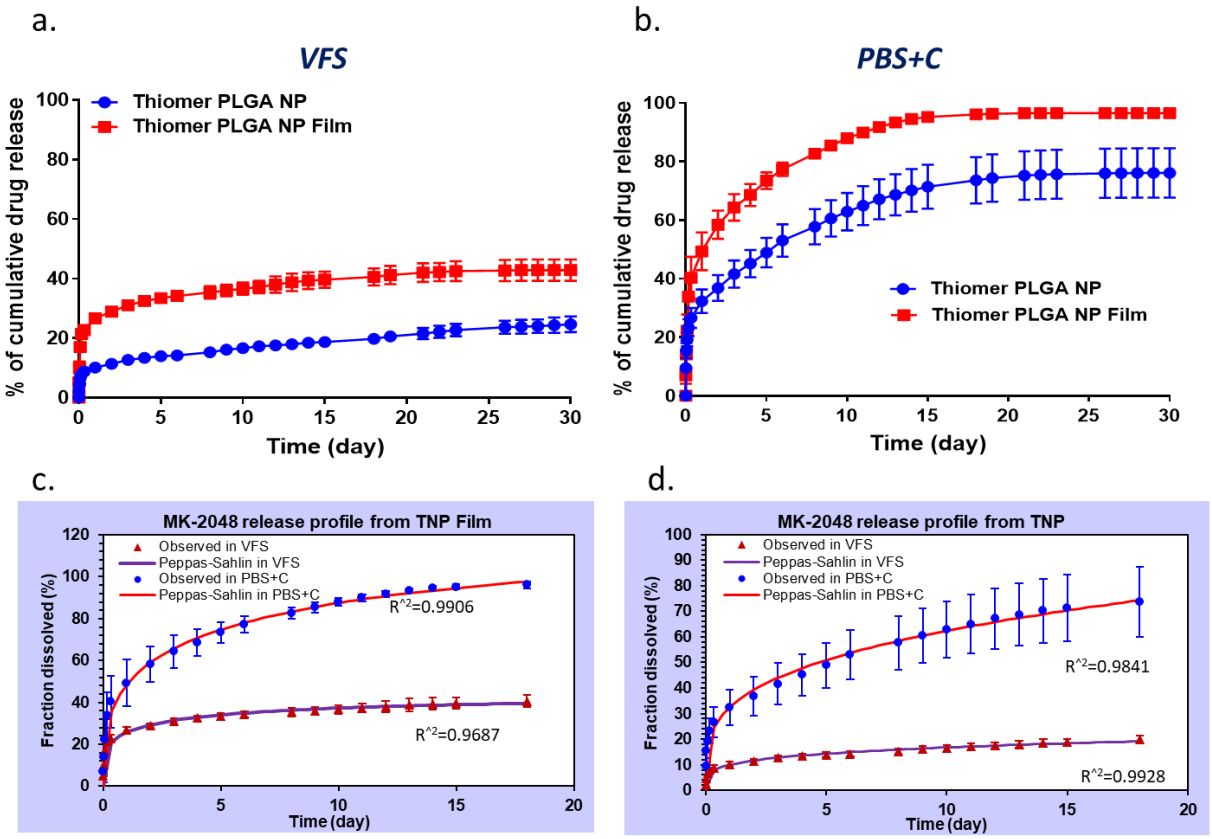


Figure 25. Drug dissolution of TNPs and TNP films

In vitro MK-2048 release from TNP and TNP film in media of **a.** VFS and **b.** PBS with 1% of Cremorphor EL (PBS+C). Peppas-Sahlin mathematical model for MK-2048 release profile from **c.** TNP Film and **d.** TNP in VFS and PBS+C. (n = 3)

4.3.6 Tissue permeability of TNP film

The tissue permeability of MK-2048 TNP film was performed by In-Line Cell, which is a flow through system and reduces the impact of solubility limitation. As compared to free MK-2048, the P_{app} ($p < 0.05$) and tissue drug concentration ($p < 0.01$) of MK-2048 TNP film were significantly increased (**Figure 26 a and b**). The PNP film was also used here as a control group. The coating of thiomer on nanoparticles showed a trend to improve the tissue permeability of MK-2048 but not statistically significant due to small sample size. This result indicates that the enhanced tissue permeability was not only because of the smaller particle size but also due to the

permeability enhancing property of thiomers. This permeability enhancement combined with mucoadhesion is likely to provide enhanced drug exposure to cervicovaginal tissues.

