NOVEL APPLICATION OF HOT MELT EXTRUSION FOR PHARMACEUTICAL VAGINAL FILM PRODUCTS

by

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The vaginal film dosage form is a highly applicable and versatile delivery platform for a variety of active pharmaceutical agents. Several behavioral studies have reported its desirability by women as a vaginal dosing platform. Traditionally, pharmaceutical films have been manufactured using solvent casting (SC) techniques. Hot melt extrusion (HME) is an advantageous manufacturing method since it is a continuous process requiring fewer steps for efficient large-scale manufacturing and batch uniformity. HME could potentially lead to lower manufacturing costs due to its simplicity, minimal processing steps, and its availability worldwide. The goal of this project was to evaluate the feasibility of HME for the manufacture of pharmaceutical vaginal films. To achieve this, drug candidates with varied chemical properties (hydrophobic or hydrophilic small molecule, protein or bacteria) were studied. The specific compounds selected were metronidazole (hydrophilic small molecule), dapivirine (hydrophobic small molecule), levonorgestrel (hormone), griffithsin (protein) and *Lactobacillus* (probiotic bacteria). Single layer films were successfully manufactured using HME. The films were fully characterized and the obtained attributes were within target specifications. Batch to batch reproducibility and within batch consistency for film manufacture using HME was studied for the dapivirine HME vaginal film product. A design of experiments was applied to evaluate the

impact of HME processing parameters on final film product attributes. In addition, the application of HME to produce multilayer vaginal films was explored. These studies demonstrated that the novel application of HME for vaginal film product manufacture is versatile, reproducible, and robust. Further this method can be applied to generate both single and multilayer vaginal film products which can be used to address a multitude of women's reproductive health needs.

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PREFACE

"The important thing is not to stop questioning. Curiosity has its own reason for existing."…. Albert Einstein.

I would like to acknowledge several individuals who encouraged me to become a better scientist, by teaching me how to ask better scientific questions while maintaining my scientific curiosity, during my Ph.D. training.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
API	Active Pharmaceutical Ingredient
ARV	Antiretroviral
ACN	Acetonitrile
ASPIRE	A Study to Prevent Infection with a Ring for Extended Use
ATCC	American Type of Culture Collections
BV	Bacterial Vaginosis
CDC	Centers for Disease Control and Prevention
CFU	Colony-Forming Unit
DMEM	Dulbecco's Modified Eagle Medium
DPV	Dapivirine
DSC	Differential Scanning Calorimetry
ELISA	Enzyme Linked Immunosorbent Assay
FDA	Food and Drug Administration
EC_{50}	Half Maximal Effective Concentration
GRFT	Griffithsin
GI	Gastrointestinal
H & E staining	Hematoxylin & Eosin Staining

HBT	Human Blood Tween (HBT) Bilayer Medium plates
HEC	Hydroxyethyl Cellulose
HIV	Human Immunodeficiency Virus
HME	Hot Melt Extrusion
HPC	Hydroxypropyl Cellulose
HPLC	High Performance Liquid Chromatography
HPMC	Hydroxypropylmethyl Cellulose
H_2O_2	Hydrogen Peroxide
IC_{50}	Half Maximal Inhibitory Concentration
IVR	Intravaginal Ring
LNG	Levonorgestrel
LOD	Limit of Detection
LOQ	Limit of Quantification
MTN	Microbicide Trials Network
N-9	Nonoxynol-9
NDA	New Drug Application
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
OD	Optical Density
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
PEO	Poly(ethylene) Oxide
PrEP	Pre-Exposure Prophylaxis
RH	Relative Humidity

SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SC	Solvent Cast
SMST	Standard Microbicide Safety Test
STIs	Sexually Transmitted Infections
TFA	Trifluoroacetic Acid
Tg	Glass Transition Temperature
Tm	Melting Temperature
TMB	Tetramethyl-Benzidine
VCF	Vaginal Contraceptive Films
VFS	Vaginal Fluid Simulant
VOICE	Vaginal and Oral Interventions to Control the Epidemic
UV	Ultraviolet
WHO	World Health Organization

1.0 INTRODUCTION

1.1 VAGINAL DRUG DELIVERY

1.1.1 Brief History of Vaginal Drug Delivery and Vaginal Films

The dynamic environment of the vagina provides a unique opportunity for drug delivery of a wide range of pharmaceutical products. This route of delivery originally primarily used for the delivery of contraceptive agents. However, in today's society, a number of vaginal products have been commercialized for a range of therapeutic indications including antimicrobials, spermicides and hormone replacement therapy. There are several advantages of the vaginal route for the delivery of pharmacologically active agents such as its large surface area and the rich blood supply of the vaginal mucosa [1]. Topical dosage administration by this route is noninvasive and can avoid first pass metabolism as well as side effects that are caused by systematic exposure [1, 2]. Vaginal delivery systems can be found in forms such as gels, creams, suppositories, intravaginal rings (IVR), inserts, douche, and films. The film dosage form is a highly applicable and widely versatile delivery platform for a variety of active pharmaceutical agents. In several behavioral studies, the advantages of vaginal films, including its discreet use, minimal product leakage, and ease of use, have been shown to contribute to its desirability by women. To date, the commercially-available pharmaceutical films are manufactured using solvent casting (SC)

techniques. In this study, the novel use of hot melt extrusion (HME) as an alternative manufacturing process for pharmaceutical vaginal films was studied.

1.1.2 Anatomy and Physiology of the Vagina

The vaginal route is suitable for local and systemic administration of pharmaceutical products. The vagina is a key organ of the female reproductive tract with unique features such as a mucous membrane, acidic vaginal environment, vaginal secretions, and distinct microflora [1, 3, 4]. The anatomy and physiology of the vagina must be taken into consideration during the development of vaginal dosage forms [4].

The vagina is a relaxed fibromuscular cylindrical organ that is situated between the bladder, urethra, and the rectum and exists as a collapsed space with easily distensible walls. It is the bridge between the vulva, the external opening of the female reproductive tract, and the cervix, the neck of the uterus. The vagina is a channel that connects the uterus to the external space. This allows menstruation to flow out of the uterus and facilitates the entrance of the seminal fluid during sexual intercourse. It also functions as the orifice for the birth canal that allows for childbirth [5].

The shape of the vagina is unique and not symmetrical or comparable to any known geometric shape [6]. Additionally, the axis and dimensions of the upper and lower vagina are dissimilar. When standing, the lower vagina, from the introitus to the pelvic diaphragm, is vertical and posterior (convex curve), while the upper vagina, from the pelvic diaphragm to the cervix, is at a 130° angle [6, 7]. This unique characteristic of the vagina allows the pharmaceutical products to remain in the vaginal space once delivered.

The shape and dimensions of the vagina vary amongst women depending on age, sexual activity, and the number of childbirths. [2]. In a study conducted by Pendergrass PB et al. the shapes of the vagina were categorized as a parallel sided, conical, heart, slug shapes and pumpkin seed [8, 9]. The average dimensions of the vagina have been reported to be 6.86 to 14.81 cm in length, 4.8 to 6.3 cm in width, and 2.39 to 6.45 cm of introital diameter [8]. Furthermore, the surface area of the vagina was found to be 87.46 cm^2, with the range of 65.73-107.07 cm^2 [10]. These measurements are an underestimation of the vaginal surface area since they do not take into account muscular folds within the vaginal tissue called rugae. The variability among vaginal shape, size and dimensions must be taken into consideration when developing a vaginal dosage form, as this affects dosage form acceptability [2].

The vaginal and ectocervical wall consist of a multilayered, non-keratinized, stratified squamous epithelial layer, a lamina propria, a muscular layer, and the tunica adventitia (vascularized tissue) [11]. The surface of the vaginal mucosal layer is covered by several rugae folds, which greatly increases the vaginal surface area [12]. The multilayer squamous epithelium can be divided into three cellular sections according to the stage of maturation: basal or germinal cell layer, parabasal layer, and an intermediate/superficial cell layer. As the cells mature, they migrate from the basal layer to the superficial layer and develop a flatter shape, larger cell volume, and smaller nuclei [13, 14]. Unlike epithelium of the vagina and the ectocervix, the endocervix is covered by a single layer of columnar epithelium and is connected to the ectocervix at the transformation zone. The transformation zone is where the multilayer squamous epithelium meets the single layer columnar endocervical epithelium. This zone is rich in immunologic activity and needs to be considered when developing vaginal dosage forms.

The typical thickness of the squamous epithelial layer varies from 0.2mm to 0.5 mm between across women and changes in vaginal thickness occur as women age. These changes are correlated with estrogen levels circulating in the blood. Estrogen levels fluctuate during different stages of the menstrual cycle and menopause [14]. A thorough understanding of the changes in the thickness of the vaginal epithelium is crucial when developing vaginal drug delivery systems since cyclical changes in the vaginal epithelium thickness have been reported to alter drug permeability and absorption [7, 14]. After the drug is absorbed by and penetrates the tissue, it enters the vaginal blood supply. The blood supplied to the vagina is received by the uterine and vaginal arteries, which are two branches of the internal iliac artery. The venous return is accomplished when the blood enters the venous plexus and is drained into the internal iliac veins [11]. Drugs that are absorbed in the vagina avoid first pass metabolism, which is one of the benefits of systemic vaginal drug delivery systems.

Hydrophilic vaginal fluid covers the epithelial surface of the vagina and can be a target or barrier for drug delivery systems, depending on drug product properties. Although the vagina does not contain any secretory glands, it accumulates a large amount of fluid. The vaginal fluid is composed of secretions from the cervical and vaginal vestibular glands, plasma transudate, endometrial, and oviduct fluids [4, 14]. Vaginal fluid contains water, cervical mucus, epithelial debris, and microorganisms, which are the major component of the innate microflora, in high amounts [2, 15]. In addition, it also contains plasma proteins, cholesterol, lipids, ions, enzymes, enzyme inhibitors, immune cells, and lipids [15]. Vaginal fluid can vary due to pathology, age, sexual arousal and throughout the menstrual cycle, in terms of amount and composition. Women who are at the reproductive age produce approximately 6g/day and have about 0.5-0.75g present in the vagina at any given time depending on the blood flow, menstrual cycle, age and sexual

activity [16]. The vaginal fluid, including cervical mucus, can have an impact on drug delivery. The cervical mucus is composed of water and mucin, which are very long, glycosylated, and hydrated proteins. The physical, chemical and rheological characteristics of the mucus change dramatically during the menstrual cycle [17]. During the beginning and the end of the menstrual cycle, the mucus is thick, hydrated and viscous; to create a protective layer that limits sperm access. Around mid-cycle, when estrogen is high (during ovulation), the mucus viscosity decreases and pH increases to create an optimal environment for the semen to access the genital tract [18]. From a drug delivery perspective, temporal and cyclical changes in the vaginal fluid and cervical mucus can alter pharmacological effects of drug products, such as drug ionization state, hence solubility, adsorption, non-specific binding, resident time, and bio-adhesiveness.

Commensal microbes cover the surface of the vagina and the ectocervix and play a crucial role in the host defense mechanism [19]. The vaginal flora is a dynamic and complex environment consisting of gram-positive, gram-negative, aerobic, and anaerobic bacteria. The predominant microbe, *Lactobacillus* spp., metabolizes glycogen released by the vaginal epithelial cells into lactic acid which contributes to the acidic environment of the vagina. The lactic acid and low pH of the vaginal fluid function as selective antimicrobial barriers against harmful pathogens [20]. Furthermore, some--but not--all, strains of *Lactobacillus* species present in the vagina produce hydrogen peroxide (H_2O_2). H_2O_2 has been found to be toxic to a nonresident pathogenic microorganism. For example, in a study examining women colonized with H_2O_2 positive lactobacilli, acquisition of bacterial vaginosis [21], gonorrhea, and HIV [22] infection were found to be decreased. These H_2O_2 producing microbes are thus associated with the maintenance of a healthy vaginal environment with a low growth rate of harmful pathogens [19, 23].

The normal pH of vaginal fluid at reproductive age is within an acidic range of 3.5-4.7. The pH value of the vagina increases during the menstrual cycle and in the presence of semen. In addition, higher vaginal pH values can be caused by and are indicators of vaginal infection. This fluctuation in pH may affect pH sensitive drugs and vaginal drug delivery systems. Furthermore, any vaginal drug delivery system needs to be safe and should not alter the vaginal microflora, since it plays a crucial role in maintaining vaginal health.

1.1.3 Vaginal Drug Delivery Systems

Vaginal drug delivery is an important route for local and systemic drug administration. Before 1918, absorption of drugs in the vagina was not well understood. However, in 1918, Dr. David Macht reported the absorption of several drugs, such as morphine, cocaine, and nitroglycerine, through vaginal administration [24]. Since this significant finding, many agents have been administered vaginally, such as antivirals, labor inducing agents, contraceptives and hormone replacement therapy [2, 4]. Additionally, local targeted vaginal products have been developed including antimicrobial, antifungal, antiprotozoal, and spermicidal agents. Several dosage forms that are commonly used for the delivery of these compounds via the vaginal route include creams, gels, inserts, foams, ointments, douches, vaginal rings and vaginal films [25, 26]. The development of these delivery systems is based on several factors, such as the drug properties, product safety, and user acceptability [14].

The vaginal route of administration provides ease of use and non-invasiveness, which can prevent tissue damage and potential infection. Administration does not typically require intervention by medical personnel, which reduces the burden of repeated hospital visits and/or appointments with health-care professionals. Furthermore, the vaginal delivery products are

usually discrete, which results in minimal interference with daily life. In addition, drugs that are administrated vaginally avoid gastrointestinal (GI) absorption, GI side effects, and hepatic first-pass metabolism [7]. The vagina's enzymatic composition is unique, and therefore, drugs designed to undergo enzymatic metabolism after administration may apply to vaginal administration. This drug delivery strategy will potentially allow for lower drug dosing levels to achieve sufficient biological effects, reduced toxicity, and evasion of side effects associated with higher dosing levels.

The vaginal route can be used to deliver drugs both systematically and locally. It is efficient in systematic delivery due to its large surface area and rich blood supply [1]. On the other hand, for vaginally-targeted drugs, direct vaginal application reached higher concentrations in the vaginal tract than those via another systemic route [27]. This is important since the vaginal administration of drugs can results at higher drug concentration at the site of action, leading to higher efficacy and treatment for vaginal associated infection [26].

Although the vaginal route offers many advantages, there are some limitations that should be considered during vaginal drug delivery development. Pharmaceutically-based limitation of this route is the variability in terms of drug absorption. There are several physiological factors that can impact drug absorption at the vaginal site. These include epithelial thickness, cervical mucus, and pH of vaginal fluid. The epithelial thickness can affect drug permeation through the tissue. Cervical mucus is a network of high molecular weight glycoproteins, through which the drug must penetrate to reach the tissue. Fluctuation in volume and composition of cervical mucus, which can be caused by age, ovulation, sexual activity, and more, can modify drug permeation through the cervical mucus. pH changes caused by an alteration in microflora, the presence of semen and phase of menstruation can affect the stability

of pH sensitive drugs [7, 25]. All of these factors can be affected by the patient's age, menstrual stage, sexual activity menopausal state, and pregnancies. The shedding of vaginal epithelium may also have negative effects on the drug retention time and absorption. In addition, drug absorption depends on the physiochemical properties of the drug such as the molecular weight, dissolution, and ionization properties [2]. These physiochemical properties can be influenced by the vaginal physical properties. For example, high molecular weight drugs may be limited in the ability to pass the multilayer vaginal epithelium based on their size. Furthermore, vaginal delivery is a gender specific route of administration and therefore is limited for women use.

Knowing the advantages and limitations of vaginal drug delivery can allow for better development of effective, safe and acceptable products for this route of administration. Ideally, a vaginal delivery system that is intended for localized delivery should distribute uniformly throughout or remain within the vaginal cavity. Any vaginal product should be compatible with the vaginal microflora and the vaginal epithelium while maintaining the low pH of the vaginal fluid. Overall, when developing a vaginal product, the ease of use, coital dependence or independence, distribution and vaginal compatibility should be considered.

1.2 VAGINAL FILM

1.2.1 Films

Vaginal drug delivery has a significant impact on the treatment of both local and systemic diseases. Technological advances and modern approaches to drug delivery systems have led to a larger variety of vaginal dosage form choices and optimized pharmacokinetic profiles. These

developments have allowed the vagina to be considered as a potential route for drug administration. Commonly-used vaginal dosage forms include semi-solid forms such as creams, foams, ointments, and gels, as well as solid dosage-forms such as tablets, IVR, and suppositories [4]. Some of these traditional dosage forms are associated with leakage, messiness, and low residence time of the active pharmaceutical ingredient due to the turn-over of the vaginal epithelium [14]. Some major challenges for vaginal formulations are related to maintenance of crucial criteria for successful vaginal delivery of the dosage form. These include an interaction of the product with the vaginal content, product dispersion throughout the vagina, the release profile of the active agent, and the effect on the targets [28].

The vaginal film delivery system offers an alternative strategy for administration of the drug by this route. Polymeric thin films, offer a vaginal drug delivery system that is thin, soft, and flexible. These films meet the demands and requirements of vaginal solid dosage forms. The films are a self-administered product, they are safe for the vaginal environment, and can deliver an adequate amount of active agents. Currently marketed vaginal films are rectangular or square shaped, with a homogenous and soft surface. They offer several advantages over traditional dosage forms. Polymeric thin films can be designed to quickly dissolve and disintegrate to release the active ingredient when it comes in contact with a small amount of fluid, as found in the vagina. Vaginal films do not require a separate applicator. Therefore, they are portable, relatively easy to use and inexpensive to manufacture [29]. Furthermore, vaginal films allow accurate dose administration and can be used to stabilize drugs susceptible to degradation in an aqueous condition [14, 29-31]. Many advantages of the vaginal film make it an acceptable dosage form among women, as was reported in several studies [30-32]. In a recently published acceptability study regarding the film dosage form, three topical prevention methods for sexually

transmitted infections were assessed for acceptability. The methods included vaginal films, soft-gel capsules, and vaginal tablets as potential vaginal microbicides. In this acceptability study, the vaginal film was chosen more often than the soft-gel and tablet as the preferred dosage form (39% and 37% vs. 25%, respectively) mainly because of the ease of administration and the fast dissolving properties [30].

1.2.2 Vaginal Film Formulation

Vaginal films are comprised of the active pharmaceutical ingredient, water-soluble film-forming polymers, plasticizers, and disintegrants [28]. The selected polymers should be non-toxic, non-irritating, and follow Generally Recognized as Safe (GRAS) guidelines. Commonly used polymers in vaginal film formulations are usually water-soluble synthetic (e.g. polyvinyl alcohol), semi-synthetic (e.g. hydroxypropyl methyl-cellulose), or natural (e.g. pullulan). The polymer selection and its associated molecular weight can have a significant impact on the mechanical properties of the film, including the mechanical strength and disintegration. The inclusion of plasticizers in the film formulation is necessary to ensure film flexibility and acceptable texture. Examples of commonly used plasticizers are glycerin and propylene glycol. In addition, disintegration agents (e.g. higher MW Polyethylene glycols) are used to enhance the speed of the disintegration and the drug release, as required for the dosage form [33].

Films can be categorized based on dissolution speed: 1) fast dissolving films (can release the active agent quickly) 2) (release the active agent less quickly) and 3) slow-disintegrating mucoadhesive films (slow release or controlled release films) [33].

1.2.3 Application of Films to Vaginal Delivery

Currently, there are two commercially available vaginal films, including the VCF® vaginal contraceptive films, and VCF® lubricating film (Apothecus Pharmaceuticals). Several additional films are under investigation, each with a distinct therapeutic goal.

Vaginal films have been investigated in recent years for contraceptive, microbicidal, antifungal, and antimicrobial applications [34-36]. In 1997, a Phase I post-coital study demonstrated safety and efficacious contraception of a vaginal film containing nonoxynol-9 (N9) (Allendale-N9) [37]. Another Phase I post-coital study showed that a contraceptive film containing benzalkonium chloride (Allendale-BZK) and a sperm immobilizer was safe and efficacious. This study reported that the spermicidal effect of this film was comparable to the VCF film, containing N9, in sperm inhibition, while maintaining less disruptive effects on the vaginal epithelial [38]. In the microbicide field, films are also investigated for vaginal delivery. For example, Akil et al. incorporated dapivirine (DPV)—a non-nucleoside reverse transcriptase inhibitor (NNRTI)—in film development using polyvinyl alcohol, HPMC, polyethylene glycol, propylene glycol and glycerin. In a Phase I study, this DPV vaginal film was found to be safe. Cervical biopsy samples obtained from women who used this DPV film had significantly reduced HIV-1_{Bal} replication when challenged ex vivo in comparison to those obtained from women with untreated vaginal samples [36, 39]. A vaginal film containing polystyrene sulfonate (PSS), a noncytotoxic antimicrobial contraceptive agent, has also been developed. The film showed *in vitro* antiretroviral activity against HIV-1 (strain IIIB; NIAID, Bethesda, MD, USA) that was comparable to PSS drug substance alone [34]. Another example of vaginal microbicide film development is the quick-dissolving film developed by Ham et al. contains NNRTI pyrimidinedione, IQP-0528. Their study revealed the potential of incorporating this microbicide

candidate in a film dosage form [40]. Peptide and protein microbicide agents have also been formulated and assessed in the vaginal film platform. For example, Sassi et al. published a study on a microbicide agent, RC-101. [41]. RC-101 is a synthetic microbicide analog of retrocyclin, which demonstrated *in vitro* activity against HIV-1. Also, vaginal films have been developed and evaluated for antimicrobial and antifungal applications. One example of an antimicrobial application of film is the metronidazole film developed by Hani et al. to treat vaginal microbial infections [42]. Likewise, a vaginal film containing clotrimazole for vaginal candidiasis has been developed [43]. Both antibiotic/antiprotozoal and antifungal films were characterized for physical and mechanical properties [42, 43].