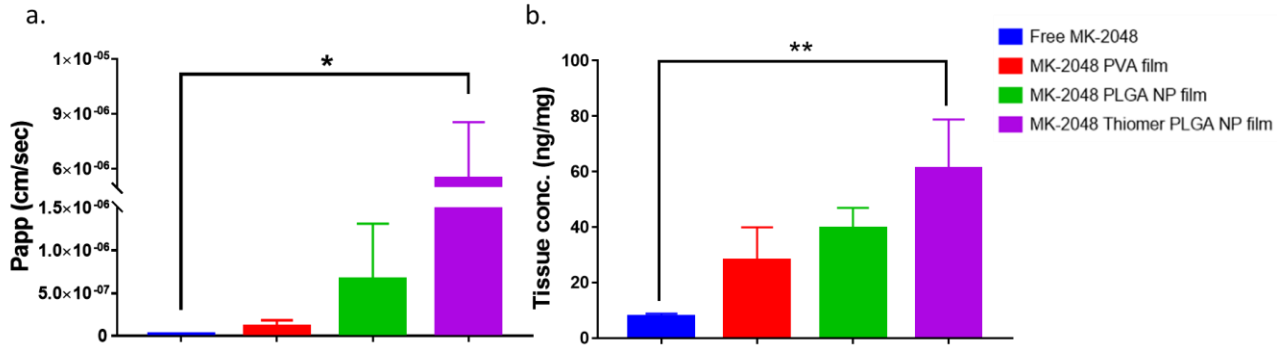


Figure 26. *Ex vivo* tissue permeability using In-Line Cell system for MK-2048 and formulated MK-2048

a. apparent permeability coefficient (P_{app}); **b.** MK-2048 levels in ectocervical tissues (n = 3, ** $p<0.01$, * $p<0.05$)

4.3.7 Impact of TNP films on the morphology of cervicovaginal epithelium

Human ectocervical tissue was exposed to free MK-2048, MK-2048 PVA film, MK-2048 PNP film and MK-2048 TNP film for 6 h. After treatment, the epithelial morphology of human ectocervical tissue was evaluated by H&E staining. As shown in **Figure 27**, there is no significant changes in the morphology of excised human ectocervical epithelium after the exposure to all the treatments. It indicates that the TNP film can improve the tissue permeability with impacts on tissue epithelial morphology.

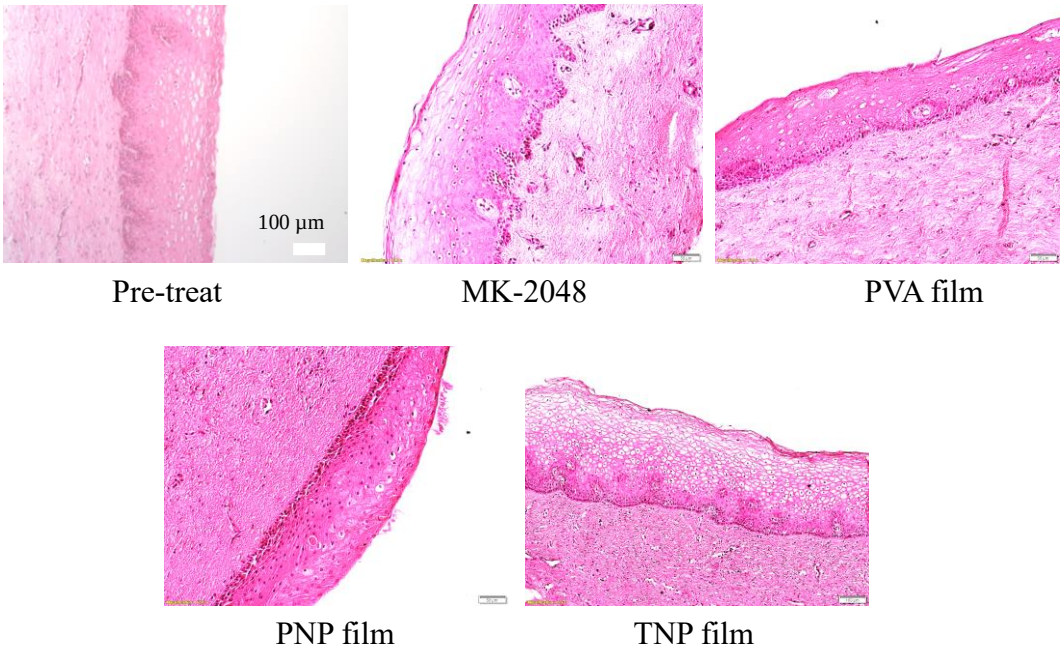
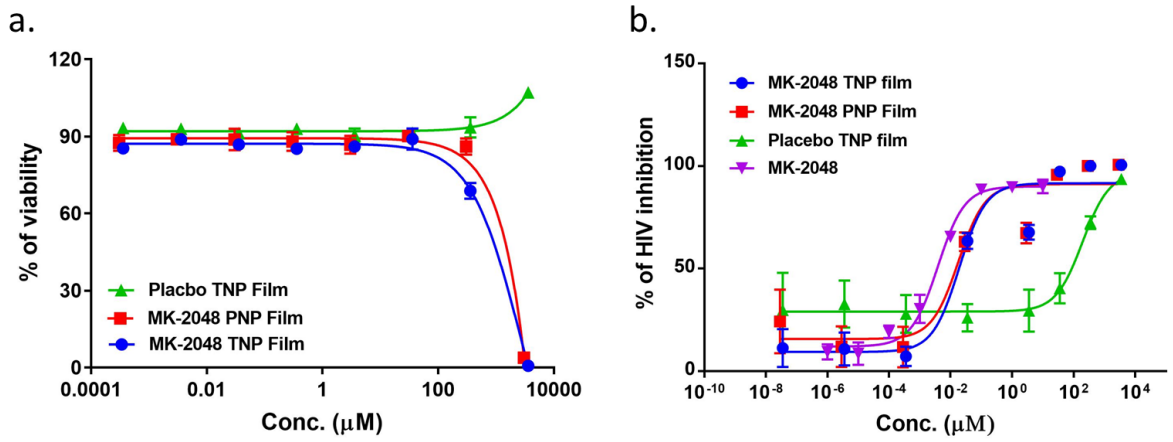


Figure 27. H&E staining for morphology of human cervical tissues.

4.3.8 *In vitro* anti-HIV activity of TNP film

Placebo TNP film, MK-2048 PNP film and MK-2048 TNP film were evaluated at concentrations of 0.0003 µM to 3580 µM in TZM-bl cells. No toxicity was observed for placebo TNP film up to 3580 µM. MK-2048-loaded TNP film and PNP film were not toxic up to 36 µM in TZM-bl cells (**Figure 28 a**). In order to ensure that the anti-HIV-1 activity of the MK-2048 was maintained after being loaded into TNP and TNP film, we tested the free MK-2048 and formulated MK-2048 against HIV-1_{BaL} infection, using the TZM-bl assay. Both TNP film and PNP film formulated MK-2048 showed comparable anti-HIV activity with the drug substance MK-2048 in the tested TZM-bl cell model. No significant difference in the anti-HIV activity was observed between MK-2048 TNP film and MK-2048 PNP film. In addition, the anti-HIV-1 activity of

placebo TNP film was also observed in the TZM-bl assay (**Figure 28 b and c**). This activity observed of the placebo TNP film is more than likely due to the polymer excipients.



c.

	Free MK-2048	MK-2048 TNP Film	MK-2048 PNP Film
IC_{50} (nM)	3.88	19.28	18.04

Figure 28. Inhibitory activity of MK-2048, PNP/TNP film-formulated MK-2048 and placebo TNP film against HIV_{BAL-1}.
a. Cytotoxicity of placebo TNP film, MK-2048 containing TNP film and PNP film in TZM-bl cells. **b.** HIV inhibition of MK-2048, MK-2048 TNP film and MK-2048 PNP film. **c.** Summary of IC_{50} of free MK-2048, MK-2048 TNP film and MK-2048 PNP film in HIV inhibition. (n = 3)

4.3.9 Compatibility of TNP film with normal vaginal flora *Lactobacillus*

The compatibility of MK-2048 PVA film, PNP film and TNP film with *Lactobacilli* was evaluated using the *in vitro* cultured *Lactobacilli* strains. MK-2048 PVA base film was safe against *L. jensenii* and *L. crisp* stains after 30-min incubation. Similar results were observed for similar dose of MK-2048 PNP loaded and TNP loaded film (**Table 15**). The results indicated that MK-2048, PVA base film, PLGA nanoparticles and thiomers coating were all compatible with the most sensitive strains of vaginal *Lactobacillus*.