1.3 VAGINAL FILM MANUFACTURING PROCESS

1.3.1 Solvent Cast

The common and traditional manufacture of vaginal films is based on solvent casting technology. This multiple step process is illustrated in Figure 1.1. The first step is to prepare the film solution, which is done by dissolving all the excipients in an appropriate solvent. The most common quick-dissolving vaginal film excipients include water-soluble polymers, plasticizers, and disintegrants. The order of excipient addition depends on the excipients' physical and chemical properties, as well as the selected solvent. The second step is the addition of the API. The API is dissolved or suspended in the polymer-plasticizer solution, in the process of mixing and homogenizing. During this mixing step, entrapment of air in the solution can occur, which can be eliminated using centrifugation or sonication for small batches. Vacuuming may be used

for larger scale production. Once the film solution is homogenous, it is transferred into a casting apparatus. After casting, the film solution is dried. The drying process can occur at ambient temperature or accelerated temperature by direct heat or vacuum oven. The drying process is defined for each specific formulation during the film formulation development step [34, 36]. Another way to prepare the film is to cast it directly into individual film molds [44]. In the film mold process the homogenous film solution is placed into the mold to dry. Once the film sheets are made, they are cut into individual unit doses using a die press. The dimensions and shape of the film can be determined as needed, depending on the pharmacological application [45].

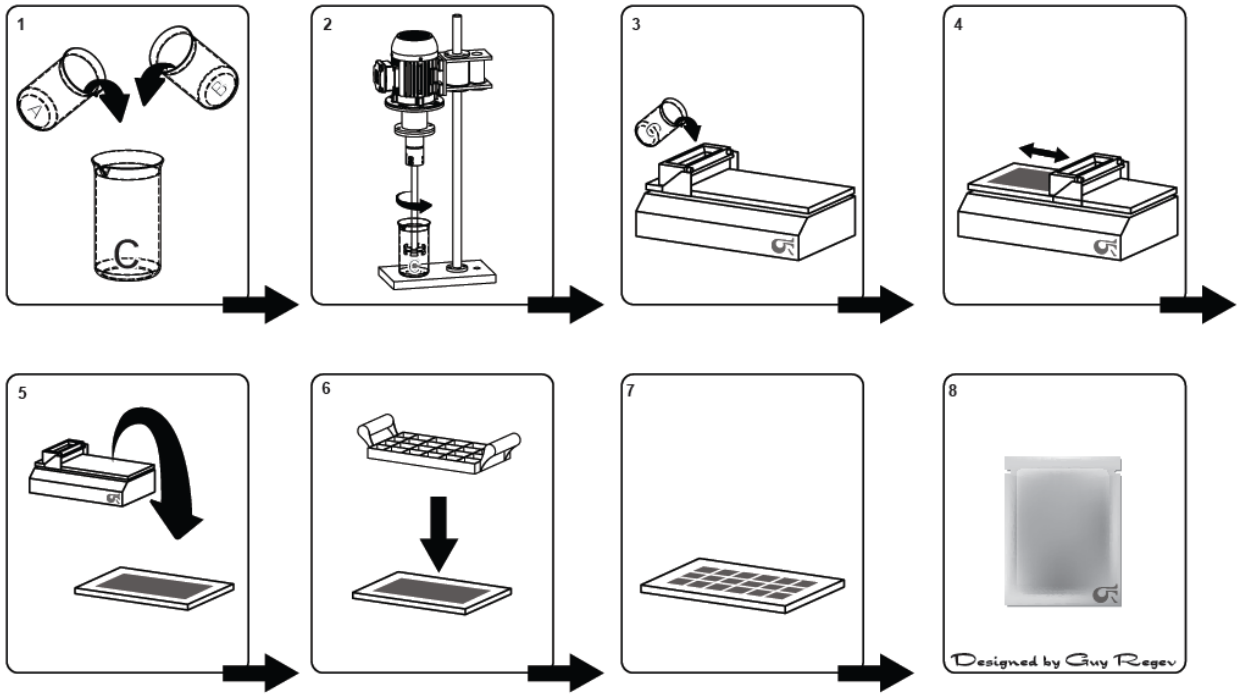

Figure 1.1: Illustration of the Solvent Cast Process for Vaginal film Manufacturing.
1) The excipients are added to the solution in multiple step process, 2) the film solution is then mixed until homogeneity is achieved, 3) film solution is transferred to the casting reservoir, 4) film solution is cast onto a substrate 5) film solution is dried and pilled from the substrate, 6) the film sheet is then transferred to the cutting station or prepared to be cut if using a continuous manufacturing casting apparatus 7) using a cutting template the dried film sheet is cut into the desire unit dose, 8) the film is packed in individual package or a multiple-film cassette. (Drawn by Guy Regev, industrial designer).

1.3.2 Hot Melt Extrusion

1.3.2.1 Introduction to Hot Melt Extrusion Technology

Hot melt extrusion (HME) is a widely applied process and investigated technology used in the plastic, rubber, and food industries. During the extrusion process, the active pharmaceutical ingredients (APIs) are melted, mixed, and dispersed into various polymer matrices. The extrusion process uses high temperature, pressure, sheer, and physical mixing of solids as opposed to traditional methods, which rely on solubilization, dispersion, and other physiochemical properties to create uniform mixtures [46, 47]. An extruder consists of two distinct parts: 1) the conveying system, which mixes and transports the material and 2) the die, which is required for shaping the extruded material. HME has been found to be an extremely useful technology in the pharmaceutical industry because of its robust production reproducibility, product uniformity, and ability to accommodate a wide range of APIs regardless of Biopharmaceutics Classification System (BSC) class [7]. HME technology was first introduced to the plastic industry in the 1960's and has since become a staple in the production of modern plastics [48]. In contrast to the solvent cast method, HME typically avoids the use of a solvent and the need to optimize the formulation solution, as HME can use only dry raw material. In addition, casted films often contain residual solvent that can lead to issues with compendial compliance. Many common-use plastic products are processed on the HME during product manufacturing, including plastic bags, pipes, and plastic sheets, as well as high-tech products such as car interiors and windshields, and space shuttle parts [48]. More recently, the pharmaceutical industry has gained interest in HME technology. The number of patented HME-applied pharmaceutical products has significantly increased worldwide. Commercialized drug products manufactured using HME have also increased considerably. Additionally, within the

past five years, the number of pharmaceutical publications regarding HME has doubled [48]. HME has been used to develop various delivery systems, including intravaginal rings (IVRs) [49, 50], modified release tablets, [51], transmucosal/ transdermal films [52], pellets [53, 54], and implantable reservoir devices [55, 56].

1.3.2.2 Hot Melt Extrusion Process and Equipment

Pharmaceutical extruders are designed specifically for the development of various solid dosage forms and wet granulations. The internal parts of the equipment cannot be reactive, additive, or absorb the pharmaceutical product. In addition, the equipment configuration must meet the cleaning and validation requirements for the pharmaceutical field [47]. There are two types of HME: the ram extrusion or screw extrusion process. While ram extrusion operates using high pressure (piston-like), the screw extrusion operates using rotating screws. The principle of the ram extruder is a positive displacement of high pressure that pushes the material through a die. In other words, a piston is used to push the mass through the die/screen that is at the end of the barrel [57]. At the beginning of the ram extrusion process, the material is introduced to the heat in the cylinder and becomes softer. After the material is soft, the ram (piston) pushes the soft material through the die for the chosen shape of the dosage form. The ram extruder is known for precise extrusion of highly valuable materials, due to lower and less frequent pressure. However, one of the drawbacks of this process is the limited control of heat uniformity caused by the limited melting capacity [47].

Screw extruders are more applicable to the pharmaceutical industry because they provide greater product uniformity than Ram extruders, due to higher shear and increase mixing. There are two types of screw extruders; single screw extruders and twin screw extruders. Single screw extrusion contains one screw rotating inside the extruder barrel and is utilized for feeding,

melting, and mixing. The twin-screw extruder contains two screws rotating inside the extruder barrel. The twin screws can be co-rotating (rotating in the same direction) or counter rotating (opposite direction) [47, 58]. The co-rotating screws can work at elevated speed allowing high output necessary for sufficient mixing. The counter rotating screws are used to generate high shear. Due to the nature of mixing using counter rotating screws air entrapment and high pressure may occur [58]. Twin screw extruders have several advantages over the single screw extruders, such as easier feeding of material, better kneading and dispersing capacities, less tendency to overheat, and shorter transit time [48, 58].

The individual parts of the twin screw extruder (Figure 1.2) include 1) the feed hopper (used to feed the raw material to the extruder barrel under controlled speed); 2) the conveying and mixing system (screws, temperature control barrel); 3) the die system for forming the shape of the dosage form; 4) the downstream auxiliary equipment for cooling, collecting and cutting the extrudate [46].

The barrel collects the materials from the feed hopper and is responsible for temperature maintenance and mixing. Mixing is carried out by the screws, which are housed within the barrel. These screws, typically made of stainless steel, continuously turn within the barrel, allowing for mixing of the raw material. Importantly, the screws can be designed with multiple different elements that allow for different functions. Three common screw elements are the mixing element, the compressing element, and the metering element. These elements are selected based on the desired final product. As the raw material passes through the barrel and encounters the designed screw elements, it is melted, mixed, and pushed through the die. [47].

The die system is attached to the end of the barrel and its shape designed to the desired shape of each dosage form. The die is generally made of stainless steel, to minimize reactivity to

the material and to allow sufficient cleaning. The die controls the shape of the dosage form, for example, a film die (also known as a flat die) is used for film manufacturing, which allows for the extrusion of the film sheet at the desired thickness. For downstream processing, a variety of cooling equipment can be used to collect the extruded product, depending on the dosage form. For example, chilled rolls are used to rapidly cool down and collect an extruded film sheet. Once cooled, the film sheet is collected, cut and packed into the desired unit dose.

In summary, the HME process is a continuous operation that melts and mixes raw material to produce a shaped extrudate. The process allows for in-process controlling and monitoring. Ultimately, the Hot Melt Extrusion process is a robust system that is currently being developed for application across a variety of fields.

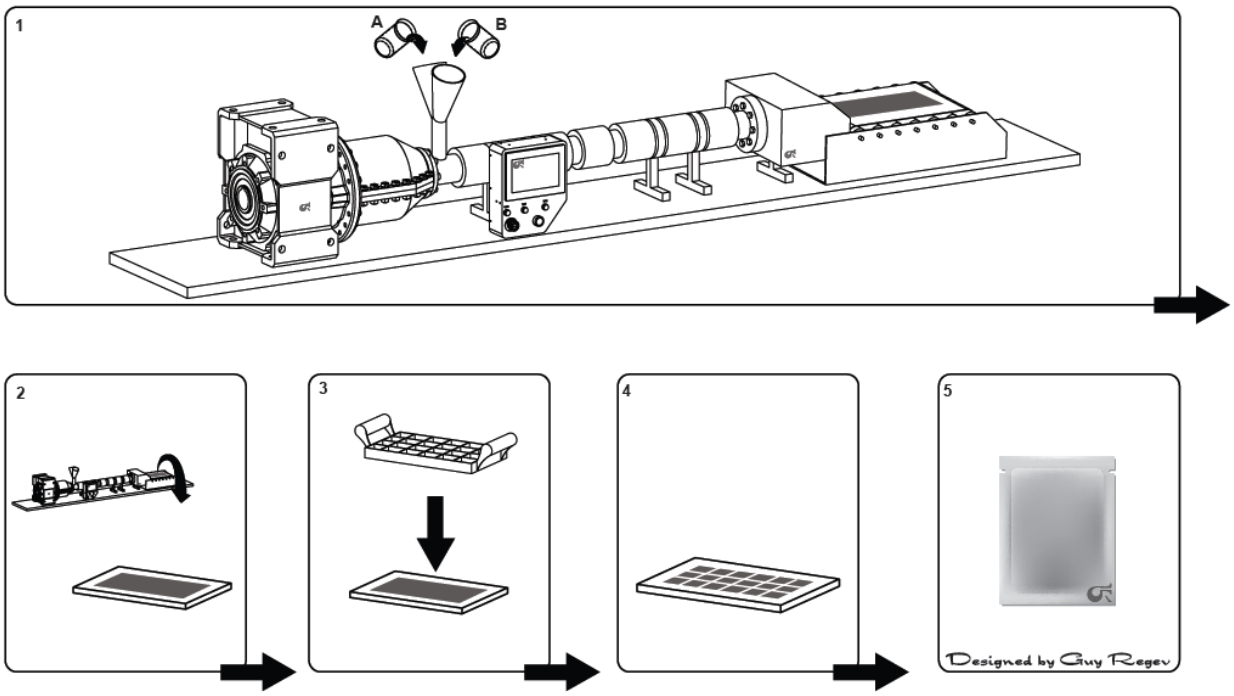

Figure 1.2: Schematic Diagram of the HME Process for Vaginal Film Manufacturing.
1) The dry excipients are mixed in a blender then the homogenous mixture is transferred to the HME feeder. From the feeder, the mixture then enters the HME through the feeding zone. The mixture is then melted, mixed and pushed through the film die. 2) The film sheet is then transferred to the cutting station or prepared for cutting if using a continuous manufacturing HME apparatus. 3) Then, using a cutting template, the film sheet is cut into the desired unit dose. 4) This shows the sheet after it was cut into the individual film units. 5) The film is packed in individual packages (illustrate in the figure) or in a multiple-film cassette (Drawn by Guy Regev, industrial designer).

1.3.2.3 Advantages and Disadvantages of the Hot Melt Extrusion Process in the Pharmaceutical Field

HME offers several manufacturing advantages over the solvent casting technique. HME can provide a solvent-free process that is suitable for moisture-sensitive drugs and hydrophobic active pharmaceutical ingredients as it can enhance solubility and bioavailability of water insoluble active agents. Eliminating the need for volatile solvents makes it easier and safer for scientists to handle. This process is more economically beneficial than solvent cast due to its reduced production time, fewer processing steps, and continuous operation. It may be applied to

sustained, modified, and targeted release drug delivery systems. Moreover, HME allows for more specific mixing than solvent cast. While solvent cast requires all components to be mixed in solvent prior to casting, HME can utilize a wide range of screw elements that can be designed to achieve specific mixing conditions for process optimization. These screws are also self-wiping, which makes cleaning and cleaning validation easier [56, 57]. Likewise, the lack of fluid dynamics involved in the process can make scale-up less challenging than solvent based manufacturing methods. Finally, because in the HME process the API has a short residence time in the mixing chamber (barrel), its exposure to these potentially harmful processes is limited. This can increase stability and reduce degradation of the final product. Although the HME provides many advantages, it also has some disadvantages. The high processing temperature can disrupt heat-labile drug products. Furthermore, there are a limited number of polymers which are currently available for the manufacture of pharmaceutical products using HME [56].

1.3.2.4 Excipients of Hot Melt Extrusion for Vaginal Film Formulation

The excipients used in the HME process have to meet the same safety and purity regulations as those set for the traditional manufacturing process, such as solvent casting. Most of the excipients that are currently being used for the HME are utilized in traditional solid dosage form manufacturing, such as transdermal patches and tablets. Excipients that are heat- resistant are preferable in HME formulations. However, the short resident time of the process may help to prevent thermal degradation of some heat sensitive excipients. Sometimes the extrusion process can be used to make formulation intermediates such as granulations, pellets, and beads. Typical film product of the extrusion process contains the final dosage form, the excipient, and the active agent. Major categories of the final film ingredients include matrix carrier; plasticizer; antioxidant; and API [47].

Matrix carriers for film dosage forms are usually composed of film forming polymers. Selection of a polymer for the extrusion process should consider polymer stability, drug-polymer miscibility, and the end function of the dosage form, which must meet the goal of the target product profile. For example, in order to create quick dissolving films, a polymer that can disintegrate and release the API quickly must be selected. Additionally, the polymer's melting point and glass transition state (Tg) should suit the process parameters of the selected product. Finally, the selected polymer should be stable at elevated temperatures and pressures, possess thermoplastic behavior, and maintain a low toxicity profile. Several polymers with film forming properties have been determined to be well suited for the HME process. For example, polyethylene oxide (PEO), a crystalline polymer that is available in a range of 100,000 to 7,000,000 DA molecular weight, is very suitable for the extrusion process of film due to its large processing window [59, 60]. Also, PEO was reported by Bruce et al. to possess a low processing temperature, which can be beneficial for heat sensitive APIs [61]. Another example of a suitable film-forming polymer is hydroxypropylcellulose (HPC), which is a non-ionic water-soluble cellulose base polymer. It has dual solubility in aqueous solutions and polar organic solvents [62]. Klucel HPC EF and Klucel HPC LF are the most widely applied polymers for film since they can be processed at temperatures as low as 150-160°C [63]. McGinity et al. and Fuisz et al. used Klucel in films. Both groups extruded the Klucel at relatively low temperatures, ranging from 50 °C to 180 °C [63, 64]. Since polymers with high molecular weight can have high melt viscosity and thus become difficult to extrude, plasticizers are often used as part of the film formulation.

Plasticizers are usually low molecular weight compounds that can be added to the formulation to increase the plasticity, soften the polymer carrier, and enhance the flexibility of

the final product. The addition of plasticizers to the formulation is usually necessary to improve the process manufacturing conditions or the physiochemical properties of the film [47]. The addition of a plasticizer can lower the Tg of the carrier polymer by increasing the free volume between polymer chains which, in turn, lowers the process manufacturing temperature [65]. A lower processing temperature can enhance the stability profile of the polymer carrier and API. Some commonly used plasticizers that have been investigated for HME formulation are triacetin, low molecular weight polyethylene glycols, and citrate [47]. It should be noted that some APIs can have intrinsic plasticizer functionality in the HME process [66]. Plasticizers can play important roles in the drug release rate of extruded dosage forms and long-term stability of the final product. Although the elevated process temperature HME can be reduced by the addition of plasticizer, polymer and API stability may still be affected.

Antioxidants are molecules that can inhibit oxidation and are used in several food and pharmaceutical products. They can also be added to formulations to enhance stability. Antioxidants can be divided into two main categories based on their mechanism of action: (1) preventative antioxidants and (2) chain-breaking antioxidants. Preventative antioxidants prevent the initiation of a free radical chain reaction. Ascorbic acid is an example of a preventative antioxidant. This is a vitamin that can undergo oxidation and react with free radicals. The free radical can impact drug degradation. The self-reduction properties of preventative antioxidants can interfere with autoxidation and protect the drug and the formulation from oxygen molecules. Common chain-breaking antioxidants are hindered phenols and aromatic amines. When a free radical is present a second radical is formed. Then chain-breaking antioxidants cause a third molecule to generate a free radical. The free radical process continues that way until the process terminates and the radical is stabilized by a chain breaking antioxidant, or the product decays

into a stable state. Vitamin E is a commonly hindered phenol that is used in many HME formulations reported in the literature [67, 68].

Excipient selection is dependent on the desired API properties. An initial assessment of the thermal, physical, and chemical properties of the API must be taken into account before designing the formulation and the manufacturing process. Additionally, the API can modify excipients, affecting their functionality and the overall formulation. For example, it was reported by Follonier et al. that the API, oxprenolol hydrochloride, resulted in a decrease in melt viscosity of the formulation that caused a soft, unusable extrudate [69]. Another example reported by Repka et al. is that hydrocortisone was lowering the Tg of the HPC films [66]. During pre-formulation, the potential effect of the excipients should be considered to ensure the API maintains efficacy.

1.3.2.5 Commercialized Pharmaceutical Products Manufactured via HME

Hot melt extruded pharmaceutical dosage forms and medical devices are currently being prescribed and/or sold as over-the-counter products. One of the most successful extruded products on the market, that is FDA approved for the treatment of HIV is called Kaletra™. The melt extrusion process of the new Kaletra™ formulation enhances the solubility of lopinavir and ritonavir thus improving its therapeutic efficacy. Furthermore, this product does not require refrigeration to maintain stability [70]. While the majority of extruded product development work is focuses on poorly soluble oral drug delivery systems, other extruded products are already commercially available. One of the most well known extruded pharmaceutical products is NuvaRing (Merck-Schering Plough) which is an intravaginal ring used for contraception. NuvaRing is a novel vaginal ring system that releases a combination of APIs for contraceptive purposes. It is composed of extruded ethylene-vinyl acetate copolymer

which releases 15 µg ethinylestradiol and 120 µg etonogestrel per day [71]. The HME is utilized for various applications during pharmaceutical product development and manufacture. For example, it can be used for shaping of the product, mixing and melting to create amorphous dispersion and granulation. Table 1 summarizes the currently marketed extruded products that are available and the HME purpose of use [48].

Table 1.1: Marketed Pharmaceutical Products Manufactured using Hot Melt Extrusion Process [48]

Product	Indication	HME purpose	Company
Rezulin (troglitzone)	Diabetes	Amorphous dispersion	Parke-Davis
Onmel (itraconazole)	Antifungal	Amorphous dispersion	Merz North America, Inc.
Zoladex (goserelin acetate)	Prostate cancer	Shaped system	AstraZeneca
Implanon (etonogestrel)	Contraceptive	Shaped system	Merck
Lacrisert (HPC Rod)	Dry eye syndrome	Shaped system	Merck
Gris-PEG (griseofulvin)	Antifungal	Crystalline dispersion	Pedinol Pharmacal
Palladone (hydromorphone HCl)	Pain	Controlled release	Purdue Pharma
Nucynta (tapentadol)	Pain	Controlled release	Jenssen
Opana ER (oxymorphone HCl)	Pain	Controlled release	Endo Pharmaceuticals
NuvaRing (etonogestrel, ethinyl estradiot)	Contraceptive	Shaped system	Merck
Norvir (ritonavir)	Antiviral (HIV)	Amorphous dispersion	AbbVie
Kaleatra (ritonavir/lopinavir)	Antiviral (HIV)	Amorphous dispersion	Abbvie
Eucreas (vildagliptin/metformin HCl)	Diabetes	Melt granulation	Novartis
Zithromax (azithromycin)	Antibiotic	Melt granulation	Pfizer
Orzurdex (dexamethanone)	Macular edema	Shaped system	Allergan
Noxafil (posaconazole)	Antifungal	Amorphous dispersion	Merck
Belsomra (suvorexant)	Medication for insomnia	Amorphous dispersion	Merck

1.3.2.6 Film Application of Hot Melt Extrusion

To date, transdermal and transmucosal films are typically manufactured using solvent casting techniques, which involves the use of aqueous or organic solvent [72]. For film production, solvent cast techniques face several problems, as reported by Guitierrez- Rocca et al. Acrylic films manufactured by solvent casting for stability evaluation, had increased tensile strength and decreased elongation and elasticity over time [73]. In addition, it was reported that the level and type of plasticizer, curing time, and temperature have a significant effect on the dissolution rate of drug from solvent casted films [74]. In the late 1990s, Aitken et al. demonstrated the applicability of HME for transdermal film product manufacturing. It was noted that the extruded

films were not solvent restricted, as solvent is not included in the formulation or the manufacturing process [72]. Additionally, this Eudragit® E100 transdermal film studied by Aitken contained, local anesthetic agent, lidocaine HCL and were extruded utilizing single screw HME. In this dissertation work, the twin screw HME is applied for vaginal film development.

HME is currently under investigation as a potential manufacturing process for transdermal and transmucusal films. Repka et al. produced hydroxypropyl cellulose (HPC) films using the HME technique. The purpose of their study was to better understand the effect of plasticizers on HPC and therefore, they incorporated several plasticizers and two model drugs (chlorpheniramine maleate and hydrocortisone) into an HPC film formulation. In their study, they investigated the effect of the plasticizers and drugs on the physical and mechanical properties of the film. Their main finding was that HPC could not be extruded without plasticizers due to high torque level [75]. Crowley et al. extruded polyethylene oxide (PEO) films containing guaifenesin (GFN) or ketoprofen (KTP) as model drugs. The purpose of their study was to investigate the thermal stability of PEO. They were able to extrude drug loaded films containing up to 30% GFN and up to 15% KTP. The physicochemical and mechanical properties of the films were investigated. Overall they were able to extrude these films and maintain PEO based tablet stability [67].

HME is a suitable and very beneficial manufacturing process for various dosage forms including film. Recently, the applicability of vaginal films for delivery of antiretroviral drugs has been explored. The benefits of using vaginal film include user comfort, ease of use, delivery of drugs that are not effective as an oral dose and delivery of drugs to the site of action. Vaginal films are traditionally manufactured by the solvent cast technique. There are currently no reported vaginal films being commercially manufactured by the HME. However, other vaginal

products manufactured by the HME, such as intravaginal rings, are reported in the literature and have also been commercialized. Research into the development of extruded vaginal films is ongoing and is important for achieving a film manufacturing process that can reduce production time, avoid solvent, allow product uniformity, allow product production reproducibility, and lead to overall lower manufacturing cost and greater scalability.

1.4 RESEARCH GOAL AND OBJECTIVES

The vaginal film dosage form is a highly applicable and widely versatile delivery platform for a variety of active pharmaceutical agents. Some advantages of the vaginal film such as discreet use, no product leakage during use, ease of insertion, rapid drug release, minimal packaging, and minimal impact on innate factors make it a very desirable dosage form [30, 32]. Traditionally, vaginal films are manufactured using the solvent cast method. However, hot melt extrusion can potentially be applied to the manufacturing of vaginal films, and has more advantages than the solvent cast method. The HME process is continuous and requires fewer processing steps for efficient large-scale manufacturing and maximum batch uniformity. Since no solvent is required, the drying step of solvent cast is eliminated, which reduces manufacturing time. This can potentially lead to lower manufacturing costs due to the simplicity, minimal processing steps, and availability worldwide for local production of HME products.

The main purpose of this study was to evaluate whether hot melt extrusion can be utilized for the development of a versatile polymeric vaginal film that is capable of incorporating and delivering biomolecules, small molecules, and combination drugs. To evaluate the feasibility of the process, a range of molecules were selected including Metronidazole (hydrophilic),

Dapivirine (hydrophobic), Griffithsin (protein), Lactobacillus (probiotic), and Levonorgestrel (hormone).

The Central Research Question of this Dissertation Work:

- Can hot melt extrusion be utilized for the development of advanced and versatile polymeric films that are capable of incorporating and delivering biomolecules, small molecules, and their combinations?

This question was addressed through the following specific aims:

Specific aim 1: To establish applicability of the hot melt extrusion (HME) manufacturing technique for the production of single-layer vaginal films for delivery of a range of individual APIs (chapter 2 and 3).

The two parts of aim 2 are:

a) Hot Melt Extrusion for Film Formulation Development of Small molecules (hydrophobic/ hydrophilic), and Active Biologics (proteins and probiotics) (chapter 2)
b) Hot Melt Extrusion as An Alternative Manufacturing Technique, for Topical Vaginal Film Application Containing Microbicide Candidate Dapivirine (chapter 3)

Specific aim 2: To utilize HME for the development of multilayer films containing small molecules (hydrophobic/ hydrophilic) and active biologics (proteins, peptides, and probiotics)

The two parts of aim 2 are:

a) Multilayer Film for Co-Delivery of Antibiotic and Probiotics for the Treatment of Bacterial Vaginosis (chapter 4)
b) Multilayer Film for the Co-Delivery of Antiretroviral and Contraceptive Agents for the Prevention of HIV and Unintended Pregnancy (chapter 5)

2.0 AIM 1 PART A: ESTABLISHMENT OF HOT MELT EXTRUSION (HME) APPLICABILITY FOR THE PRODUCTION OF SINGLE-LAYER VAGINAL FILMS FOR THE DELIVERY OF A RANGE OF INDIVIDUAL ACTIVE PHARMACEUTICAL INGREDIENTS

2.1 INTRODUCTION

Many investigated and marketed vaginal products support women's health, whether it is through promoting vaginal sanitation (e.g. VCF® Vaginal Cleansing Film), providing efficacious contraception (e.g. NuvaRing) or preventing sexually transmitted infections such as HIV (e.g. dapivirine intravaginal ring). A variety of manufacturing processes have been implemented to produce these vaginal products including compression, solvent casting, spray drying, and fusion molding. In this chapter, the application of HME for manufacturing of vaginal films was explored utilizing a range of active pharmaceutical ingredients (APIs) that aim to bolster overall vaginal health. A variety of APIs, which encompass a wide range of physicochemical characteristics, were chosen to versatility of the HME process. The panel of drug candidates selected for this study included both small and large molecule drug candidates that range in hydrophobicity. These molecules included dapivirine (small hydrophobic molecule), metronidazole (small hydrophilic molecule), Griffithsin also known as GRFT (large molecule protein) and *Lactobacillus* bacteria (microorganism). These APIs range in therapeutic

application; from preventing HIV acquisition and transmission to treating sexually transmitted infections or maintaining vaginal microflora.

Dapivirine is (DPV) a small molecule, hydrophobic microbicide candidate with a log of 5.27. It is a potent, clinically-advanced HIV-1 replication inhibitor which is being developed to be licensed as a topical microbicide candidate by the International Partnership for Microbicides (IPM). DPV is classified as a non-nucleoside reverse transcriptase inhibitor (NNTRI) with an EC_{50} of 0.3ng/mL and an EC_{90} of 0.9ng/mL against wild type and resistant mutant HIV [76]. It binds with high affinity to the allosteric binding pocket on the reverse transcriptase enzyme near the catalytic site and inhibits HIV-1 replication [63]. It has a high melting point of 220° C and is not heat sensitive, and therefore can be utilized as a model hydrophobic molecule for the HME application. DPV has been formulated as an intravaginal ring (IVR), vaginal gel, and solvent casted film all of which have been tested in clinical trials [39, 76].

The DPV intravaginal ring (IVR) is clinically advanced and was determined to be safe for long term use and effective for HIV prevention in a Phase III clinical study (MTN-020, ASPIRE) [76-78]. In another Phase I clinical study, a solvent casted (SC) DPV vaginal film was found safe and effective in an *ex vivo* model (challenged with $HIV\text{-}1_{Bal)}$ using cervical biopsy samples obtained from women who had used the film (MTN-02) [39]. Its high potency, chemical characteristics, and applicability to vaginal film development make it a desirable drug candidate for this HME based project.

Metronidazole is a hydrophilic (log -0.46), heat stable (melting point 160.5°C) small molecule that is commonly used as an antibiotic agent for the treatment of vaginal microbial infections. A metronidazole 0.75% vaginal gel is approved by the US Food and Drug Administration (FDA) as a treatment for bacterial vaginosis (BV) which is commercially

available over the counter [62]. Metronidazole is also marketed as a prescription medication under several names: MertoGel®, Vandazole®, and Nuvessa®. Metronidazole diffuses passively into the cytoplasm of anaerobic bacteria, where it partially reduces cytoplasm proteins, such as ferredoxin, which creates free radicals. The resultant free radicals interact with intracellular DNA, resulting in the inhibition of bacterial DNA synthesis and ultimately, bacterial death. Additionally, metronidazole was reported to have minimal antibiotic resistance [79]. Taken together, metronidazole is the most common antibiotic prescribed for vaginal infections, such as BV, and is currently marketed for vaginal use as gel dosage form. Due to its proven effectiveness against wide range of vaginal pathogens, its unique physicochemical properties and favorable resistance profile, it was selected as the hydrophilic small molecule for HME applicability.

Griffithsin, also known as GRFT, is a novel HIV entry inhibitor and a potent microbicide candidate. GRFT is a lectin that binds to mannose-rich glycans on the viral gp120 attachment protein of HIV to prevent infection [80]. It is able to bind to the clusters of oligomannose N-linked glycans on the HIV envelope protein gp120 [80, 81]. In addition, GRFT can inhibit the binding of HIV to dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) receptor and thus, the resultant transfer of HIV-1 to CD4 target cells [82]. GRFT is not only effective at HIV prevention, it also inhibit herpes simplex virus 2 (HSV-2) [83], hepatitis C virus (HCV) [84] and severe acute respiratory syndrome coronavirus (SARS-CoV) [85]. GRFT is a small homodimer lectin that contains 121-amino acids with an approximate molecular mass of 12.7 kDa. It was identified in a genetic screen for antiviral activity and originally isolated from a red alga (*Griffithsia* sp.). Kouokam et al. and O'Keefe et al. reported that GRFT was found to be safe to human cervical explants and in an *in vivo* rabbit model [81,

86]. The high molecular weight and large structure of this protein along with its low vaginal toxicity and proven efficacy against HIV infection provide a novel molecule for this project.

Lactobacillus species are the predominant bacteria that comprise the vaginal microflora. They play an important role in the healthy balance of the vaginal micro-environment. These bacteria suppress the growth of harmful pathogenic bacteria in the vagina through competitive inhibition and provide protection from various pathogens due to the production of lactic acid and hydrogen peroxide (H_2O_2) [19]. Additionally, *Lactobacillus* is known to produce powerful antimicrobial proteins, such as bacitracin. These antimicrobial proteins disrupt the cell wall and peptidoglycan synthesis of gram positive and gram negative bacteria leading inhibition of harmful pathogens [87]. The lactic acid produced by *Lactobacillus*, a by-product of the metabolism of glycogen released by vaginal epithelial cells, allows the vaginal fluid to remain acidic (healthy vaginal pH 3.5-4.7). The lactic acid and low pH of the vaginal fluid create a selective antimicrobial barrier against harmful pathogens that can cause vaginal infections such as BV [20]. Furthermore, H_2O_2, produced by specific *Lactobacillus* species, maintains a healthy vaginal environment due to its toxicity to nonresident pathogenic microorganisms [19, 23, 88].

Lactobacillus-containing products can be applied locally in order to maintain vaginal colonization of H_2O_2-producing *Lactobacillus* and can be defined as probiotic products. [89]. Probiotics are live microorganisms that offer benefits when administered to the host [90]. Vaginal probiotics may be composed of one or multiple species of *Lactobacillus*. The sensitivity to environmental conditions, the complexity of the species as well as the known benefits to vaginal health makes bacteria a clear albeit challenging selection for evaluation using HME manufacturing.

Within the scope of this dissertation work, each of the individual APIs described above was utilized to test the versatility of the HME process with respect to the vaginal film product manufacturing. These APIs represent hydrophilic, hydrophobic, small and large molecules and a microorganism. With this work, each API was formulated as a film dosage form manufactured using HME. Obtained film formulations were characterized with respect to physicochemical characteristics and bioactivity.

2.2 MATERIALS

Dapivirine was provided by International Partnership for Microbicides (Silver Spring, MD). Metronidazole was purchased from Spectrum (Gardena, CA). Recombinant Griffithsin was manufactured by Kentucky Bioprocessing LLC (KBP; Owensboro, KY) and provided by Dr. Kenneth Palmer, from the University of Louisville School of Medicine. PolyOx N10, N80 (Polyethylene oxide, MW, 100,000 and 200,000 respectively), and Polyethylene glycol 4000 were purchased from Dow Chemical Company (Midland, MI). Klucel EF (HPC MW, 100,000) was purchased from Ashland, Inc. (Bridgewater, NJ). Polyethylene glycol 400 and vitamin E acetate were purchased from Spectrum (Gardena, CA). Ultrapure water was obtained from an in-house MilliQ water purification system. Phosphate buffered saline 10X molecular biology grade was purchased from Mediatech, Inc. (Manassa, VA). Acetonitrile (ACN) and trifluoroacetic acid (TFA) were obtained from Fisher Scientific (Pittsburgh, PA). Columbia Sheep's Blood agar plates (BA) and Human Blood Tween (HBT) Bilayer Medium were purchased from Becton Dickinson and Co. (Sparks, MD).

2.3 METHODS

2.3.1 Polymer Screening by Thermal Analysis

Several film-forming polymers were screened for melting temperature (Tm) and glass transition (Tg) temperature. Differential scanning calorimetry (DSC 1 Mettler-Toledo, STARe system) equipped with GC 200 gas controller was used to evaluate thermal properties of film excipients. Nitrogen gas was used as a purge, with a flow rate of 50 mL per minute. Approximately 4-9 mg of sample was weighed and sealed in an aluminum pan. All polymers were screened with temperature increasing at 10°C per minute.

2.3.2 Film Manufacturing

The vaginal films were manufactured using twin screw HME technology. The extrusion barrel temperature was set based on the API and the designed formulation (see table 2.1). All excipient powders and the API were blended for 15 minutes using a bench top mixer to ensure content uniformity of the drug in the final extrudate product. The powder blend was transferred to the HME feeder to allow a controlled feeding rate. At the end of the extrusion, the product was collected by rolling the film sheet using a chilled roll system. Once the film sheet was collected, it was cut using a die press into final unit doses and packed individually in aluminum foil pouches.

Table 2.1: Summary of the Formulation and HME Parameters for Dapivirine, Metronidazole, Griffithsin and *Lactobacillus* Films

	Dapivirine film formulation	Metronidazole film formulation	Griffithsin film formulation	*Lactobacillus* film formulation
Excipients	PolyOx N80, Hydroxypropyl cellulose EF, Polyethylene glycol 4000, Polyethylene glycol 400, vitamin E	PolyOx N10, Polyethylene glycol 4000, Polyethylene glycol 400, vitamin E	PolyOx N10, Polyethylene glycol 4000, Polyethylene glycol 400, and vitamin E	PolyOx N10, Polyethylene glycol 4000, Polyethylene glycol 400, and vitamin E
Conditions	Increased temperature from 115 °C to 134 °C	Increased temperature from 110 °C to 120 °C	Constant temperature of 65 °C	Constant temperature of 65 °C
Die melt (°C)	140 °C	125 °C	65 °C	65 °C
Screw speed (RPM)	180	150	100	40
Film size (inches)	1x2	1x2	0.5x1	0.5x1
Pre-formulation steps			GRFT solution was lyophilized to remove the aqueous storage solution	

2.3.3 High Performance Liquid Chromatography (HPLC) Analysis

2.3.3.1 Hydrophobic Molecule: Dapivirine HPLC Detecting Assay

A high performance liquid chromatography (HPLC) system (Dionex Ultimate 3000, Thermo Scientific) equipped with an auto-sampler, a quaternary pump, and a diode array detector was used to quantify DPV. This gradient method utilized a reversed phase chromatography using a C18 column (Acclaim 150x4.6mm) at a wavelength of 290 nm with a retention time of 10.3 minutes. The mobile phase consisted of A) 0.1% trifluoroacetic acid in water (v/v) and B) 0.1% trifluoroacetic acid in acetonitrile (v/v), pumped at a flow rate of 1 mL/min. The mobile phase

gradient was maintained as follows (minutes;%B): 0;30, 6.6;45, 12.6;54.0, 13.2;80, 15;30. The lower limit of detection (LOD) was 0.025 µg/mL and the lower limit of quantitation (LOQ) was 0.0825 µg/mL. Linearity of the detector response curve was at a range of 0.1 µg/mL to 100 µg/mL. Chromeleon software was used to capture data from the HPLC system.

2.3.3.2 Hydrophilic Molecule: Metronidazole HPLC Detecting Assay

A high performance liquid chromatography (HPLC) system (Waters Corporation; Milford, CA) equipped with an auto-sampler, a quaternary pump controller, and a diode array detector was used to quantify metronidazole. This isocratic method used a reversed phase chromatography using a C18 column (Zorbax Eclipse C18 4.6x100 mm) 150x4.6 mm). The mobile phase consisted of 0.1% trifluoroacetic acid in water (v/v) and 0.05% trifluoroacetic acid in acetonitrile (v/v), at a flow rate of 1mL/min. Metronidazole was quantified by UV detection at 275 nm and the average retention time of metronidazole was approximately 2.5 minutes with 0.32 µg/ml for the lower limit of detection (LOD) and 1.056 µg/mL for lower limit of quantitation (LOQ). Linearity of the detector response curve was at a range of 1 µg/mL to 200 µg/mL. Empower software was used to capture the data generated by the HPLC system.

2.3.3.3 Protein: GRFT HPLC Detecting Assay

The High Performance Liquid Chromatography (HPLC) system (Waters Corporation; Milford, CA) equipped with an auto-sampler, a quaternary pump controller, and a fluorescence detector was used. GRFT was quantified using an excitation wavelength of 273 nm and an emission wavelength of 303 nm. The GRFT gradient was applied via a C18 column (Phenomenex Jupiter 5µ 300Å 4.6x250 mm). The mobile phase consisted of 0.1% trifluoroacetic acid in water (v/v) and 0.05% trifluoroacetic acid in acetonitrile (v/v) pumped at a flow rate of 1 mL/min. The

gradient was maintained as follows (minutes;%B): 0;12, 15;20, 16;50, and 20;12. The LOD was found to be 0.3µg/mL and the LOQ 1.0 µg/mL. The linearity of the detector response curve was at a range of 10 µg/mL to 500 µg/mL. Empower software was used to capture data from the HPLC system.

2.3.3.4 Large Molecule: Bacteria Detection via Bacterial Viability and Colonization Test

Columbia agar with 5% sheep's blood (Becton Dickinson and Co., Sparks, MD) was used to evaluate the bacterial viability. Bacteria-loaded films were dissolved in sterile 1X phosphate-buffered saline (PBS), and serial dilutions were performed. Each of the dilutions were plated onto agar plates and incubated at 37 °C with 6% CO_2 for 48 hours. After incubation, the period the bacteria colony forming units (CFU) were counted and recorded.

2.3.4 Film Characterization

2.3.4.1 Appearance, Thickness, and Mass

Prior to packaging, film appearance was visually evaluated based on texture, color, an appearance of bubbles, holes, tears, evenness, and uniformity. The average film thickness (mm) was measured using calipers. Film mass (mg) was measured using an analytical balance.

2.3.4.2 Puncture Strength

The puncture strength of HME films was measured using a texture analyzer (TA.XT.Plus®). The film was placed tightly on the TA-108S5 fixture and was punctured by a 1/8'' ball probe at a rate of 1.0 mm/sec and a trigger force of 1.0 g. The maximum force required to rupture the film was recorded. The following equation was used in order to calculate the puncture strength

$$Puncture\ strength\ \left(\frac{g}{mm}\right) = \frac{Force\ at\ break\ (g)}{Thickness\ of\ the\ film\ (mm)}$$

2.3.4.3 Disintegration

In vitro disintegration (seconds) was determined using the Texture Analyzer instrument (TA-XT.Plus). The disintegration was assessed by the amount of time for the film to break in the presence of both external force, using 1/8'' ball, and 15 µL of MilliQ water applied directly to the film.