Table 15. Compatibility of the vaginal nanoparticle loaded film with the normal vaginal flora Lactobacillus

Log (T_{30min} plate count $-T_0$ plate count)			
	L. jensenii LBP 28AB	L. jensenii ATCC 25258	L.crisp ATCC 33197
MK-2048 PVA film	-0.684	0.108	-0.426
MK-2048 PNP film	-0.363	-0.18	-0.402
MK-2048 TNP film	0.048	0.007	-0.397

4.4 DISCUSSION AND CONCLUSION

We have developed a safe and effective topical microbicide approach using nanoparticle loaded vaginal film for sexually transmitted HIV-1 prevention. In this study, we demonstrated that MK-2048 can be encapsulated within TNPs without compromising its anti-HIV activity. MK-2048 dose was selected based on a clinical evaluated intravaginal ring containing vicriviroc and MK-2048, which is equivalent to 30 mg in a $2'' \times 2''$ film (human size)[316]. Unlike vaginal ring, polymeric film is a thin and discrete dosage form. To achieve 30 mg of MK-2048 in the film, the percentage of TNPs incorporated in films was up to 26.45%. We observed that the incorporation of TNPs led to significant change in the film structure including decreased puncture strength, water content and increased disintegration (**Table 14**). The major component of TNP is a hydrophobic polymer PLGA, which imparts high hydrophobicity to the film and a possible factor influencing the mechanical properties of the film. The similar result was also observed in the tenofovir PLGA/stearylamine nanoparticles in PVA based film[317]. However, the decreased puncture strength of TNP film was still above the minimum threshold value (0.8 kg/mm)

considered enough to maintain structural integrity during product development and handling[318].

Unlike the currently developed nanoparticle films using non-mucoadhesive polymeric nanoparticles such as PLGA[319, 320], Eudragit[224] or PLGA/stearylamine[116], TNP is a polymeric nanoparticle with mucoadhesive coating that exhibits significantly prolonged tissue residence. The coating of thiomer on the surface of PLGA nanoparticles was confirmed by the increased particle size, positive zeta potential and thiolation in the nanoparticles (**Table 13**). In our study, film was selected as the delivery platform since it is considered a suitable and acceptable dosage form for topical microbicide. The PVA film is fast dissolving film that can be disintegrated fast when exposed to vaginal fluid in 30 mins to allow the release of nanoparticles loaded in film. The major role of film in this product is to aid nanoparticle intravaginal delivery and reduce the leaking of nanoparticles which has been observed in previous study using the suspension of nanoparticles in saline or gel for intravaginal drug delivery[126]. Thus, TNPs plays an important role in prolonging the tissue mucoadhesion. To better understand the mucoadhesion of TNPs, the incline flow through model was used. This model is a simple and quantitative technique that has been utilized for evaluating and rating the bioadhesiveness of polymers and dosage forms[315]. Results shown in **Figure 22 b** clearly demonstrate that the thiomer coating on the surface of PLGA nanoparticles exerted significantly higher tissue mucoadhesion than PLGA nanoparticles. Two possible reasons are the interaction between the positive charge on the particle surface and the negative charge of mucin, and disulfide bond formation between thiomer and mucin.

In addition, TNPs showed significantly enhanced cellular uptake compared to PNPs at 2 h and showed no significant difference after 4 h in cellular uptake for both nanoparticles (data

not shown). These results indicate that TNPs not only exhibit increased mucoadhesion but also are able to be taken by vaginal epithelial cells faster than the uncoated nanoparticles (**Figure 23**). The underlying mechanism of enhanced cellular uptake by TNP is most likely due to the positive charge on the surface of TNP that electrically interacts with the epithelial cells which have negative charge[321]. This was also confirmed in the *ex vivo* tissue permeability study, where the P_{app} of TNP film formulated MK-2048 was significantly higher than that of the free MK-2048 ($p<0.05$). We also observed that the TNP film formulated MK-2048 had a trend in permeating into tissue faster than PNP film (**Figure 26**). These results together indicate that the improved cellular uptake and tissue permeability of TNP film formulated MK-2048 were not only due to the particle size at nanoscale but also because of the modification with thiomers.