2.3.4.4 Moisture Content

The residual water content of the films was measured using a Karl-Fisher apparatus (Metrohm, 758 KFD Titrino).

2.3.4.5 *In Vitro* Release

2.3.4.5.1 Dapivirine, Metronidazole and Griffithsin Film Dissolution Assays

The drug release evaluation from the extruded film (dapivirine, metronidazole, and griffithsin films) was conducted using USP apparatus IV flow-through dissolution system (SOTAX CE7 Smart, Sotax, Switzerland) equipped with auto-sampling and UV systems. The assay for each individual film is summarized in table 2.2. These studies were conducted at 37°C for 60 minutes with a flow rate of 16 mL/min.

Table 2.2: Dissolution Assay Condition Summary

Condition/Film	Dapivirine film formulation	Metronidazole film formulation	Griffithsin film formulation
Dissolution media	1% Cremophor	MilliQ water	Phosphate-buffered saline
Method of detection	UV detection at 290 nm using the UV system attached to the Sotax apparatus	Sample collection and HPLC analysis	Sample collection and HPLC analysis

2.3.4.5.2. Lactobacillus Dissolution Assay

An assay utilizing the flow cytometry LSRII (BD Biosciences) was developed. *Lactobacillus* was separated from the film particles based on the size and granularity. A film was submerged into a mixture of MilliQ H_2O, and 1 mL sheath fluid (BD Biosciences) and samples were collected for analysis at predetermined time points. These samples were analyzed by the flow cytometry LSRII with the forward scatter (FSC), and side scatter (SSC) parameters turned on. FSC correlates with cell size, and SSC correlates with the granularity of the samples.

2.3.5 Product Specific Characterizations

2.3.5.1 Dapivirine Film: *Lactobacillus* Toxicity

Lactobacillus crispatus and jensenii (L. crispatus and *L. jensenii)* were used for the Standard Microbicide Safety Test (SMST) of the DPV films. Films were dissolved in a bacterial suspension of *L. crispatus and L. jensenii*. The suspension was incubated for 30 minutes at 37 °C and bacterial viability was determined. Samples were taken at the beginning of the incubation at time points 0 minutes and after 30 minutes of incubation. Viability was determined by standard

plate count. Compatibility of the film with the *Lactobacillus* was measured as log differences in bacterial viability between time points.

2.3.5.2 Dapivirine Film: Bioactivity and *In Vitro* Cellular Toxicity

Anti-HIV activity testing was performed using a TZM-bl cell based assay as previously described [36]. DPV film was dissolved in 2 mL of saline and ten-fold serial dilutions were made. The DPV drug substance stock solution (1µM) that was used as a control, was mixed in DMSO and serially diluted in DMEM with 10% BSA supplemented with antibiotics (which is the medium used in the TZM-bl assay). All dilution samples were added in triplicates to the plated TZM-bl cells. Afterward, HIV-1_{Bal} was added and cultured for 48 hours. The amount of infected cells was detected by the addition of a chemiluminescent developer of luciferase, BrightGlo (Promega), to each well. For the background control and the maximal luciferase activity, cells alone and cells infected with HIV-1_{Bal} were used respectively. The IC_{50} was calculated using GraphPad Prism software (V6.07). For cellular toxicity evaluation, a similar set up of the experiment was conducted. Except that for the toxicity study, the cells were not exposed to the virus. In this study, the cells were exposed to CellTiter-Glo, and luminescence signal was measured. Cellular viability was determined based on the deviation from the cell-only control [91].

2.3.5.3 Metronidazole Film: Bioactivity

To test the bioactivity of metronidazole film, *G. vaginalis* (ATCC 14018) was plated on HBT plates to form a lawn. A metronidazole film was placed in the middle of the plated bacteria and plates were incubated at 37 °C with 6% CO_2 for 48 hours. Post incubation, the plates were

visually inspected for the formation of a zone of inhibition. Placebo films and metronidazole drug substance were prepared and tested in the same manner as the controls.

2.3.5.4 GRFT: Size Determination

In order to ensure that GRFT did not undergo fragmentation during the film manufacturing process sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The tested samples were GRFT powder post-lyophilization (before HME film manufacturing process), GRFT films, and GRFT drug substance. The SDS-PAGE protocol was developed and shared with our lab by Dr. Kenneth Palmer's laboratory. For this assay, BioRad precast Mini-PROTEAN® TGX Any kD gels, BioRad Precision Plus, 1X Tris/Glycine/SDS Running buffer, and ProteinTM KaleidoscopeTM Standards were utilized. GRFT samples were diluted to 1-8µg of protein. 30µL of these samples were combined with 30µL of Laemmli sample loading buffer. Samples were boiled for 5 minutes following 5 minutes of cooling on ice. Each 30 µL sample contained 0.033mg/mL-0267 mg/mL protein (according to the dilution level of the solution). In each well of the gel, 15 µL of sample was loaded and electrophoresed. Post electrophoresis, the detection of the protein was conducted by Coomassie Blue staining (Bio-Safe Coomassie Stain). Gel images were collected with a Gel DocTM EZ System (Bio-Rad).

2.3.5.5 GRFT: gp120 Binding Evaluation

Enzyme-linked immunosorbent assay (ELISA) was used to test the gp120 binding activity of the lyophilized GRFT (before HME film manufacturing process), GRFT films, and GRFT drug substance. The gp120 solution (HIV-1 gp120 CM was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (Cat# 2968) was bound to a 96 well plate (Nunc MaxiSorp 96-well plates) overnight at 4°C. After overnight incubation at 4°C, the excess gp120

was removed and a blocking solution was added for two hours incubation at room temperature and washed. The various dilutions of GRFT samples were then added to a 96 well plate and incubated for one hour. The plate was then washed, and primary goat anti-GRFT antibody was added and washed after one-hour incubation. The secondary antibody HRP-labeled rabbit anti-goat was added for one hour and washed. Tetramethyl-benzidine (TMB) substrate was applied to the wells for 3 minutes incubation until the blue color was developed. In order to stop the reaction, sulfuric acid was added (yellow color). gp120 binding was detected at an optical density (OD) of 450 nm.

2.3.5.6 *Lactobacillus* Bacterial Film: Detection of Hydrogen Peroxide Production

Hydrogen peroxide detection was determined using a Tetramethyl-benzidine (TMB) plate. Bacterial powder and bacteria-loaded film were tested using the same method. Bacteria-loaded films were dissolved in sterile 1X PBS. The film solutions were placed on TMB plates. The plates were placed in an anaerobic box at 37 °C for 48 hours. The plates were then exposed to air. The horseradish peroxidase present in TMB plate oxidizes TMB in the presence of hydrogen peroxide produced by the *Lactobacillus* to form a blue pigment [23]. The presence of blue color was visually compared to positive and negative controls. The *L. crispatus* (ATCC# 33197) is a positive control for this test since it known for the ability of hydrogen peroxide production while *L.iners* (ATCC # 55195), is a negative control since it does not produce hydrogen peroxide.

2.3.5.7 *Lactobacillus* Bacterial Film: Detection of Lactic Acid Production

Litmus milk (BD, B11343) assay was used for the detection of lactic acid production. Bacteria powder, bacteria-loaded film, and *L. Crispatus* (ATCC # 33197) were evaluated for lactic acid production. One film was dissolved in a 5 mL litmus milk solution. Each sample was incubated

at 37°C with 6% CO_2 for 48 hours. Litmus milk-bacterial solutions were visually inspected for color change. The litmus milk changed from a purple color to a whitish color with the production of lactic acid. The color change was recorded as (+) for color change and (-) for no change.

2.3.5.8 Statistical Analysis

All results were presented as the mean ± standard deviation (SD). Student's *t*-test was used to compare the difference in mean values between the film data. Two-way ANOVA was used to compare the mean values of the stability values. P values ≤0.05 were considered statistically significant. Statistical analyses were conducted using GraphPad Prism software (V6.07).

2.4 RESULTS

2.4.1 Polymer Screening

Polyethylene oxide (PEO), hydroxypropyl cellulose (HPC), polyvinyl pyrrolidone (PVP), hydroxyethyl cellulose (HEC), hydroxypropylmethyl cellulose (HPMC) were screened for melting temperature and glass transition state. Results are summarized in table 2.3 [62]. PEO demonstrated the lowest melting point and glass transition state among all the screened polymers (65-70°C and -30°C, respectively). Therefore, PEO was selected as a main film polymer for this project. Additionally, HPC had a similar melting temperature to HEC with much lower glass transition state of 0°C and therefore was selected as the second polymer carrier for this project.

Table 2.3: Polymers Screened for Hot Melt Extrusion Process

Polymer	Melting point Tm, (°C)	Glass transition temperature Tg, (°C)	Description
Low molecular weight Polyethylene oxide (PEO)	65-70	-30	Selected for this project
Hydroxypropyl cellulose (EF) (HPC)	135-140	0	Selected for this project
Polyvinyl pyrrolidone (PVP-K90)	150-160	165	Softens at 150 °C
Hydroxyethyl cellulose (HEC)	135-140	120	Softens at 135-140 °C, decomposes at 280 °C
Hydroxypropylmethyl cellulose (HPMC)	190-200	140	Browns at 190°C and chars at 200°C

2.4.2 Film Manufacturing

The DPV film formulation contained 37.0% (w/w) Polyox N80, 37.0% (w/w) Klucel EF, 20.0% (w/w) PEG 4000, 2.0% (w/w) PEG 400, and 2.0% (w/w) vitamin E and 1% DPV (see Figure 2.1). The metronidazole film formulation contained 39.5% (w/w) Polyox N80, 26.3% (w/w) PEG 4000, 2.6% (w/w) PEG 400, 2.6% (w/w) vitamin E and 25% metronidazole (see Figure 2.2). The GRFT formulation contained 52.6% (w/w) Polyox N80, 53.1 % (w/w) PEG 4000, 8.8% (w/w) PEG 400, 3.5% (w/w) vitamin E, and 1% GRFT (see Figure 2.3). The *Lactobacillus* film formulation contained 52.6% (w/w) Polyox N80, 53.1 % (w/w) PEG 4000, 8.8% (w/w) PEG 400, 3.5% (w/w) vitamin E, and 3% bacterial-load (see Figure 2.4). Complete film characterization results are detailed in table 2.4.

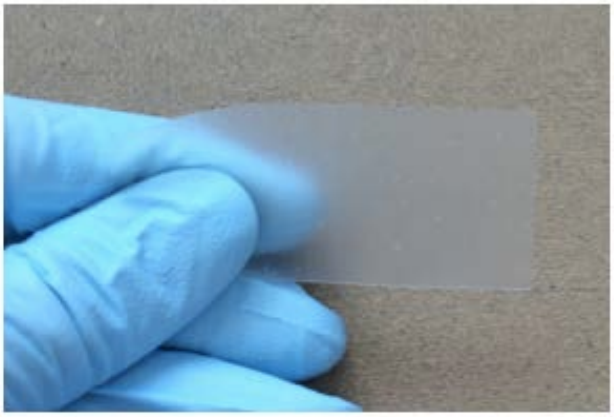

Figure 2.1: Picture of DPV Vaginal Film (2’’x1’’).

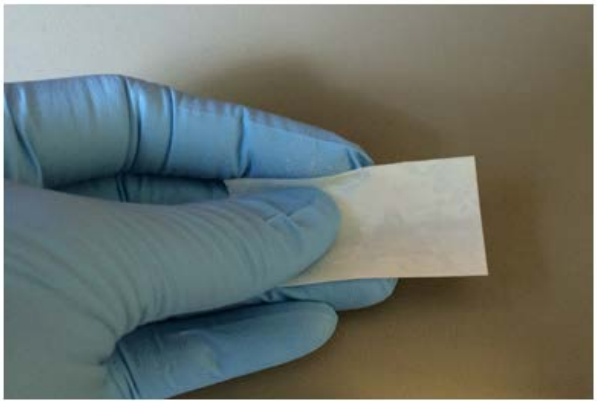

Figure 2.2: Picture of Metronidazole Vaginal Film (2’’x1’’).

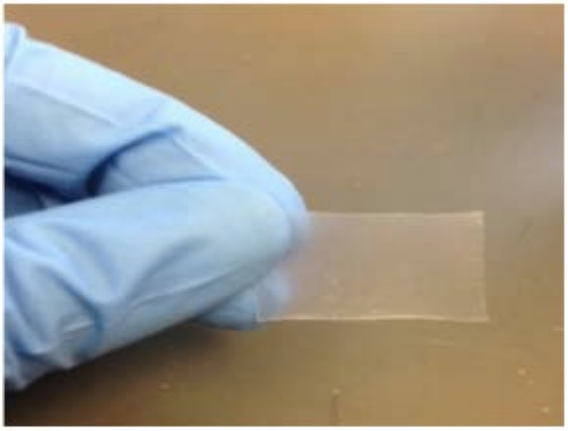

Figure 2.3: Picture of GRFT Vaginal Film (2’’x1’’).

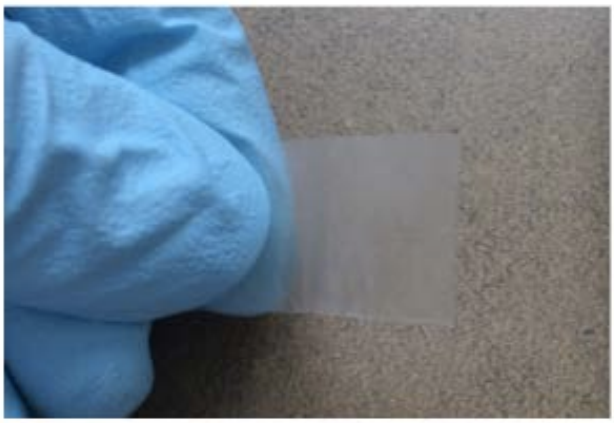

Figure 2.4: Picture of Probiotic Vaginal Film (2''x1'').

2.4.3 Film Characterizations

Each film was evaluated for appearance, mass, thickness, drug content, puncture strength water content and disintegration. The data is summarized in the following sections (see table 2.4). The average weight and thickness of the DPV film was 147 ± 10.1 mg and 0.2 ± 0.01 mm, respectively. DPV drug content was 1.3 ± 0.1 mg/film with less than 2% water content present in the film. Disintegration studies demonstrated a quick disintegration time of 46.6 ± 8.1 seconds, which indicates the quick dissolving nature of the film.

The average weight and thickness of metronidazole film was recorded to be 205.76 ± 14.2 mg and 0.2± 0.02 mm, respectively. Metronidazole drug content was quantified via HPLC analysis and was determined to be uniformly distributed, with total drug content being 50.0 ± 4.5 mg/film. Additionally, the residual water present in the metronidazole film was found less than 1%. The disintegration analysis confirmed the quick (75.8 ± 13.6 seconds) disintegration of the metronidazole film.

The average weight and thickness of GRFT film was 129.4 ± 25.9 and 0.1 ± 0.01 mm,

respectively. The total average drug content of GRFT in the film was 1.2 ± 0.06 mg/film, less than 1% water content and disintegration time of 75.8 ± 13.5 seconds.

The average weight and thickness of the bacterial-loaded film was 133.9 ± 8.7 mg and 0.1 ± 0.008 mm, respectively. The bacteria colonies were uniformly distributed throughout the film and the bacterial CFUs were found to be 10^7. The water content present in the bacterial film was found to be less than 1%. The disintegration time of the film was recorded to be 64.2 ± 0.3 seconds. The results from the dissolution evaluation suggested that the release of the bacteria from the film increased in a time dependent manner. The peak bacterial release was observed after 30 minutes in contact with the aqueous environment (Figure 2.5). In addition, the viability of the bacteria was monitored during the dissolution assay and viability was confirmed throughout the experiment.

Overall all films were determined to be thin, smooth and soft while containing the targeted drug loading. Additionally, the film had quick disintegration profiles with rapid release of the API when in contact with the dissolution media selected.

Table 2.4: Summarized Films Characteristics with All Types of API Containing Films

Parameter/ Film type	DPV film	Metronidazole film	GRFT Film	*Lactobacillus* bacterial film
Appearance	Off white, translucent, smooth	White, transparent, smooth	Clear, transparent, smooth	Clear, transparent, smooth
Weight (mg)	147.3 ± 10.1	205.8 ± 14.2	129.4 ± 25.9	133.9 ± 8.7
Thickness (mm)	0.2 ± 0.01	0.2 ± 0.02	0.1 ± 0.01	0.1 ± 0.008
Ave API per film (mg)	1.3 ± 0.1	50.0 ± 4.5	1.16 ± 0.06	10^7 (CFU)*
Puncture Strength (kg/mm)	3.4 ± 0.5	3.7 ± 0.2	5.1 ± 0.9	5.1 ± 0.9
Water Content % (w/w)	<2.0	<1	<1	<1
Disintegration (seconds)	46.6 ± 8.1	75.8 ± 13.6	61.7±0.9	64.2 ± 0.3
Dissolution (Cumulative Release after 15 minutes, %)	78.9 ± 4.3	70.9 ± 7.8	57.7 ± 6.3	10.3

* CFU=colony forming unit

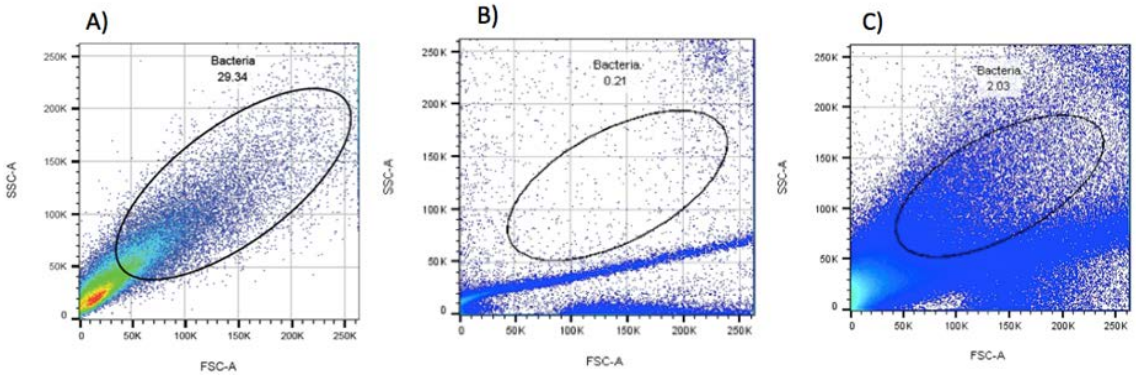

Figure 2.5: Diagram Depicting Flow Cytometer Separation of *L.jensenii* and Polymeric Film.

A) Bacteria population, B) Placebo film, and C) Film containing bacteria at T=45minutes. The dissolution test was conducted using an LSRII flow cytometer. The peak bacterial release was observed after 30 minutes.

2.4.4 Product Specific Characterizations

2.4.4.1 Dapivirine Film Compatibility with *Lactobacillus*

The Standard Microbicide Safety Test (SMST) was used to evaluate the DPV film compatibility with two *Lactobacillus* species present in the vagina, and found that DPV did not hamper bacterial viability. A small fluctuation in bacterial viability observed after the exposure to the film product (table 2.5). However, these changes were not significant since a log value must take place to be considered as a significant change in bacterial viability.

Table 2.5: Compatibility of the DPV Extruded Film with *L.crispatus and L.jensenii*

The compatibility of the *Lactobacillus* was measured as the log difference in bacterial viability before and after exposure to the film product. No loss of bacterial viability was observed after 30 minutes of incubation with the film.

Lactobacillus	**Dapivirine Film (CFU)**	**Placebo Film (CFU)**
L. crisp ATCC 33197	-0.138	0.011
L. jen LBP 28AB	0.168	0.005
L. jen ATCC 25258	0.162	0.084

2.4.4.2 Dapivirine Film Bioactivity and *In Vitro* Toxicity

The inhibition of the HIV infection was assessed for the DPV HME film using the TZM-bl cellular assay. The half maximal inhibitory concentration (IC_{50}) obtained for DPV HME film was 2 nM (Figure 2.6). DPV API substance had similar anti-HIV activity, while the placebo film had minimal anti-HIV activity due to the presence of the polymer in the film formulations. In addition, there was no loss in cellular viability as a result of the exposure of the cell to the DPV HME film. The cell viability remained greater than 80% throughout the assay.