Thiomers have been shown to exhibit enhanced permeability through opening the intercellular junctions[322]. This could be another possible mechanism for the improved tissue permeability of MK-2048 after formulated in TNP films. However, the difference in permeability of MK-2048 between TNP film and PNP film is not statistically significant. This might because the In-Line Cell system doesn't include the mucus layer and mucus clearance, which may have overestimated the tissue permeability for PNP films. It also could be because the particle size of PNP is smaller than that of TNP as shown in **Table 13**, which plays an important role in the tissue penetration. *In vivo* study needs to be conducted to further investigate the advantageous vaginal drug delivery of TNP films. The tissue was also evaluated with the epithelial histology after film exposure. No histological changes were identified at the ectocervical epithelium layer (**Figure 27**). This result indicates that TNP films can improve the tissue penetration without impact on tissue morphology. This could be due to the fact that

thiomer is not absorbed from the mucosal membrane and can reversibly open the tight junctions and interact with the glutathione to improve the paracellular transport[323].

We were able to demonstrate the sustained release of MK-2048 formulated in TNP and TNP film. Mathematical models were applied to evaluate the release profile of MK-2048 in TNP and TNP films. The biphasic release profile of MK-2048 TNP and TNP film perfectly fits the Peppas-Sahlin model (**Figure 25 c and d**). This reveals that the drug release mechanism of the TNP based formulation is a combined diffusion and polymer degradation. The burst release may be related to the physical adsorption of MK-2048 on the surface of TNPs, and the sustained release stage of MK-2048 may be more due to the polymer degradation. However, higher burst release of MK-2048 was observed with TNP film compared to TNPs in both dissolution media for first 4 h. After an initial mild burst release, nearly linear sustained and parallel release profile of MK-2048 were obtained for TNP and TNP film. It is possible that the stress induced during manufacturing could lead to release of MK-2048 on the nanoparticle surface into the film solution. Further the amount of processing time, nanoparticles are suspended in film solution could trigger drug release from the nanoparticles into the solution. Due to these reasons, TNP film exhibited higher burst release.

It is possible that some excipients (eg. nonoxynol or surfactants[287]) could cause alterations in cervicovaginal tissue permeability or negatively affect the protective bacteria in the vagina leading to increased susceptibility to viral infections. Thus, it is necessary to check the safety of excipients utilized in microbicide products. Since the safety of thiomers hasn't been evaluated for vaginal delivery, we investigated the compatibility of TNP film with three major lactobacillus strains including the most sensitive one *L. jensenii LBP 28Ab* (**Table 15**) . In addition, the cytotoxicity of TNP film to vaginal epithelial cells was also evaluated. Both results

demonstrate that TNP film could be safe for vaginal administration (**Figure 23 a**). More long-term *in vivo* studies are warranted to confirm its safety. The *in vitro* anti-HIV activity of TNP film formulated MK-2048 was further investigated using the TZM-bl assay. It was noticed that TNP film and PNP film formulated MK-2048 showed higher IC_{50} than free MK-2048. This is because MK-2048 was encapsulated into the polymeric nanoparticles, and it needs to be released from the nanoparticle first to take action in inhibiting HIV infection.

In conclusion, we developed a mucoadhesive nanoparticles-based film for vaginal delivery of MK-2048 as a promising sustained-release topical microbicide for HIV prevention. MK-2048 loaded TNPs were optimized for achieving high encapsulation efficiency and drug loading capacity, and further incorporated into the quick-dissolving PVA film platform. MK-2048 TNP presented improved tissue mucoadhesion and cellular uptake that may be considered to be advantageous over other non-mucoadhesive nanoparticles under development for topical microbicides. In particular, the sustained release profile of MK-2048 TNP film over 30 days potentially enables less dosing frequencies and favorable to improve user adherence. MK-2048 loaded in TNP film exerts comparable bioactivity without affecting tissue epithelial morphology, cytotoxicity and normal vaginal microflora lactobacillus. These results highly promote further investigation of TNP loaded films as topical microbicides and support their potential as an alternative strategy to the vaginal ring system.