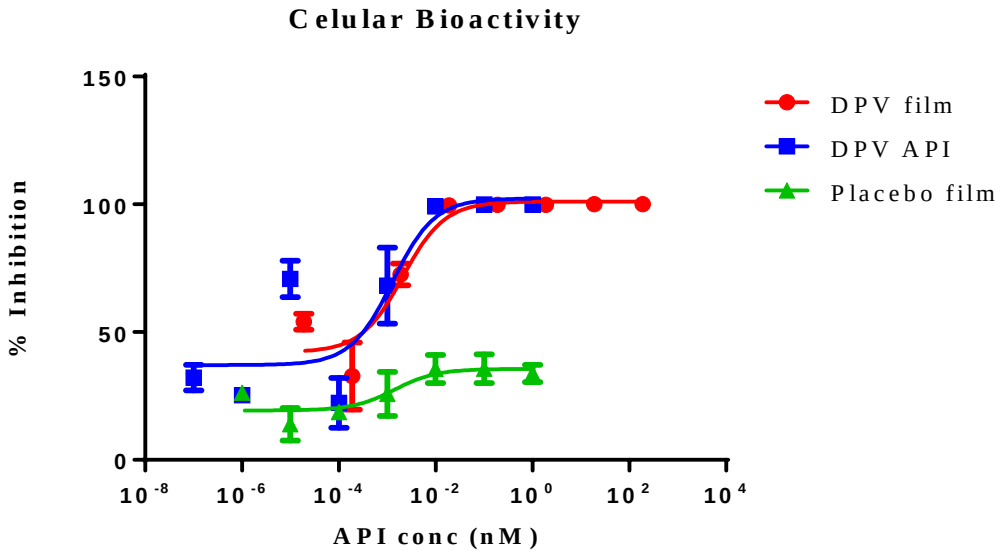

Figure 2.6: *In Vitro* Bioactivity Evaluation of DPV HME Films in TZM-bl Cellular Assay. DPV HME film, drug substance, and placebo films. DPV HME film maintained similar IC_{50} of 2.0 nM compared to the DPV drug substance. The placebo film demonstrated minimal anti-HIV activity. This is due to the presence of the placebo polymers in the cellular assay.

2.4.4.3 Metronidazole Film Bioactivity

Bioactivity was evaluated using lawn of *Gardenlla vaginilis* (*G. vaginilis*) on HBT plates. The metronidazole film demonstrated a 1.5 cm zone of inhibition within the *G. vaginilis* lawn, which was comparable to the metronidazole drug substance's zone of inhibition. This confirms that *G. vaginilis* growth is inhibited by the presence of both the metronidazole drug substance alone and within the film. On the other hand, *G. vaginilis* was not affected by the placebo film see Figure 2.7.

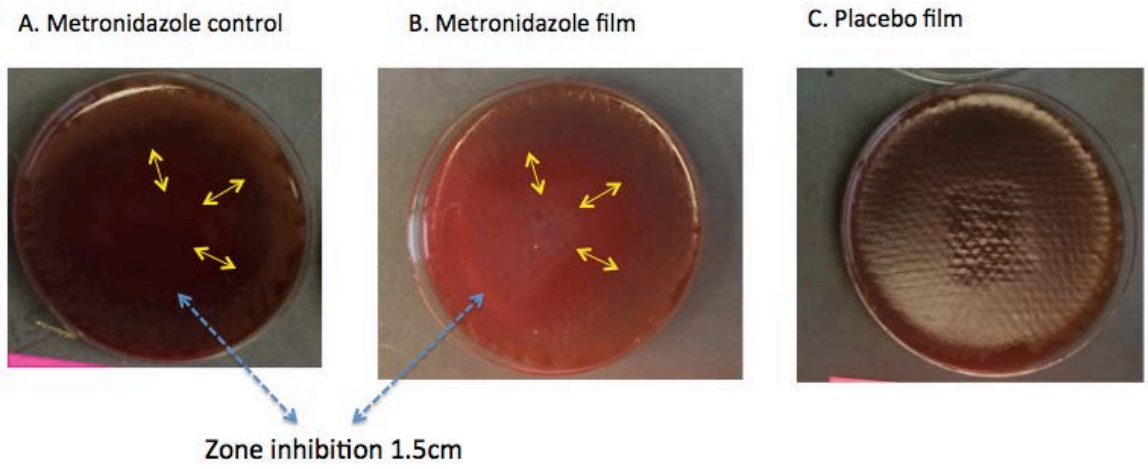

Figure 2.7: Metronidazole *In Vitro* Antimicrobial Evaluation.

A) Metronidazole drug substance inhibited the growth of *Gardenlla vaginilis* B) Metronidazole film inhibits the growth of *Gardenlla vaginilis* and resulted in 1.5cm zone of inhibition, similar to the drug substance alone. C) Placebo film did not inhibit the growth of *Gardenlla vaginilis.*

2.4.4.4 GRFT SDS-PAGE and GRFT gp120 Binding Assay (ELISA)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to evaluate GRFT HME film extrudate (Figure 2.8). GRFT post extrusion (see lanes 4 and 5) displayed bands between 10 kDa and 15kDa. The GRFT control (lane 7) and lyophilized GRFT (lane 8) also displayed bands between 10 and 15 kDa. GRFT contains a 12.7 kDa monomer, which was visible on the gel and therefore confirms that the HME process did not affect the native GRFT structure. The banding pattern of GRFT between 10-15 kDa is common among proteins with similar molecular masses using this gel (BioRad precast Mini-PROTEAN® TGX).The placebo extruded film (lanes 2 and 3) were run on the gel as negative controls, and did not show GRFT bands. Additionally, the binding ability of GRFT was evaluated using ELISA assay. GRFT maintained binding ability to gp120 binding pocket after the HME extrusion process.

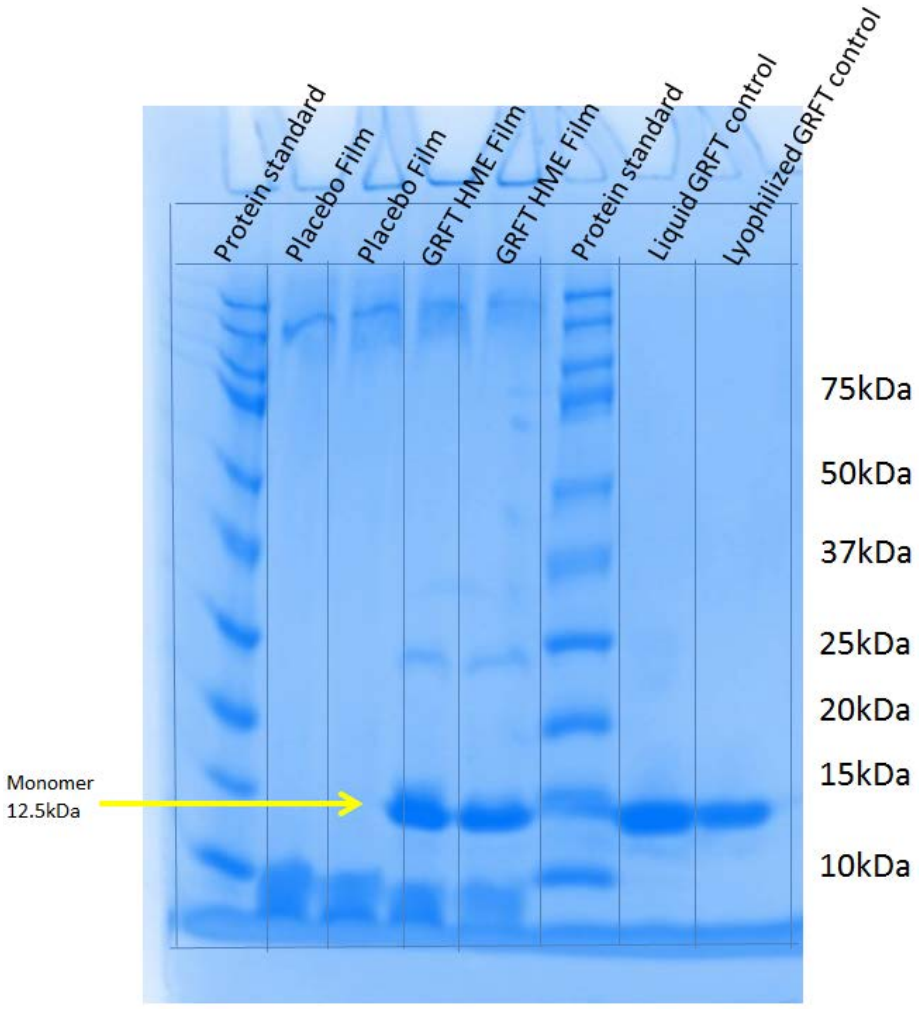

Figure 2.8: SDS-PAGE Gel for Molecular Masse Comparisons of GRFT.
Lanes 1 and 6 contain the protein molecular weight markers. Lanes 2 and 3 contain placebo HME film. Lanes 4 and 5 contain GRFT HME film. Lane 7 contains GRFT drug substance. Lane 8 contains lyophilized GRFT.

2.4.4.5 *Lactobacillus* Bacterial Film Detection of Hydrogen Peroxide Production

Hydrogen peroxide production was determined using a Tetramethyl-benzidine (TMB) plate. In this assay, the presence of blue color confirms H_2O_2 production by *Lactobacillus* and was visually compared to positive and negative controls. *L.crispatus* (ATCC 33197) was used as a positive control due to its known hydrogen peroxide production, while *L.iners* (ATCC # 55195) served as a negative control. Figure 2.9 depicts the plate post incubation and exposure to the air,

and shows the *Lactobacillus* from the HME film produced a blue color present in the zone of growth similar to the *L.crispatus* positive control zone. On the other hand, no blue color was produced in the zone of *L.iners* growth. Therefore, it can be concluded that the *Lactobacillus* retained the ability to produce hydrogen peroxide post extrusion.

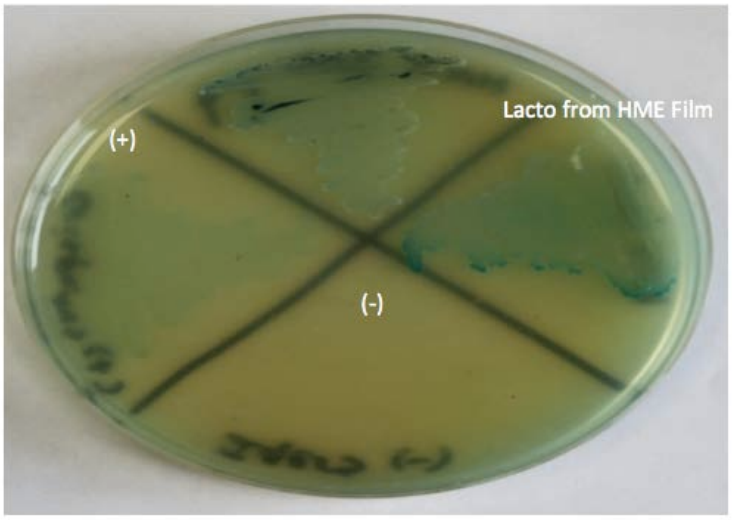

Figure 2.9: HBT Plate for the Hydrogen Peroxide Production Evaluation of Bacteria Loaded HME Films.

On the Left: (+) positive control *L.crispatus* produces lactic acid and a blue color change in the zone of growth. Bottom: (-) negative control *L.iners* does not produce hydrogen peroxide indicated by the lack of the blue color in the zone of growth. Top right: the *Lactobacillus* from the film maintained hydrogen peroxide production post extrusion process indicated by the blue color.

2.4.4.6 *Lactobacillus* Bacterial Film Detection of Lactic Acid Production

Production of lactic acid was determined by litmus milk assay. Litmus milk-bacterial solutions were visually inspected for color change. The litmus milk changed from a purple color to a whitish color with the production of lactic acid. The color change was recorded as (+) for color change and (-) for no change. The results from a litmus milk assay performed after the film manufacturing process showed a decrease in pH, which confirmed that the bacteria produced lactic acid after release from the film. Both the HME bacterial-loaded film and the control

sample (refer to Figure 2.10 A and B) were assigned (+) for color change and the litmus milk negative control (refer to Figure 2.10 C) was assigned (-).

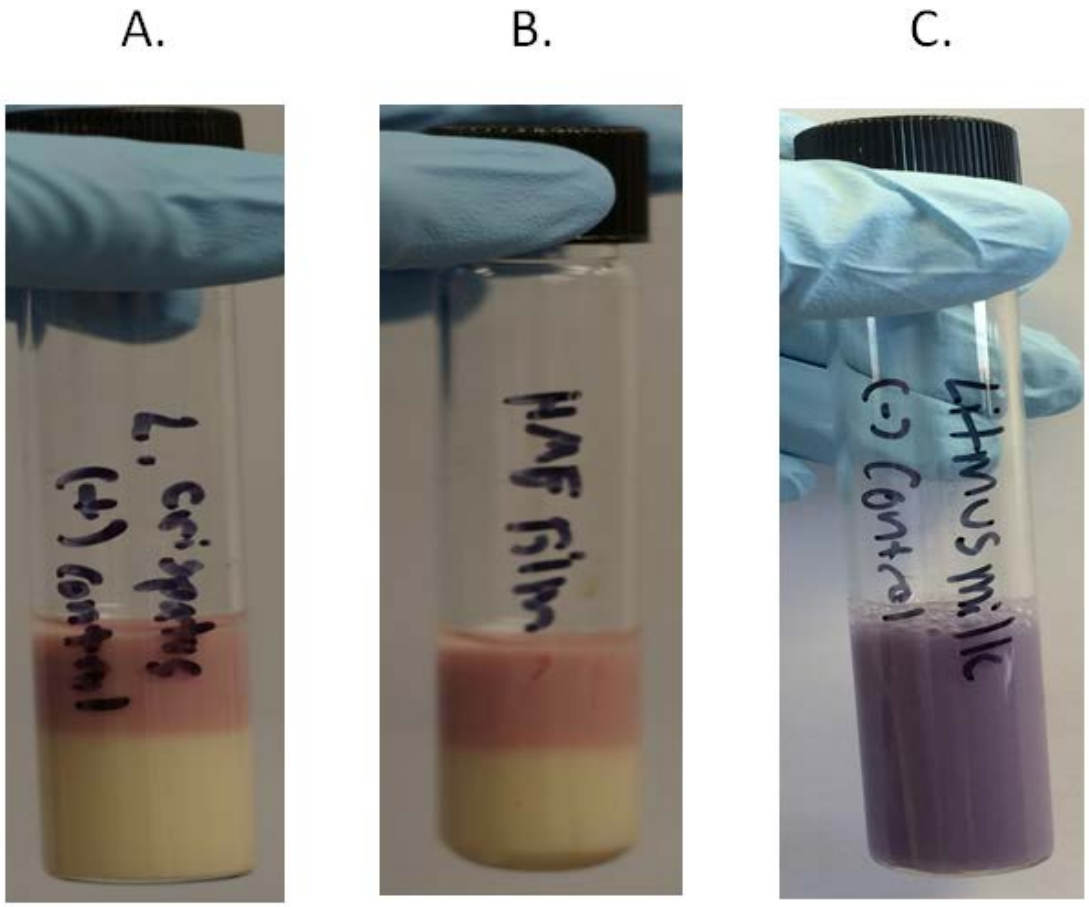

Figure 2.10: Litmus Milk Assay for the Detection of Lactic Acid Production by *Lactobacillus* Post HME Film Manufacturing.

A) *L. crispatus (*ATCC# 33197) positive control B) *Lactobacillus* from the film formulation. In both A and B samples a purple color was converted into a whitish color, confirming lactic acid production. C) Litmus milk (negative control) showed the expected lack of color change.

2.5 DISCUSSION AND CONCLUSIONS

2.5.1 Overall Characterization of the Film

Hot melt extrusion (HME) is a widely versatile technique that has gained attention in the pharmaceutical industry as it can be applied to many dosage forms [48]. Since HME has not yet been applied to the vaginal film drug delivery system, this project explored the feasibility of HME for film manufacturing. In this current project, a different formulation composition was selected for the film development of each active pharmaceutical ingredient (API). Polymers were screened for Tm and Tg. This was done to better select the polymers for the film formulation and HME process conditions. The extrusion process must take place when the polymer is in the melt state and therefore it was important to evaluate the Tg and the Tm of the polymers. PEO, HPC, or both were selected as the film forming polymers of all films since they have low glass transition states (Tg) (-30°C and 0°C respectively) and low melting point (-65°C and 135°C respectively). In addition, they are both approved for pharmaceutical and food use. Interestingly, HPC and HEC found to have similar Tm but different Tg. The Tg of HPC found to be lower than the Tg of HEC (0° C vs. 120 °C). This is due to the structural differences between these two polymers. The Tg depends on the structure of the cellulose ethers and the amount of the cellulosic hydroxyl group on the molecule. The more cellulosic hydroxyl groups, the lower the Tg of the polymer. HPC contains more cellulosic hydroxyl groups and therefore has lower Tg. Lower Tg allows these polymers to soften and be processed at significantly lower temperatures which remove the need for a plasticizer. Therefore, PEO and HPC demonstrated lower melting point and lower Tg, which resulted in lower temperature for the HME processes. Films were successfully manufactured for each individual API utilizing the HME. After film

manufacturing, the films were evaluated for physiochemical and biological properties. Film dissolution analyses were used to ensure the rapid release of the APIs from the film formulation. The drug dissolution test confirmed that within one hour, all four of the APIs were released from the film. More specifically, for each film, over 50% of all the total active agent was released from the film within the first 10 minutes of the test. The rapid drug release can be attributed to the film forming polymer, PEO, and the presence of PEG4000, which is a strong disintegration agent [171]. The rapid release allows the APIs to reach the vaginal canal within minutes from the time that the film is administered, ensuring rapid availability to achieve the therapeutic effect.

All the HME films were evaluated for the water content present in the film. In all the films, except the DPV film, water content was found to be less than 1%. The water content of the DPV film was less than 2%. In both cases, the water content is considered to be low. The low residual water in the films was due to the total lack of water in the formulation. HME is a “dry” process, and therefore, low residual water was expected. The slightly higher water content present in the DPV film (2%) could be a result of the HPC in the formulation. HPC is a hygroscopic polymer that can absorb moisture from the air during the manufacturing process. The water content found in the film is considered to be low compared to other films manufactured by solvent cast [92]. This is a desirable attribute for the formulation because the amount of water in the film can have an impact on the film structural stability over time and is necessary to minimize microbial growth.

2.5.2 Product Specific Characterization

After conducting the traditional film characterizations, each film was appropriately characterized based on the specific API they contained. These characterizations are each discussed below.

The ultimate goal of the DPV candidate was to effectively inhibit the HIV-1 replication while maintaining the safety profile after the formulation process. The DPV film retained potent anti-HIV activity (IC_{50} = 2 nM), similar to the DPV drug substance alone. These results confirmed that the DPV film formulation and the HME process did not affect the anti-HIV activity of DPV. The DPV film was tested for cellular toxicity and compatibility with *Lactobacillus*. The DPV film was found to be safe to the TZM-bl cells and did not show harmful effects to *Lactobacillus* present in the microflora, confirming that the safety profile was maintained. Taken together, these results confirm that HME is capable of manufacturing a highly effective and non-toxic DPV film.

Metronidazole, an anti-microbial agent, was selected as the hydrophilic molecule for this project. The metronidazole film was tested for bioactivity using a HBT plate containing a lawn of *G. vaginalis.* The metronidazole film demonstrated a zone of inhibition similar to the metronidazole drug substance. This test confirmed that metronidazole retained its antimicrobial activity after the HME process. This demonstrated that the hydrophilic small molecule metronidazole could be incorporated into the HME film while maintaining anti-microbial properties.

The selected large molecule protein for this project was GRFT, and since proteins known to be shear sensitive, it was predicted to be prone to degradation during the HME process. The GRFT film was tested for size (SDS-PAGE) and binding activity (ELISA) to confirm GRFT functionality post extrusion process. The GRFT was found to be intact and maintained its binding ability to the gp120 post HME processing. The GRFT was able to maintain its desired properties and function due to the relatively low temperature (65 °C) and high screw speed (100 rpm) which allowed for minimal residence time of GRFT in the screw barrel during the extrusion

process. The short residence time limited the exposure of GRFT to shear stress created during the HME process. In addition, since the formulation components consisted of a thermoplastic polymer, GRFT was protected during the extrusion process. These studies demonstrated that the GRFT maintained its structure and functionality. Therefore, it can be concluded that the HME can be used for the manufacturing film containing large molecules protein such as GRFT.

The *Lactobacillus* species was selected as the microorganism for the process evaluation. The production of bacteria loaded film was challenging since bacteria are sensitive to environmental changes such as exposure to high heat and shear. The bacterial functionality and release from the film was evaluated and is discussed below.

Production of lactic acid and hydrogen peroxide by the bacteria is a fundamental requirement, as this product function maintains a low vaginal pH to prevent vaginal infection. Bacteria contained in the films were able to maintain viability and produce both hydrogen peroxide and lactic acid following the extrusion process, confirming that bacterial-loaded films can be produced by HME manufacturing process.

In this chapter, an *in vitro* dissolution assay was developed for the bacterial loaded films. *In vitro* release testing is an important analytical tool used to investigate and assess the drug product during prototype development. The release profile, which reveals the dosage form release mechanism and kinetics, is used to determine a scientific approach for drug product development [93]. Despite the many benefits of developing probiotic-containing dosage forms, compendial or regulatory standards have yet to be developed for *in vitro* release testing. Therefore, the bacterial detection method, flow cytometry, was applied to *in vitro* release. Flow cytometry has been widely used in the food industry for the rapid detection of viable contaminants and physiological states of yeast and bacteria [94]. In addition, flow cytometry has

also been applied to pharmaceutical science and nanoparticle dosage form development [95]. Taken together, the goal of this assessment was to develop an *in vitro* release assay for the detection of bacteria in the film dosage form utilizing flow cytometry.

In summary, a variety of chemically and functionally diverse APIs were incorporated into vaginal films produced by HME technology. The resultant films maintained acceptable film characteristics and desired bioactivity levels. This project sought to establish a novel, highly functional, and widely applicable manufacturing technique for the production of vaginal films, and HME has proven such a process.

2.6 ACKNOWLEDGMENT

The project was kindly supported by the Bill and Melinda Gates foundation and National Institute of Allergy and Infectious Diseases (NIAID) at the National Institute of Health through grant numbers OPP1110953 and U19 AI082639, respectively. I would like to acknowledge the following people: Phillip Graebing at the University of Pittsburgh for developing and validating the GRFT HPLC method; Dr. Brid Devlin of the International Partnership for Microbicides (IPM) for providing DPV drug substance; Dr. Kenneth Palmer at the University of Louisville School of Medicine for providing GRFT drug substance; Dr. Bernard Moncla at Magee-Womens Research Institute for performing the DPV-*Lactobacillus* compatibility testing; Dr. Charlene Dezzutti at Magee-Womens Research Institute for performing the DPV-TZB-bl cellular assay.

3.0 AIM 1 PART B: HOT MELT EXTRUSION AS AN ALTERNATIVE MANUFACTURING TECHNIQUE FOR TOPICAL VAGINAL FILM APPLICATION CONTAINING MICROBICIDE CANDIDATE DAPIVIRINE

3.1 INTRODUCTION

Human immunodeficiency virus (HIV), one of the world's most significant public health burdens, infects women disproportionately more than men in certain parts of the world such as sub Saharan Africa [96, 97]. Heterosexual intercourse is the major risk factor for HIV contraction in women. Except abstinence, the use of condoms is the only method for prevention of heterosexual transmission of HIV. Condoms require male compliance to achieve effectiveness [98, 99]. Therefore, an urgent need exists for a woman-controlled HIV prevention method. Pre-exposure prophylaxis (PrEP) is a prevention method for those at risk for HIV acquisition. PrEP can be used orally or topically. Topical application allows sufficient antiretroviral *(*ARV) activity at the site of action, which is required to prevent the HIV transmission and offers limited systemic side effects. Currently, several ARV based topical dosage microbicides, are under investigation [14].

Microbicides have advanced into clinical trials. One of the most clinically advanced microbicide drug candidates is dapivirine (DPV), a hydrophobic, non-nucleoside reverse transcriptase inhibitor (NNRTI). DPV inhibits reverse transcriptase, which is a crucial enzyme

for HIV replication. DPV has been formulated into several dosage forms, including the intravaginal ring (IVR), gel, and film for clinical trial testing. In February 2016, the New England Journal of Medicine published the results of a Phase III DPV IVR clinical trial conducted in Africa (MTN-020; ASPIRE). In summary, the results were promising, showing DPV efficacy and safety among female users that were adherent to the product. In fact, the IVR DPV ring is under licensure and NDA process. However, the overall incidence of HIV-1 infection remained high due to low user adherence of the product [100]. In a Phase I clinical trial (FAME-02 and FAME-02b), the DPV gel and a novel DPV film were found to be safe. Effectiveness was suggested through DPV tissue levels and ex vivo challenges studies. Biopsy samples collected from women after product (DPV gel or DPV film) administration were challenged ex-vivo with HIV-1_{Bal} and inhibition of HIV infection measured. Both the DPV gel and film delivered sufficient concentrations of DPV to block HIV-1_{Bal} infection *ex vivo*. In this study, women's preference for the film was attributed to the lack of leakage during and after film usage, which was a complaint from users of the DPV gel [39]. This observed film acceptability has also been demonstrated in several other studies [30-32, 39]. One of these studies, the PAS II study, which compared the film dosage form with a vaginal soft gel capsule, and vaginal tablet among African women, also reported the film as the preferable dosage form [30].

Quick dissolving polymeric thin films are a dosage form that offers several advantages over traditional vaginal dosage forms. Most polymeric thin films quickly dissolve, disintegrate, and release the active ingredient when in contact with a small amount of fluid, as found in the vagina. Vaginal films are small and do not require an applicator, making them portable, relatively easy to use, and inexpensive to manufacture [29]. The films are a low volume dosage form and therefore can reduce the potential cause of alteration or diluting of the important

protective innate factors of the vagina such as cervicovaginal secretions fluids and microflora. Furthermore, vaginal films allow accurate dose administration and can be used to stabilize drugs susceptible to degradation in the dosage form [14, 29, 30, 36].

The marketed vaginal films and the microbicide vaginal films that have been tested in clinical trials, to date, have been manufactured utilizing a solvent casting method (NTC01989663 and NTC02280109) [39, 101]. However, hot melt extrusion (HME) offers potential benefits for manufacturing of vaginal films since this technique requires fewer processing steps for efficient large scale manufacturing, continuous operation for maximum batch uniformity, and typically eliminates the need for use of solvent and subsequent drying steps [47].

HME can be utilized to make polymeric film formulations containing both hydrophobic and water sensitive active pharmaceutical ingredient (APIs) while, solvent cast technique can be challenging for formulations of hydrophobic and water sensitive APIs due to the presence of the water in the formulation. Furthermore, the associated solvent-related stability risks that can occur during the shelf life of the formulation are avoided. Additionally, the HME manufacturing process and the HME formulations can have an impact on the film's final product attributes. These attributes can impact film properties, drug release, and drug accumulation in target tissues. The objective of the present study was to evaluate HME as an alternative process for DPV vaginal film manufacturing. To evaluate this objective, HME and SC films were compared for physical characteristics and a direct comparison of drug-tissue accumulation was also conducted. HME process analysis was evaluated to better understand the robustness of the process and the effect of the process on film final attributes.

3.2 MATERIALS

Dapivirine used in this study was provided by International Partnership for Microbicides (IPM). PolyOx N10, N80 (Polyethylene oxide, MW, 100,000 and 200,000, respectively) and Polyethylene glycol 4000 were purchased from Dow Chemical Company (Midland, MI, USA). Klucel EF (Hydroxypropyl cellulose (HPC) MW, 100,000) was purchased from Ashland, Inc. (Bridgewater, NJ). Polyethylene glycol 400 and Vitamin E acetate was purchased from Spectrum (Gardena, CA, US). Cremophor EL was purchased from Sigma-Aldrich (St. Louis, MO). Ultrapure water was obtained from in-house MilliQ water purification system. Acetonitrile (ACN), trifluoroacetic acid (TFA), methanol, methyl-tert-butyl ether (MTBE) were obtained from Fisher Scientific (Pittsburgh, PA).

3.3 METHODS

3.3.1 Preparation of the Dapivirine Polymeric Films by HME

Polymeric thin film matrices were manufactured via HME technology utilizing a twin screw NANO 16 mm Leistritz extruder (Somerville, NJ). The formulation and extrusion conditions are summarized in table 3.1. All excipient powders were blended for 15 minutes using a bench top mixer to ensure content uniformity of the drug in the film unit dose. The extruder screws were set to 180 rpm. The extruded films were rolled and cut into 1x2 inches unit doses using a die cutter.

Table 3.1: Formulation Ingredients and Processing Conditions of Hot-Melt Extruded Films
(Refer to chapter 1, HME Process Description 1.3.2.2)

Component	Function
PolyOx N80	Film forming polymer
Hydroxypropyl cellulose	Film forming polymer
Polyethylene glycol 4000	Plasticizer
Polyethylene glycol 400	Plasticizer
Vitamin E acetate	Antioxidant
DPV	API
Barrel -Zone 1	125 °C
Barrel -Zone 2	130 °C
Barrel- Zone 3	135 °C
Barrel- Zone 4	135 °C
Die/melt temperature	140 °C
Screw speed	180 rpm

3.3.2 Development of an HPLC Stability Indicating Assay

Refer to chapter 2, method section 2.3.3.1- Titled: Hydrophobic Molecule: Dapivirine HPLC Detection Assay.

3.3.3 Drug Content in HME Films

Film samples were weighed and dissolved in 50% acetonitrile, followed by vortexing at 1000 rpm for 15 minutes, until the films were completely dissolved. These samples were then centrifuged at 10,000 rpm for 10 minutes. The supernatant was then filtered using a 0.22 µm PTFE filter to remove the polymer debris within sample. The DPV drug content present in the film was quantitated using HPLC as described above. The drug concentration of the film samples was calculated by measuring the peak area of the sample and comparing it to the peak area of the calibration curve with R^2=0.999.

3.3.4 *In Vitro* Drug Release

Refer to chapter 2, method section 2.3.4.5.1- Titled: Dapivirine, Metronidazole and Griffithsin Film Dissolution Assays.

3.3.5 Film Disintegration and Residual Water Content

The film disintegration evaluation was conducted by submerging a film in 3 mL milliQ water and mixing using an orbital shaker. Visual assessment was conducted to monitor disintegration, measured as the time until complete film structural loss. The residual water content of the films was measured using a Karl-Fisher titration apparatus (Metrohm, 758 KFD Titrino).

3.3.6 Puncture Strength

Refer to chapter 2, method section 2.3.4.2- Titled: Puncture Strength.

3.3.7 Differential Scanning Calorimetry (DSC) Studies

The crystalline properties of DPV after the extrusion process were tested using a Mettler Toledo DSC 1 STARe System, equipped with a GC 200 gas controller. Approximately 4-8 mg of each sample (pure drug, physical mixture, and extruded film) was weighed and placed in aluminum crucibles sealed with Mettler Toledo sealer. Analysis was conducted under a temperature range of 25 °C to 250 °C at a ramp rate of 10 °C per minute, using N_2 (50 mL/min) as the segment gas.

3.3.8 *In Vitro* Anti-HIV Activity and Cellular Toxicity

Refer to chapter 2, method section 2.3.5.2- Titled: Dapivirine Film: Bioactivity and *In vitro* Cellular Toxicity.

3.3.9 Compatibility with *Lactobacillus*

Refer to chapter 2, method section 2.3.5.1- Titled: Dapivirine Film: *Lactobacillus* Toxicity.

3.3.10 Stability Assessment

To test the physical stability of DPV HME films, films were placed on stability at 25 ºC/65% relative humidity (RH) for 24 months and at 40 ºC/75% RH for 6 months. The films were tested at predetermined time points. At each time point, mass, thickness, appearance, microscopy, water content, puncture strength, disintegration, drug content, and dissolution were tested. In addition, *Lactobacillus* compatibility and *in vitro* anti-HIV activity (TZM-bl cell based model) were tested at specific periods throughout the stability study. A JMP fit model was used for repeated measure analysis (in keeping with FDA guidelines).

3.3.11 *Ex vivo* Permeability and Toxicity Evaluation

3.3.11.1 Tissue Exposure Studies

Direct comparison between HME and SC film was conducted in order to understand if the HME process impacts the tissue accumulation of the DPV. The human ectocervical tissue was obtained

from the University of Pittsburgh Health Sciences Tissue Bank as per approved IRB protocol PRO09110431. Tissue samples were collected from healthy volunteers undergoing routine hysterectomy. The tissue was prepared by removal of excess stroma by a longitudinal slice through the specimen using a Thomas-Stadie Riggs tissue slicer. The thickness of the tissue was measured by a digital caliper. A section of the tissue was collected for histological evaluation to represent the pre exposure state of the tissue. The tissue exposure studies were performed using an in-line- flow-through cell system. Epithelial tissue was placed within the flow-through system, with the epithelial layer faced towards the donor compartment. A 6 mm biopsy punch of DPV HME film or DPV solvent-casted film was dissolved in 450 µL of vaginal fluid stimulant (VFS) and placed in the donor compartment, on top of the tissue. The receptor medium, Dulbecco's Modified Eagle Medium (DMEM) was used for the receptor compartment, at a flow rate of 50 µL/min. The excised human ectocervical tissue was exposed to each film solution for 6 hours while the fraction collector (Gilson) collected receptor samples. In order to maintain biological temperature, the system was kept at 37 °C. At the end of the 6 hour period of tissue to film exposure, the tissue was collected and cut into halves: one half was used for histological evaluation and the other half was used for the determination of DPV concentration by liquid chromatography-mass spectrometry (LC-MS).

3.3.11.2 Tissue Processing and Staining

All tissue (pre and post experiment) were individually placed in embedding cassettes and treated with formalin for 12-24 hours. Following formalin treatment, tissue cassettes were kept in 70% ethanol for at least 24 hours prior to processing. The tissue cassettes were then incubated in 95% ethanol for 1 hour, 100% ethanol for 1 hour (3 times), xylene for 1 hour (2 times), and paraffin for 3 hours. Using the embedding station (Lecia EG 1160), the tissue cassettes were

embedded into paraffin blocks. Tissue blocks were sectioned at a thickness of 5µm using a microtome (Olympus CUT 4060) and placed onto glass slides for Hematoxylin and Eosin (H&E) staining. Morphological changes were evaluated using a microscope (Zeiss Axioskop 40) and imaged.

3.3.11.3 Tissue Extraction and UHPLC- Mass Spectrometer Analysis

After the permeability assay, tissues were homogenized (Precellys 24 Homogenizer). The homogenized tissue was then subjected to liquid extraction. DPV was extracted from the homogenized tissue using acetonitrile, methanol, methyl-tert-butyl ether (MTBE), 25% ammonium hydroxide solution (NH_4OH) solution, 0.9% sodium chloride (NaCl) solution, and MilliQ water (H_2O). After liquid extraction samples were dried under nitrogen (N_2), they were reconstituted in an injection solvent containing (7:3 acetonitrile to water) for analysis. After sample preparation, samples were injected into a Waters Acquity ultra-high performance liquid chromatography (LC-MS) system connected to a Thermo Quantum Access Advantage MAX triple quad mass spectrometer (with electric spray ionization source) for analysis. The method utilized a Phenomenex Hyperclone 3u BSD C8 150x4.6 mm column. The mobile phases consisted of (A) 5 mM ammonium formate (NH_4FA) buffer: ACN (40:60) and (B) 5 mM NH_4FA buffer: ACN (20:80) with a 1 mL/min flow rate. The range for the standard curve was 0.2-50 ng/mL and was determined to be linear [36].

3.3.12 HME Process Evaluation

A *Quality by Design (QbD)* study to evaluate the impact of the HME process parameters on the final film product and to assess the HME process reproducibility was conducted.

Response surface methodology, specifically a Box Behnken Design (BBD) was applied. In this study, multiple center points were used to detect the curvature of factor effects and estimate pure error (refer to table 3.2). The three center points (run 8,14,15) were processed using three separate lots of raw materials. This design incorporated each factor (X_1, X_2 and X_3) at three evenly spaced levels (-1, 0 and +1). The three different levels (low, medium, and high) were tested for barrel temperature, screw speed, and feeding rate. The full model for each response (Y) was represented by the following equation:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2$$

The schematic design and the design matrix utilized in this study are represented in Figure 3.1 and table 3.2.

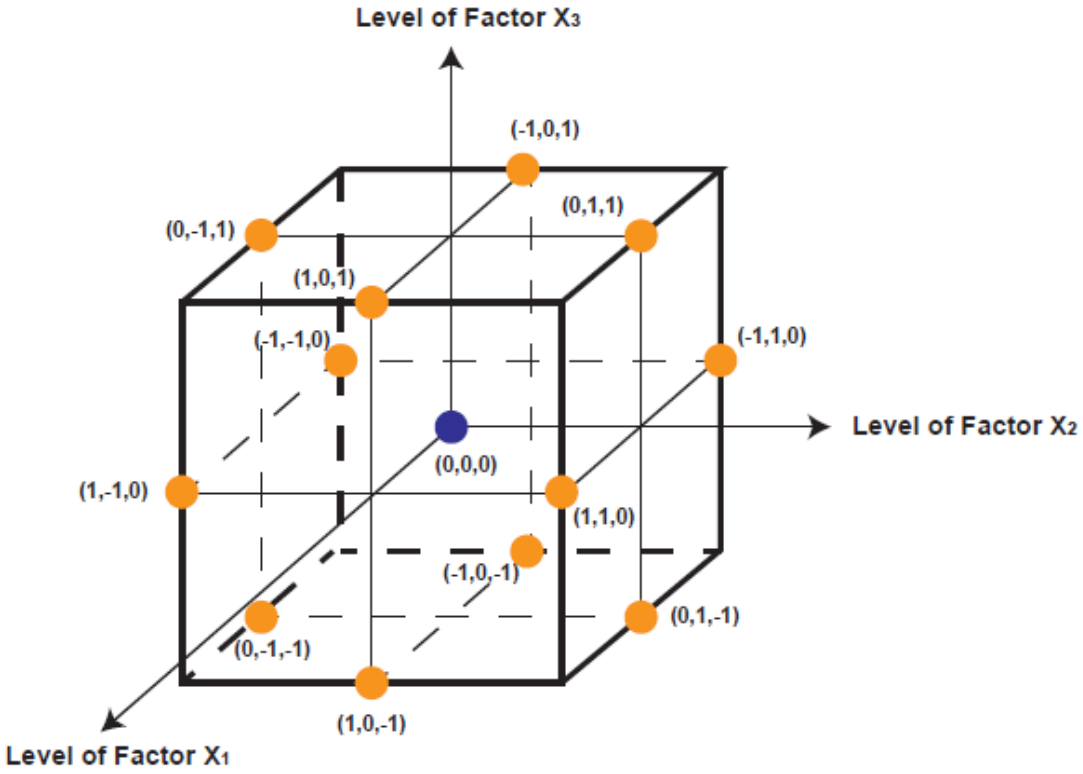

Figure 3.1: Schematic Diagram for a Three Factor Box-Behnken Design Space.
(Drawn by Guy Regev, industrial designer)

Table 3.2: Summary of the Study Design Based on Box-Behnken Design (BBD) Response Surface Methodology

Runs 1-13 were made with the same master mix. Run 14 was made with alternate lot of key excipient(s) but the same formulation. Run 15 was made with same lots of materials as A1 but mixed separately.

Run Order	Barrel Temp (zone1) (°C)	Screw Speed (rpm)	Feed Rate (g/h)	Excipient Lot
1	105	150	250	A1
2	105	210	250	A1
3	105	180	230	A1
4	105	180	270	A1
5	125	150	230	A1
6	125	210	230	A1
7	125	150	270	A1
8	125	180	250	A1
9	125	210	270	A1
10	145	150	250	A1
11	145	210	250	A1
12	145	180	230	A1
13	145	180	270	A1
14	125	180	250	B1
15	125	180	250	A2

3.3.13 Statistical Analysis

All results are reported as mean ± standard deviation (SD). The comparisons between HME and SC films data was evaluated using the Student's *t*-test. The stability data was analyzed using a two-way ANOVA and the drug tissue concentrations were analyzed using one-way analysis of variance. A p value ≤ 0.05 indicates statistical significance. Statistical analyses were conducted using GraphPad Prism software (V6.07) and JMP pro 13.

3.4 RESULTS

3.4.1 Film Characteristics

HME DPV film characterizations began with visual determination of the film as soft, flexible, and translucent. The average mass and thickness were 147.3 ± 10.1 mg and 0.15 ± 0.01 mm, respectively. The target drug content (1.25 mg/film) was selected based on the clinically advanced DPV vaginal gel formulation, which was reported to be safe and well tolerated (IPM003) [39, 102, 103]. During HME film development, the film thickness was adjusted to meet the target dose in 1"x 2" film. DPV drug content was found to be uniformly distributed (relative standard deviation < 4%), with total drug content being 1.3 mg/film in 1"x 2" film. The residual water in the film was found to be approximately 2%, lower in comparison to the solvent casted film (4.5%). To evaluate the mechanical properties of the film, puncture strength was tested. The puncture strength of HME films (3.4 kg/mm) was similar to that of SC (3.6 kg/mm); however, disintegration time of the HME film was significantly faster (HME film: 0.78 minutes), with more efficient drug release observed in *in vitro* studies (>90% within 20 minutes) (see table 3.3). To determine crystallinity or amorphous state of DPV post extrusion, DSC analysis and microscopy images were utilized (See Figure 3.2 and 3.3). Thermal analysis using DSC showed the thermal behavior of DPV before and after the extrusion process**.** A polymorphic transition of DPV drug substance was detected at 105°C and the melting point at 220°C (Figure 3.2). No thermal endotherm peak was observed for DPV after the extrusion process. Microscope images confirm no crystals formed in the film post extrusion process (Figure 3.3).

Table 3.3: Comparison of DPV HME Film with DPV SC Film

	DPV HME Film	DPV SC Film	p- value
Weight (mg)	147.1 ± 10.09*	92.7 ± 4.93	0.0011
Thickness (µm)	0.158 ± 0.01*	0.073 ± 0.005	0.0002
Drug Content (mg/film)	1.31 ± 0.05	1.31 ± 0.03	0.9999
Puncturestrength (kg/mm)	3.43 ± 0.47	3.58 ± 0.50	0.7242
Water content % (w/w)	2.4 ± 0.08*	4.5 ± 0.17	0.0001
Disintegration time (min)	0.78 ± 0.15*	6.33 ± 0.577	0.0001
Bioactivity	2.0 nM	2.0 nM	p- value

* A p value ≤ 0.05 was considered statistically significant. In terms of film performance, student t-test showed significant faster disintegration time (min) for the HME film ($p \leq 0.0001$).