4.5 ACKNOWLEDGEMENT

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5.0 MAJOR FINDINGS AND FUTURE DIRECTIONS

5.1 MAJOR FINDINGS

HIV continues as a global health pandemic and the vast majority of the infections are through sexual transmission. Women are undergoing the disproportional burden of the HIV infection, which is attributed to the physiological vulnerability and unequal socioeconomical status[4]. Millions of new HIV infections in women every year urges development of effective female-controlled prevention strategies to decelerate the spread of HIV. Vaginal polymeric film is one of the potential female-controlled topical microbicides. This product has been developed in coitally-dependent manner and demonstrated highly acceptability, favorable safety and pharmacokinetic profiles in the clinical trials[55]. Since poor user adherence hampered the effectiveness of vaginal microbicide gels, the idea of designing film dosage form into the coitally-independent fashion draws more attention in the microbicide field.

To develop efficient coitally-independent vaginal films, one of the particularly important aspects is the ability of the dosage form to be retained in the vaginal compartment and provide extended delivery of the drug. Therefore, mucoadhesion and extended release profile are two major components to be considered in the design of a coitally-independent film. The work presented in this dissertation is based on the hypothesis that increased mucoadhesion and extended drug release can be achieved by the film matrix through modifications via polymer

incorporation or nanotechnology. To test this hypothesis, three film platforms: (1) mucoadhesive film (Chapter-2), (2) extended-release film (Chapter-3) and (3) mucoadhesive nanoparticle-based film (Chapter-4) were developed and evaluated.

One strategy toward development of an extended-release vaginal film is to prolong the residence of the film in the vaginal compartment. Compared to the extended-release topical microbicide of the IQP-058 osmotic tablet, this strategy is favorable to all the antiretrovirals for HIV inhibition whose mechanism in HIV inhibition occurs either in the vaginal compartment or in the subepithelium to the targeted immune cells. To achieve this goal, thiolated chitosan was incorporated into a clinically evaluated polyvinyl alcohol (PVA) polymeric vaginal film with formulation modifications. The PVA film was selected in our study because its safety, acceptability and feasibility of delivering antiretroviral drugs have been demonstrated in our previous work[55, 226]. The most important finding from Chapter 2 is that after inclusion of the thiolated chitosan, the tissue mucoadhesion of this film was significantly increased. Of note, thiolated film was able to be retained in the vaginal compartment for at least 7 days as shown in the *in vivo* nonhuman primate (NHP) model. In contrast, most of the current marketed or in-development mucoadhesive vaginal films, gels or tablets which contain mucoadhesive polymers such as cellulose derivatives, Carpobol, chitosan, and/or alginate have vaginal retention time up to only three days[324-328]. Therefore, the film we designed prolonged vaginal retention and is promising to be developed into weekly-used products. Another major finding from this study is the feasibility and pharmacokinetic (PK) profile of delivering the combination of dapivirine (DPV) and levonorgestrel (LNG) in the bioadhesive film. The co-delivery of DPV and LNG to the vagina via bioadhesive film altered their local and systemic PK profiles of each drug when

administered alone. This finding warrants more investigations to explore the possibility of drug-drug interaction for this co-delivery.

To further extend the drug release rate from the film, the hydrophobic biodegradable PLACLs with different chemical composition were synthesized and incorporated into the bioadhesive film (Chapter 3). The major findings from this study is the incorporation of PLACL significantly improved the mechanical property of the polymeric film and extended the drug release without significant impact on the tissue mucoadhesion. Another important finding is that the tissue permeability of MK-2048 increased after being formulated in the thiomer PLACL80 film. This property could potentially improve the efficacy of MK-2048 in HIV inhibition since the action step of MK-2048 in interrupting HIV integration occurs inside the vaginal subepithelial tissue.

We also leveraged nanotechnology to design a monthly administrated vaginal film (Chapter 4). The innovation of our design lies in the modification of nanoparticle surface with thiolated chitosan to improve the tissue mucoadhesion. Three major findings are demonstrated in this study. First, the tissue retention of the mucoadhesive nanoparticles was prolonged. This property can provide sufficient time for the penetration of nanoparticles to achieve the sustained delivery of ARVs to the vaginal mucosa. Second, the surface modification of thiomer on the nanoparticle accelerated the cellular uptake of nanoparticles into vaginal epithelial cells. This characteristic of the nanoparticle enables the delivery of ARVs to the vaginal mucosa level and provide the sustained protection window from HIV infection to the immune cells in the vaginal subepithelial layer. Third, the nanoparticle system provided one-month sustained drug release profile under two conditions (non-sink condition and maintained sink condition) *in vitro*.