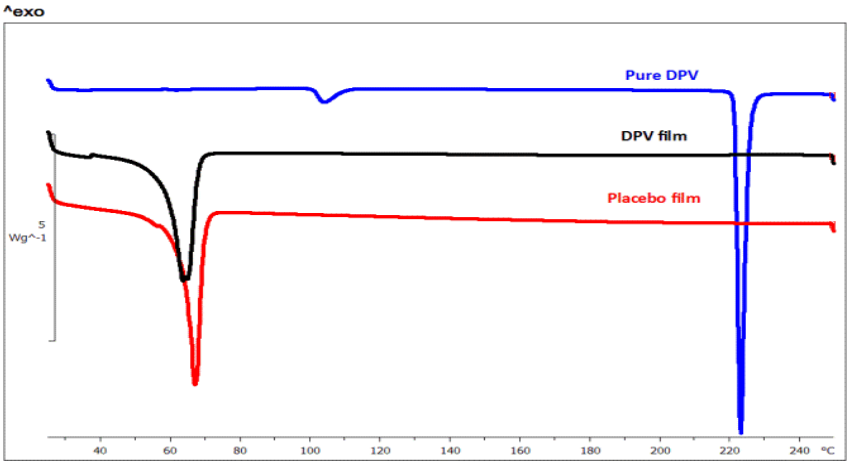

Figure 3.2: Thermal Analysis Evaluation for DPV Before and After the Extrusion Process.

Thermal analysis of DPV drug substance (blue), DPV HME film (black), and HME placebo film (red) were evaluated. No endothermic peak was observed in the DPV HME film analysis compared to the DPV drug substance. As expected, no thermal peak of DPV observed in the placebo film. Polymeric melting point was observed at 65°C, corresponds to PEO thermal behavior.

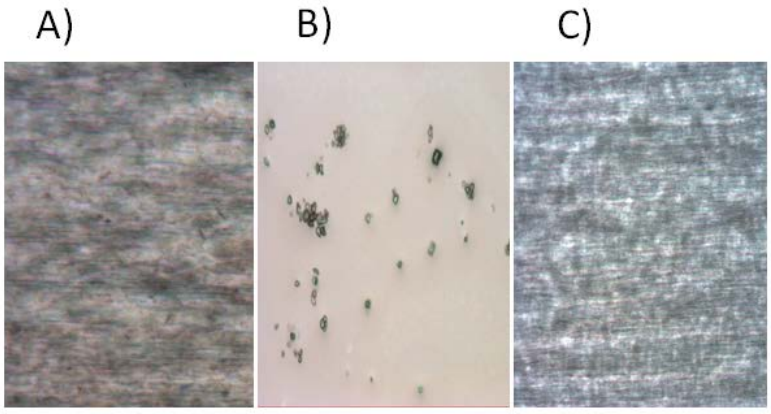

Figure 3.3: Microscopic Images for Crystals Detection in the Extruded Film.

No DPV crystals were detected in the HME film. A) HME DPV film B) Pure DPV-crystals C) Placebo HME film.

3.4.2 *In Vitro* Cellular anti-HIV Dapivirine Film Activity and Toxicity

To ensure that the extrusion process and film formulation did not affect the anti-HIV activity of DPV, bioactivity was evaluated using a TZM-bl cellular assay. The IC_{50} of the DPV film was found to be similar to the DPV drug substance of 2 nM (see Figure 3.4). Furthermore, *in vitro* cellular assay showed no cellular toxicity of the HME film, placebo film, and DPV drug substance.

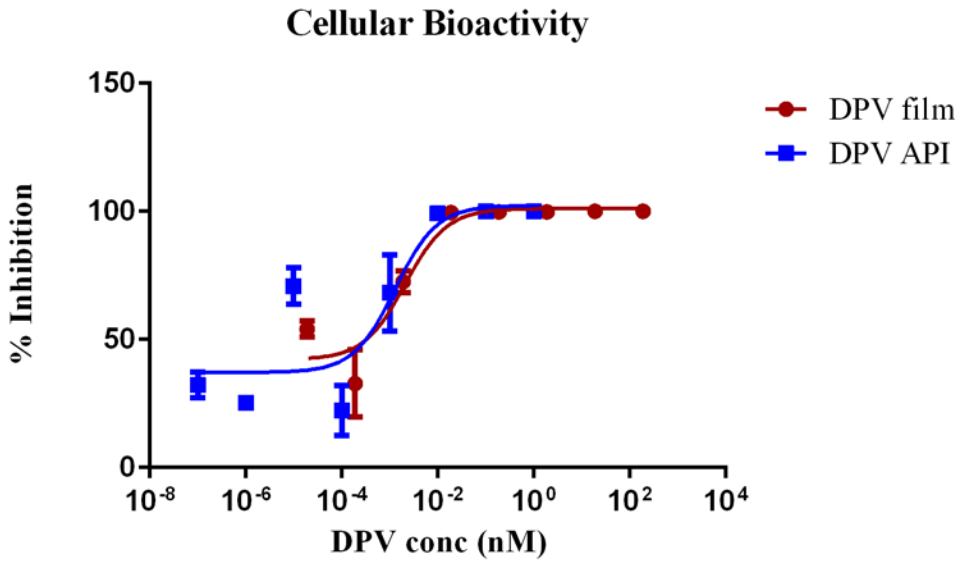

Figure 3.4: *In Vitro* Assessment of DPV Films and DPV Drug Substance in a TZM-bl Cellular Model.

DPV HME film maintained similar IC_{50} to the DPV drug substance after the formulation and extrusion process.

3.4.3 Compatibility with *Lactobacillus*

Compatibility of the film and *Lactobacillus* was assessed, as *Lactobacilli* play a crucial role in the vaginal innate defense mechanism against harmful pathogens, including HIV, by maintaining

a low pH and producing hydrogen peroxide. Therefore, it is important to evaluate the compatibility of the DPV film with the *Lactobacillus* using the Standard Microbicide Safety Test (SMST). The compatibility of the *Lactobacillus* was measured as log difference in bacterial viability before and after exposure to the film product. Small decreases in bacterial colonies' forming unit were observed as shown in table 3.3. However, a decrease in log value must take place to be considered a significant loss of bacteria and film toxicity to the *Lactobacillus*. Therefore, it can be stated that no significant loss of bacterial viability was observed after 30 minutes of incubation with the film.

Table 3.4: Compatibility of the DPV Extruded Film with *L. jensenii and L.crispatus*
Negative values indicate a loss of viability. The loss of viability must be < 1 log10 to meet safety standards.

Lactobacillus strain tested	Dapivirine Film
L. jensenii LBP 28AB	-0.169
L. jensenii ATCC 25258	-0.032
L. crispatus ATCC 33197	-0.127

3.4.4 Stability Assessment

The prototype film was placed on 24 months stability studies were in to two conditions, 25 °C/ 60%RH and 40 °C/ 75% RH. To date, 12 months stability evaluations have been conducted, and the film was found to be stable at both conditions. Based on the FDA guidance's, two-way ANOVA was conducted. Time was defined as a continuous variable and condition as a nominal variable. Figure 3.5-3.8 depicts film weight, drug content, dissolution, and puncture strength obtained from the stability study at the predetermined time points. In general, no significant change in film characterizations (e.g. film weight, thickness, Ps) and drug content occurred over time, when comparing the different time points to time zero. The water content of the film

remained unchanged and below 2%. Furthermore, DPV anti-HIV activity in the HME film was maintained throughout the stability study at both conditions. Additionally, no cellular toxicity was observed at any time point, confirmed by the TZM-bl cellular assay. Finally, no loss of *Lactobacillus* viability was observed over the course of the stability study utilizing the SMST.

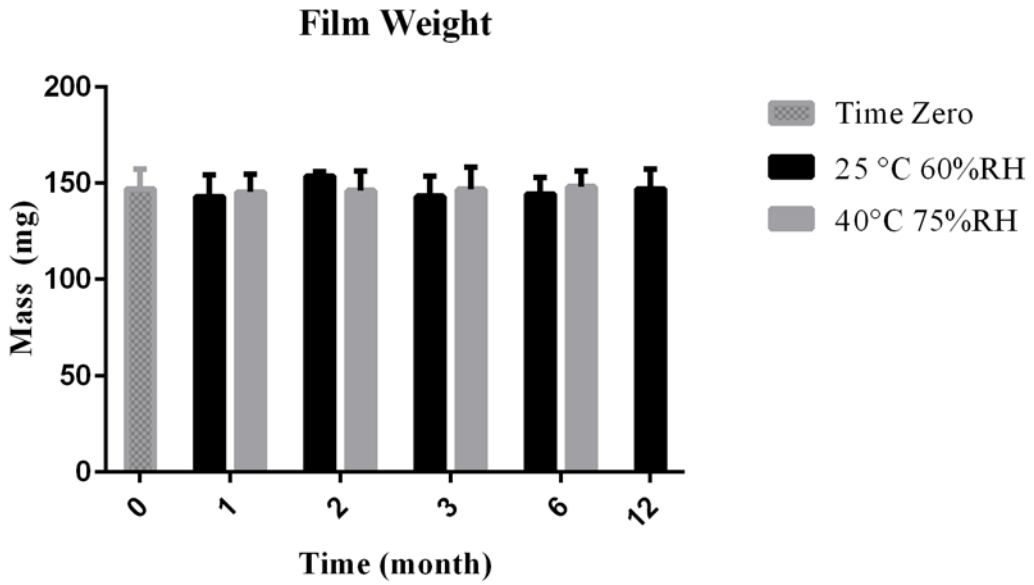

Figure 3.5: DPV Film Weight Over the Course of the Stability Study.
No changes were observed in the film mass over time determined by measuring film weight during predetermined time point. No effect of time (F=0.8107), condition (F=0.8527), or time*condition interaction (F=0.7757) were observed.
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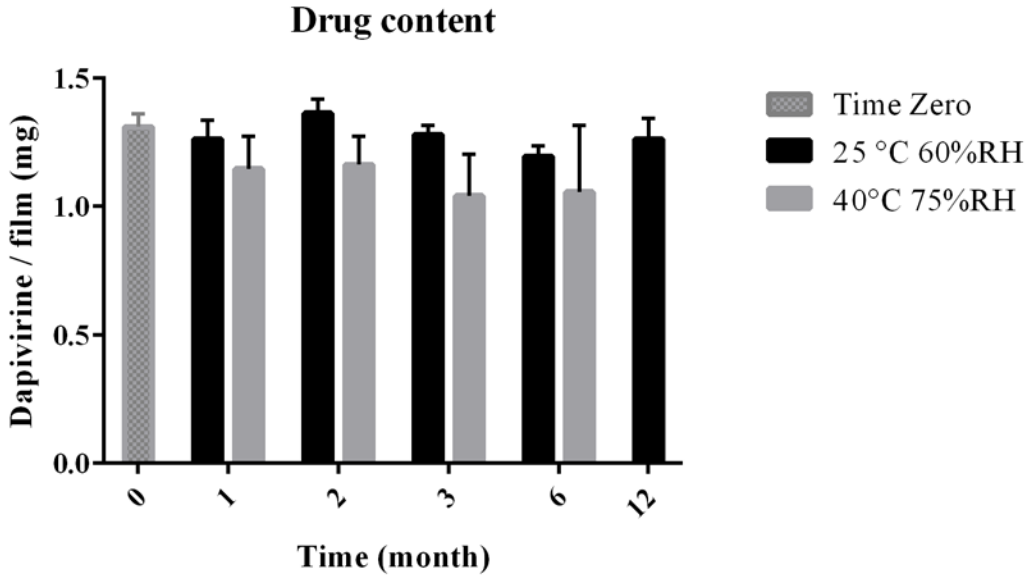

Figure 3.6: Drug Content Results of DPV Film Monitored During the Course of the Stability Study.

No changes were observed in the DPV drug content over time determined by HPLC during the stability study. No effect of time (F=0.0790), condition (F=0.0241), or time*condition interaction (F=0.7151) were observed.

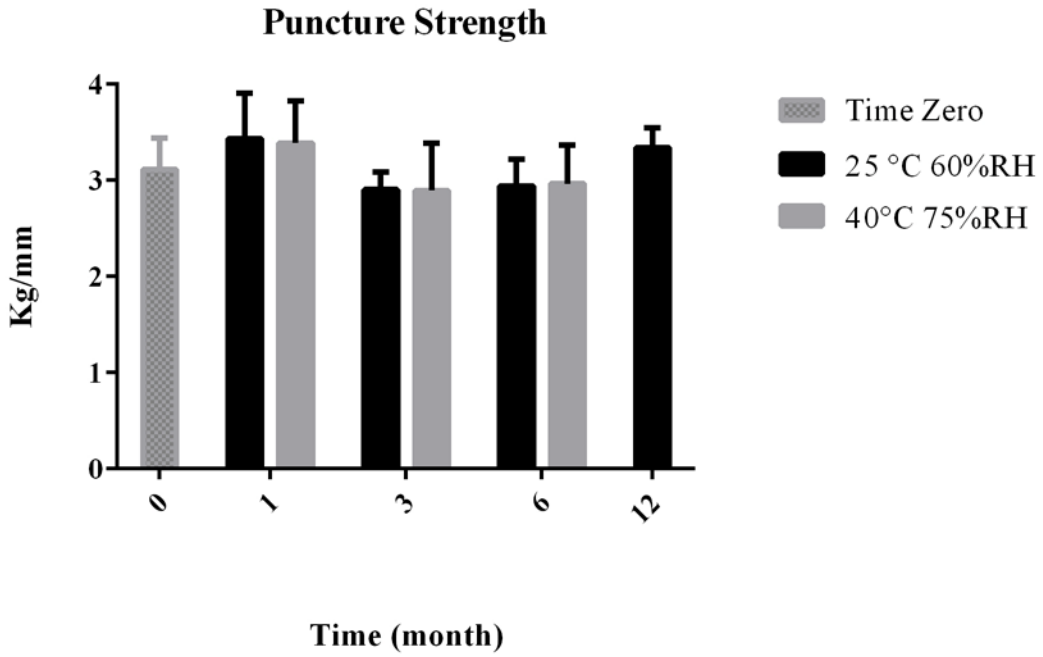

Figure 3.7: Puncture Strength of DPV Film Monitored During the Course of the Stability Study.

No changes were observed in the puncture strength of DPV film over time. No effect of time (F=0.3144), Ps (F=0.7125), or time*Ps interaction (F=0.2157) were observed.

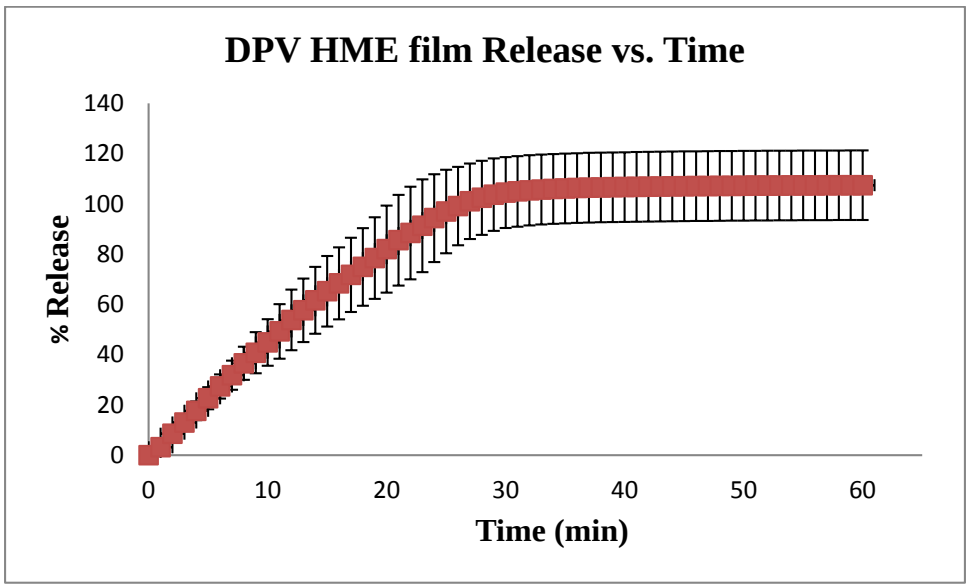

Figure 3.8: *In Vitro* Drug Release of DPV Monitored Using the SOTAX Dissolution Apparatus.

Over 90% of the DPV was released within the first 20 minutes of the study.

3.4.5 *Ex Vivo* Permeability and Toxicity Evaluation

3.4.5.1 Dapivirine Exposure Evaluation in Human Excited Cervical Tissue

Excised human ectocervical tissue was exposed to DPV HME vaginal film and DPV SC vaginal film for 6 hours. The DPV drug was extracted from the tissue and analyzed using LC-MS. The objective of this experiment was to evaluate if the formulation and the manufacturing process impacted the penetration of DPV into the tissue after exposure to the film. The results confirmed that DPV was present in the tissue after the exposure to both HME and SC film (table 3.4). In addition, evaluations were conducted to determine the safety of DPV HME film to the epithelial layer of the vaginal canal. H&E stained tissue pre- and post-exposure are presented in Figure 3.9. The epithelial layer of the tissue was found to be intact and maintained structural integrity. No morphologic changes were detected after the exposure of the HME film and the SC film to the tissue, when compared to the pre-exposed tissue. This suggests that the film did not change the epithelial layer of the vaginal canal.

Table 3.5: DPV Amount and Concentration in the Human Excited Tissue After Six Hours Exposure

	DPV HME Film	DPV SC Film
Amount found in the tissue (μg/g)	5.818 ± 2.717	1.489 ± 0.774

t-test, P=0.0567, marginal significant) However, no statistical significant difference was found. DPV accumulation in tissue treated with HME film, showed strong trend of accumulation. ($p \leq 0.05$ to be considered significant, n=3

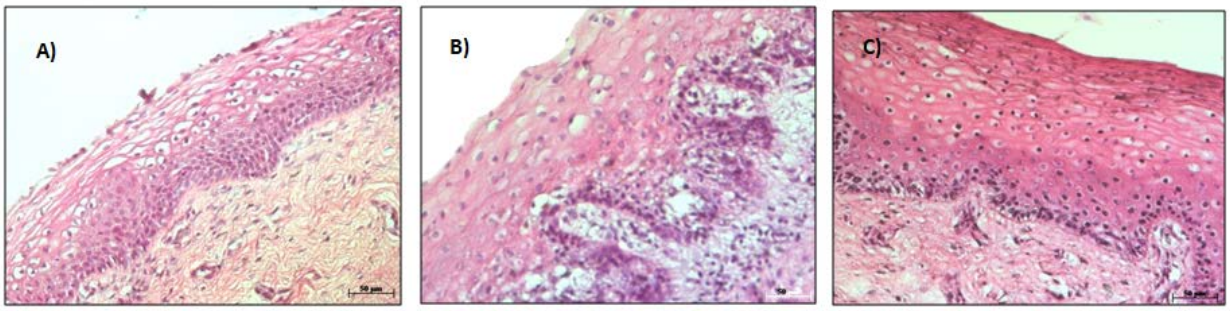

Figure 3.9: Representative Images of H&E Staining of Epithelium Pre and Post Exposure to the SC and HME Films.

H&E tissue staining was utilized for this study. Hematoxylin was used to stain the nuclei of the cells in purple and the rest of the cell structures are colored red by the eosin stain. No significant changes in the tissue morphology were observed post exposure to the films compared to the pre- exposed tissue. Tissue images of A) pre exposed tissue to the film treatment, B) tissue exposed to SC DPV film for 6 hours and C) tissue exposed to HME DPV film for 6 hours.

3.4.6 HME Process Evaluation

HME process analysis was conducted to evaluate the process robustness and the impact of the HME parameters on vaginal film manufacturing. In this work, the center points were evaluated using 3 separate lots of raw materials and were compared (0,0,0). The center points were found comparable at all response aspects with RSD <15 % (see table 3.5). Additionally, evaluation of the three independent variables: screw speed, barrel temperature, and feeding rate, demonstrated that only screw speed had a significant effect on the film attributes including the drug content (mg DPV/ mg film) of the film, film weight (mg), film thickness (mm), puncture strength (kg/mm), and dissolution rate (mg/√t). Two-way ANOVA for analysis demonstrated that the drug content (mg DPV/ mg film) reduced significantly ($p= 0.0075$) at high screw speed (1-speed) compared to other screw speed (-1, 0 speed) conditions (see Figure 3.10). For the film weight analysis, as screw speed decreased, a non-linear increase in weight was observed ($p<0.0001$) (see Figure 3.11). Thickness was found to be much greater for low screw speed ($p<0.0001$) (see Figure 3.12) and the puncture strength was statistically lower at the low screw speed ($p=0.0065$)

(see Figure 3.13). Interestingly, the dissolution rate was found to be the lowest at the target screw speed and greater at the extremes of screw speed (p=0.001) (see Figure 3.14). For this assessment the screw speed was assigned to be the independent variable and the film attribute as the dependent variable.

Table 3.6: Summary of the Center Points Values.
Values found to be homogeneous (RSD < 15 %).