Collectively, these results demonstrate the feasibility of producing film with extended release property. The incorporation of polymers or nanoparticles manipulated the function of the film by achieving prolonged vaginal residence and extended drug release profile. Besides, the excellent safety and efficacy of these three film platforms were further confirmed their potential as coitally-independent topical microbicide dosage forms. Currently, these encouraging data has led to the *in vivo* evaluation of these films in the NHP model. Moreover, the films we designed have multiple functions such as the ability of prolonged tissue retention, enhanced tissue permeability or cellular uptake, and extended drug release. These characteristics make them to be more advanced than other currently developed coitally-independent or sustained-release microbicides dosage forms such as the vaginal ring, osmotic tablets or nanofibers.

5.2 IMPLICATIONS

The current research in the development of microbicides for HIV prevention is focused on overcoming the low user adherence observed in the microbicide clinical trials. In line with this focus, the work presented in this dissertation provides three strategies which can potentially improve the user adherence to topical microbicides. We took advantage of the film dosage form and designed it as coitally-independent delivery system by multipurpose technology, empowering it with extended-release profile via matrix modification, or utilizing nanotechnology. These contribute to the advancement of the film dosage form in the development of topical microbicides. In addition, the strategy of developing the multipurpose film (DPV/LNG film) is positioned as a promising tool to improve the product compliance. To the best of our knowledge, we are the first to deliver hormone, like LNG, by film dosage form,

which indicates the feasibility of film as a delivery platform for contraceptives. Given the diversity of female populations who would use a topical microbicide, more options may lead to enhanced product compliance and consequently higher product efficacy. Both designed films, combining the merits of film dosage forms and less dosing frequencies, provide promising alternatives to the vaginal ring for coitally-independent application.

From a dosage form perspective, the findings of this work indicate that the function of polymeric films can be tuned via the matrix modification. The marketed and clinical evaluated vaginal films are limited to fast-dissolving films. Sufficient residence in the vaginal cavity is very challenging in the design of vaginal product due to the fast-mucosal clearance. Through the application of functional polymers and nanotechnologies, the versatility of the film has been expanded. The one-week *in vivo* retention of the film was achieved though the incorporation of the thiolated chitosan. To our knowledge, this data presented in this dissertation is the first *in vivo* demonstration of the long-term vaginal retention for thiomer incorporated dosage forms. Furthermore, the local pharmacokinetic profile of nanoparticle encapsulated antiretroviral drugs can be potentially improved by the surface modification of thiolated chitosan due to the prolongation of their residence in mucosal tissue. In addition, the incorporation of thiomers in the film allows the mucosal penetration of the antiretroviral drugs to target HIV residing in the subepithelium. Therefore, the introduction of thiomers to the vaginal dosage forms is very important to improve the vaginal retention time for most of the conventional vaginal mucoadhesive drug delivery systems.

Besides the application in the microbicides field and vaginal drug delivery, the film platforms designed in this dissertation may also be utilized in the delivery of drugs such as hormone, antibacterial, antifungal and others for the treatment of reproductive related diseases,

as well as applied for other mucosal routes such as nasal, buccal, and ocular delivery. First, the polymeric films are considered as an emerging platform for drug delivery. Its feasibility of delivering various drugs from small molecules (either hydrophilic or hydrophobic) to macromolecules including biologics, has been demonstrated in previous studies[217, 222, 226, 329-331]. Second, films have been used for mucosal drug delivery for decades[332]. Although these films presented in this dissertation are designed for vaginal drug delivery, the polymers used in these platforms are also compatible with other mucosal tissues because the excipients used in our designed films have been applied for nasal, buccal, or ocular films before. In addition, the mucin existing in different mucosal organs consistently present cysteine domain[82], thus the mucoadhesion behavior of these films is also applicable to nasal, buccal and ocular routes.

5.3 LIMITATIONS

Three coitally-independent film platforms have been tested in a comprehensive characterization regarding to the evaluation of their physicochemical property, biocompatibility, and bioactivity *in vitro* and *in vivo*. The data presented in this dissertation are important from the product development perspective and have shown the promising feasibility of these platforms as coitally-independent topical microbicides. Looking forward, these films are still in their infancy. There remains challenges and opportunities to facilitate their clinical translation considering from the product development and dynamic nature aspects.

With regard to the product development, the extended-release film is targeted for weekly or monthly administration. In this dissertation, weekly vaginal retention of the bioadhesive film

was demonstrated *in vivo.* *In vitro* drug dissolution data demonstrated that extended release could be further improved by incorporation of PLACL or through encapsulation of the drug into nanoparticles. However, the *in vitro* drug dissolution method described in this dissertation was not a biologically relevant method. Thus, the *in vivo* drug release time frame was not able to be accurately predicated based on the *in vitro* data for these film platforms. To circumvent this limitation, NHP studies have been proposed to evaluate the *in vivo* drug release and local PK profiles for film platforms. This data will guide the determination of the drug loading dose and dosing time frame for film optimization.

Pigtailed macaques utilized in this dissertation to evaluate the bioadhesive vaginal film are a validated NHP model for the evaluation of vaginal films as topical microbicides due to their anatomical and reproductive similarities to humans[333]. By using this model, the vaginal retention and local PK profile of the DPV/LNG bioadhesive films were evaluated. The promising *in vivo* data provided further guidance in the development of more extended-release vaginal film. However, due to the cost and limited availability of macaques, the number of sample size in the macaque studies was small and resulted in large variability in findings. More studies with larger sample sizes are needed in the future macaque studies to evaluate the vaginal films.

In addition, the vaginal residence of films could be affected by dynamic natures of vaginal mucosa. As discussed in Chapter 1.2, the mucosal properties including the mucin volume, viscoelasticity, clearance, and thickness can be varied by the change of hormonal levels. Also, the menses and vaginal sexual practice could influence the residence of film on the vaginal mucosa. These will introduce inter and intra patient variability regarding to the residence of films in the vaginal compartment *in vivo*, thereby to influence the determination of the dosing time

frame of the final products. Therefore, it is important to consider these physiological factors into the *in vivo* NHP studies.

One final factor which could potentially impact film interaction with mucus is oxidation of the thiolated chitosan. This could occur either during storage or *in vivo*. In the vagina, there are hydrogen peroxide producing *Lactobacilli* which can serve as a source for oxidation. As mentioned in Chapter-2, thiolated chitosan can be oxidized when it is exposed to the oxygen, which could reduce the amount of the available free thiol group and result in decreased mucoadhesion. From the product development perspective, it will be important to conduct thorough packaging compatibility studies. Packaging with limited permeability to oxygen will be necessary for the storage of thiomer containing films.

Collectively, the data presented in this dissertation are important and necessary in the initial development of film platform. However, the study limitations described here will guide the future investigations to improve the film platforms and advance these products into the clinic.

5.4 FUTURE DIRECTIONS

This dissertation work has set the stage for several future directions which can contribute to the advancement of these designed film platforms to clinical investigation. With regard to the development of MK-2048 coitally-independent film, long-term stability such as 24-months studies of both films need to be conducted to support the advancement of both products into animal and clinical studies. In addition, given the encouraging *in vitro* data presented in this dissertation, it is very important to investigate whether these properties including safety, mucoadhesion, sustained drug release profile, and enhanced tissue permeability can still be

achieved *in vivo*. Therefore, the future studies should be focused on the *in vivo* evaluation in the NHP model. As mentioned in the limitation section, the dose loading levels and dosing frequency also need to be determined for the development of coitally-independent product. Studies need to be conducted to evaluate the impact of multiple factors such as coitus, menses and dosing at different time of menstrual cycle, on the vaginal residence of films and the local pharmacokinetics of MK-2048. Additional studies will be required to evaluation the elimination rate of PLACL and PLGA to generate a complete understanding of product safety. Such studies could be conducted using animal models. Although PLACL and PLGA are biodegradable polymers, their degradation rates are longer than one week or a month. Weekly or monthly dosing frequency may result in polymer accumulation in the human body, which could raise toxicity concerns[334].

On the other hand, these film prototypes are very promising and can be utilized in other applications. This dissertation only presented the feasibility of formulating the hydrophobic small molecules in these films. To test the versatility of the film platforms, other APIs including hydrophilic, small and large agents should be incorporated into the films for further evaluations such as the API-excipients compatibility and physicochemical properties. Since the films are designed as extended-release prototypes, the release profiles for different APIs from films need to be investigated. Given this, in order to better predict the drug release profile *in vivo*, a valid biological relevant dissolution method should be developed. This will be very helpful to inform the optimization of film formulations to meet the needs of desired drug release profiles.

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