Factor 1 A:Screw Speed	Factor 2 B:Barrel Temp	Factor 3 C:Feed Rate	Response 1 Drug Content %	Response 2 Weight mg	Response 3 Dissolution mg/sqrt(t)	Response 4 Puncture Strength Kg/mg	Response 5 Thickness mm
0	0	0	0.00732	309.6	0.366	3.86	0.232
0	0	0	0.00908	277.4	0.275	3.59	0.203
0	0	0	0.00946	332.0	0.331	3.43	0.262
		Average	0.00862	306.3	0.324	3.63	0.232
		Std. Dev.	0.00114	27.5	0.0459	0.219	0.0292
		% RSD	13%	9%	14%	6%	13%

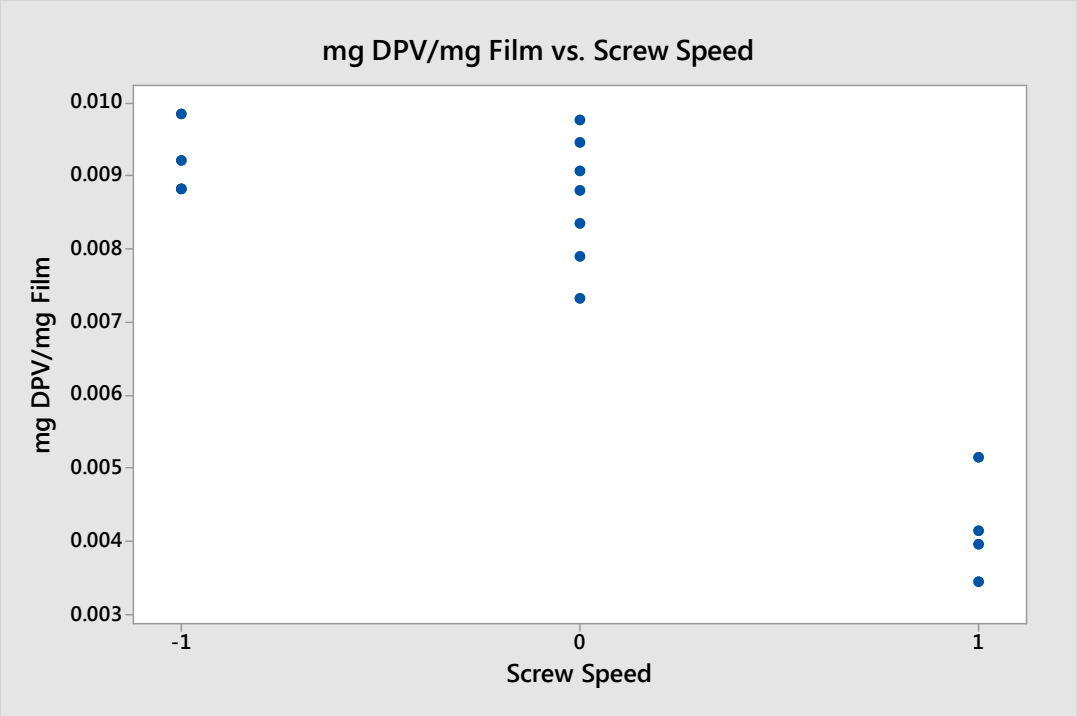

Figure 3.10: Scatterplot of Drug Content Assay vs. Screw Speed.
The higher the screw speed (1) the lower the drug content in the film was found.

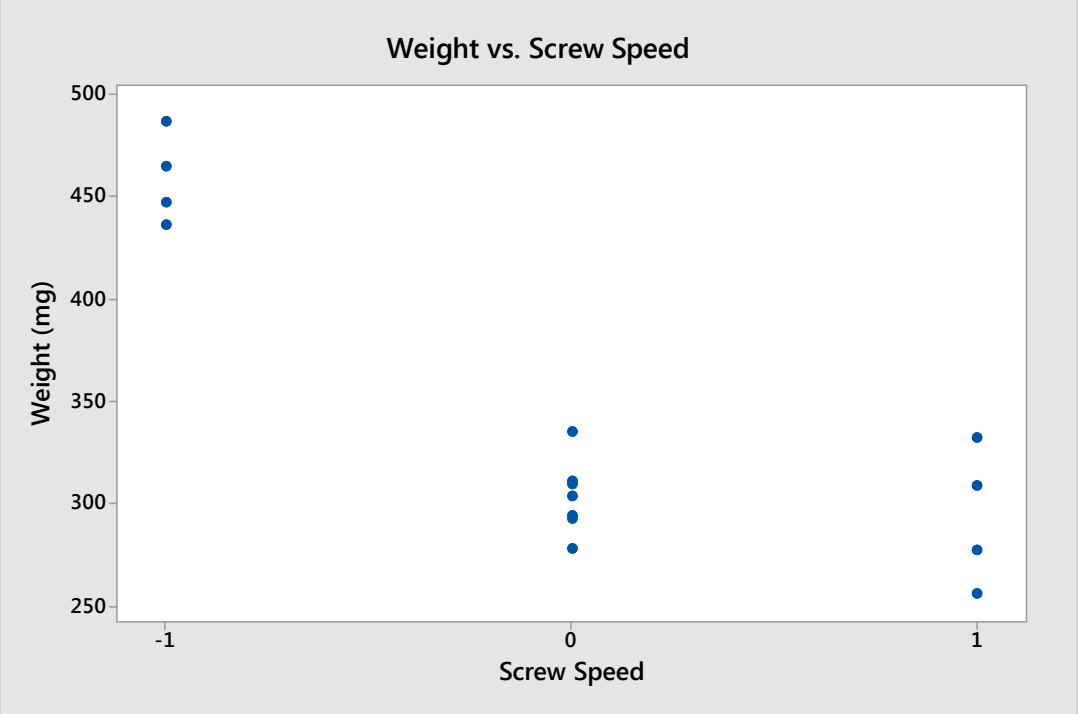

Figure 3.11: Scatterplot of the Film Weight Assay vs. Screw Speed.
The screw speed had a significant effect on the film weight. A non-linear decrease in film weight was observed as increase screw speed was tested.

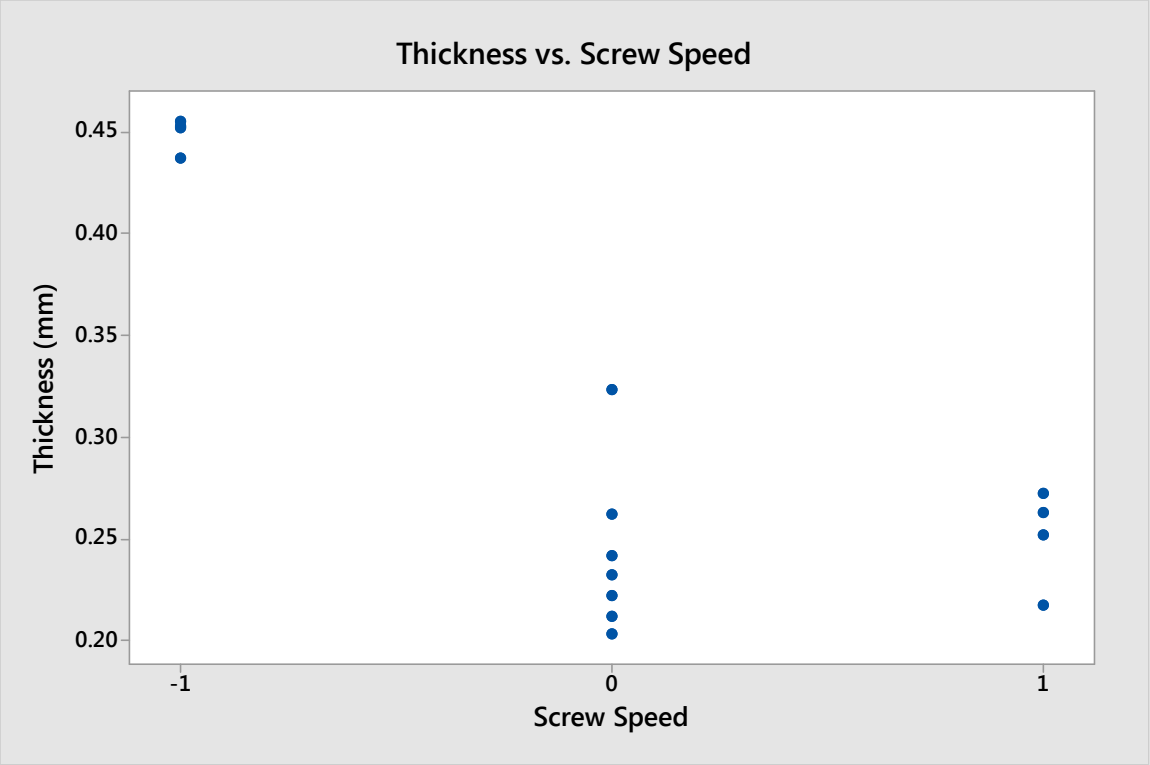

Figure 3.12: Scatterplot of the Film Thickness Assay vs. Screw Speed.

The screw speed had a significant effect on the film thickness, the lower screw speed resultant in thicker the films

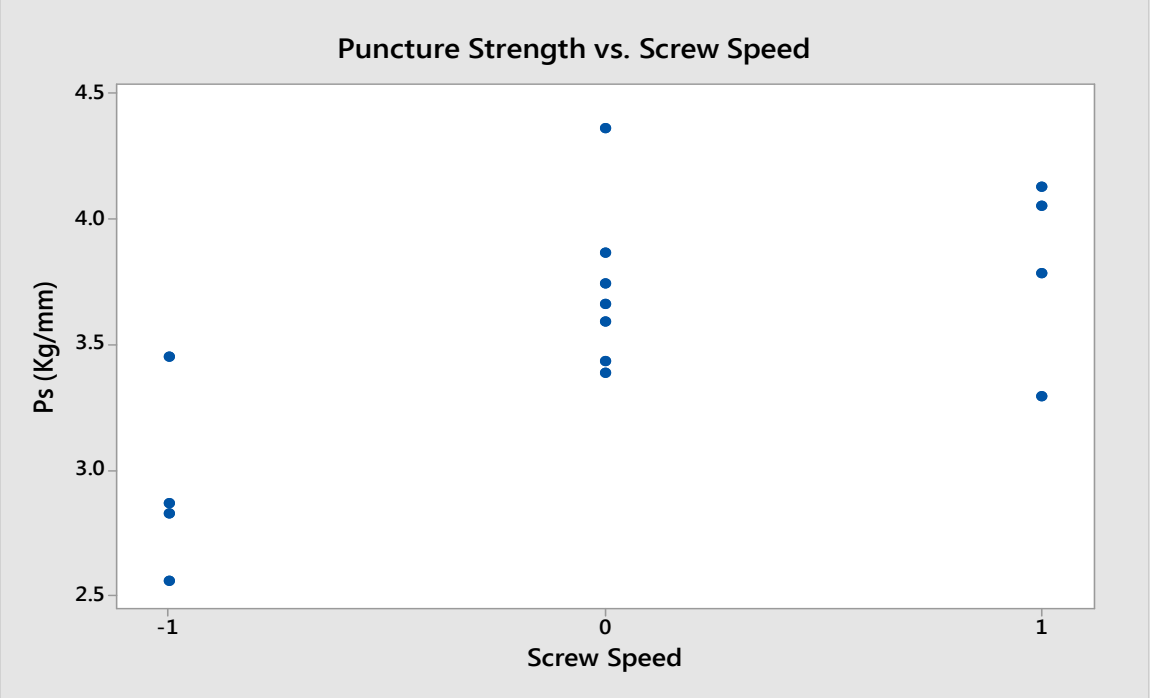

Figure 3.13: Scatterplot of Puncture Strength Assay vs. Screw Speed.

At the low screw speed the low puncture strength was observed.

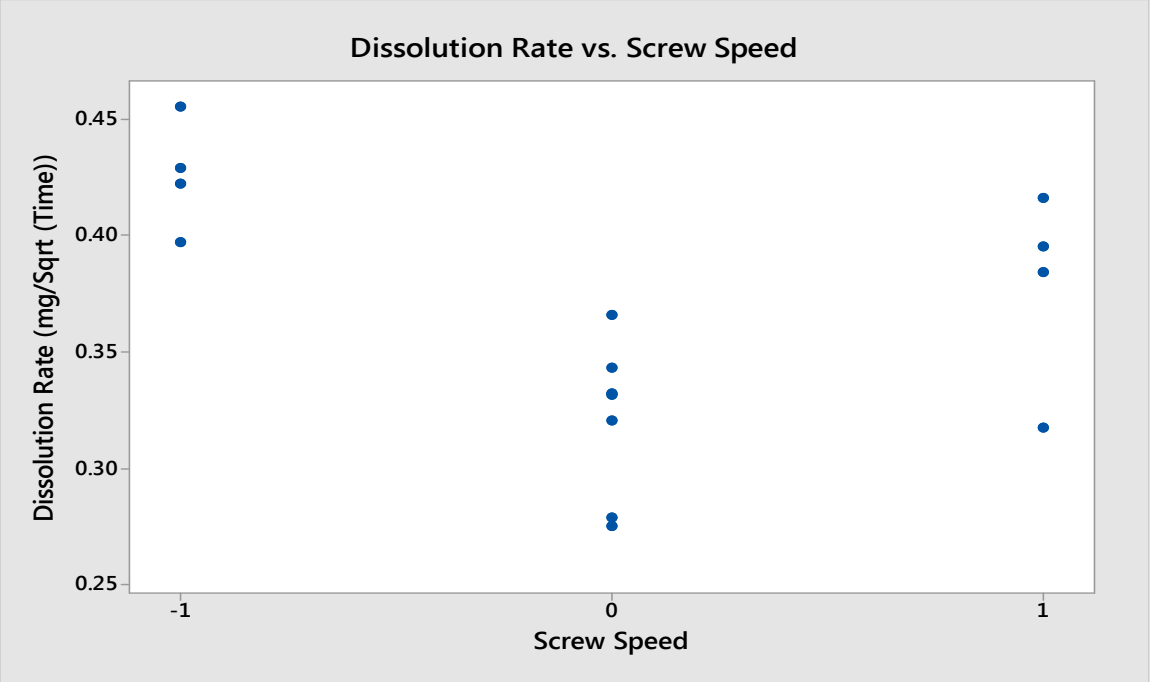

Figure 3.14: Scatterplot of Dissolution Rate vs. Screw Speed.

The dissolution rate observed at the target screw speed was the slowest compared to the lowest and the highest screw speed.

3.5 DISCUSSION AND CONCLUSIONS

The vaginal film has the potential for vaginal administration of microbicide drug candidates, including DPV, a potent HIV-1 nonnucleoside reverse transcriptase inhibitor (NNRTI). Our lab demonstrated the feasibility of vaginal film as a drug delivery strategy for DPV to the vagina [36]. The DPV vaginal film has been determined safe, acceptable, and capable of conferring adequate protection in a Phase I clinical study [39]. However, the previously investigated DPV films were manufactured using the SC technique, as are the two currently marketed pharmacological vaginal film products. As an alternative manufacturing technique, HME can provide numerous benefits over SC during film manufacture. Advantages include fewer

processing steps for efficient large batch manufacturing, continuous operation to allow maximum uniformity, and elimination of solvent in the manufacturing process. HME could potentially lead to lower manufacturing costs due to its simplicity, minimal processing steps, and widespread availability, which in turn, could lead to local production based on market needs [55, 56]. This study represents the first time that the feasibility of HME for the manufacture of a vaginal microbicide film containing a clinically advanced anti-HIV drug candidate, DPV, has been explored.

Film-forming polymers polyethylene oxide (PEO) and hydroxypropyl cellulose (HPC) were selected for the development of an HME DPV vaginal film. These film forming polymers are desirable for this project since they are both approved for pharmaceutical and food use and have a low soften temperature (Tm) (PEO Tm =65 °C, HPC Tm = 135-140 °C) which allows HME manufacture at relativly low process temperatures while maintaining the final soft extrudate. The HME manufacture should take place when the polymer is in the molten state. This indicates when the process temperature is maintained above the melting temperature of the polymer. The other main excipients used in the final prototype formulation were the plasticizers and antioxidant. Plasticizers enhance film flexibility and antioxidants improve polymer stability under high heat and shear produced by the HME process. The final DPV films were off-white, translucent, flexible, and smooth. The loading dose of DPV was selected based on the currently clinical advanced DPV vaginal gel formulations (IPM003) [39, 102, 103]. In the DPV vaginal gel studies, the vaginal DPV dose increased up to 0.05% in every 2.5 mL product that was applied, and the maximum amount of DPV that was delivered was 1.25mg. Considering this, 1.25 mg of DPV was the selected dose for the HME films. However, it is important to note the HME can be used to produce film containing larger amounts of API per film.

After film prototype manufacturing and processing, the film's physicochemical properties, bioactivity, and toxicity were investigated. Film drug content was found to be uniformly distributed in the 1''x 2''film, at a concentration of 1.3 mg/film. The drug level in the HME film was found to be similar to the level reported for the DPV in the solvent cast film [36, 39]. Rapid release of DPV from the film was confirmed by the rapid dissolution profile, which was also similar to the solvent cast film. However, the HME film disintegration rate was significantly faster than the solvent cast film. The *in vitro* dissolution assay showed that over 90% of DPV was released from the film within 20 minutes of the study. A nonionic surfactant, 1% cremophor, was used for this study as the dissolution medium to maintain the sink condition of hydrophobic DPV. The water content in HME film found to be 2.0%, which is lower than the solvent cast DPV film (4.5%), but expected because HME formulations do not contain water. It is important to maintain relatively low levels (2%) of water in the film in order to maintain film stability over time. In addition, the small amount of water present in the film helped maintain film flexibility. Finally, the puncture strength of the HME films (3.4 kg/mm) was also similar to that of SC.

The anti-HIV activity of DPV was tested using the TZM-bl cell based assay. In this model, the IC_{50} of HME film was found to be similar to that of the DPV drug substance and to the solvent cast film reported in the literature (IC_{50}=2.0 nM). DPV is an NNRTI that inhibits HIV reverse transcriptase within the target cell. Using the same cell based assay, DPV HME film was tested for toxicity and found to be safe since greater than 80% of cells remained viable throughout the assay.

The films were stored at 25 °C/60 % RH and 40 °C/75 % RH for 12 months. Stability studies are important to determine any effect on the final product efficacy and integrity caused

by physical, chemical, biological and microbiological changes of the final film product. Based on stability studies, the shelf life and suggested storage conditions of the product can be established while maintaining the safety and efficacy of the product. The stability evaluation demonstrated that the DPV film remained stable throughout predetermined time points. No significant changes were detected for drug content, bioactivity, compatibility with *Lactobacillus*, water content, puncture strength, mass and thickness of the film, and film appearance. This suggests that the formulation and the HME process can be used for the development of a stable delivery platform for DPV.

No crystals were detected in the DPV HME film and therefore it was determined to be in the amorphous state while the DPV in the SC film is suspended in the film in the crystalline state. The crystalline form has strong bonds at the molecular level thus more stable than the amorphous form. As a result, the crystalline form requires more energy to break and dissolve after film administration. Additionally, the DPV HME film had significant faster disintegration time (min) compared to the solvent cast film (t-test, $p< 0.0001$).

Since anti-HIV activity must take place in the target cells that are located in the subepithelial tissue, a DPV film exposure study took place to evaluate permeability of DPV through the tissue. In this study, DPV HME film and DPV SC film were investigated using human excised ectocervical tissue utilizing an in-line- flow-through cell system. DPV permeates the tissue via passive diffusion, therefore faster disintegration can result in higher DPV concentration that can lead to increase DPV accumulation in the tissue [104]. Based on the chemical (amorphous) state of the drug and the faster disintegration time it was predicted that the DPV from the HME film would have a greater accumulation of DPV in the tissue. Results have shown no significant difference of DPV accumulation between HME and SC film (t-test,

p=0.0567, marginal significance). Although no statistical significant difference was found, the accumulated DPV in the tissue treated with HME DPV film showed a strong trend to be larger. This suggests that the HME process of DPV film had slight effect on the permeability properties of DPV. These studies are important as they demonstrate the ability of DPV to permeate into the target tissue after the HME process. The DPV presence in the tissue post-release from the HME film is crucial to inhibit reverse transcriptase, the crucial enzyme in HIV replication.

Vaginal product-related toxicity can enhance HIV transmission through damaged epithelia. Therefore, it is important that a vaginal product is safe for the natural vaginal environment, especially for pre-exposure prophylaxis products, as their use prevents sexual transmission diseases at the site of infection. Safety evaluations were crucial to this study, because this is the first HME manufactured DPV vaginal film that has been developed. DPV HME film was found to be compatible with *Lactobacillus*, common beneficial bacteria present in the microflora. The selected strains were *L. jensenii and L. crispatus*, which play an important role in defense mechanisms against harmful pathogens including HIV. Additionally, the DPV HME film was tested for cellular toxicity and found to be safe using TZM-bl cellular assay. Lastly, the safety evaluation of DPV HME film using human excised ectocervical tissue was conducted. No morphologic changes were observed after the exposure of the tissue to the HME film when compared to pre-exposure tissue. HME film was composed of PEO, HPC, PEG and vitamin E, which are known to have low cytotoxicity and are commonly used in delivery systems. Together, these assessments provide evidence for the safety of the DPV HME film to the vaginal microenvironment.

Since this was the first evaluation of the HME for vaginal film manufacturing, it was important to establish reproducibility and consistency of this process. A Box-Behnken design

with three factors was used in this evaluation. All the design points used for this study were edge points of a cube which form a sphere and the center of the design space. The vertices of the cube were not evaluated since they represent the extreme conditions of the HME process and are not feasible or safe for the study. The Box-Behnken design was selected for this analysis. It is ideal for evaluating the robustness of a process with no expected deviations; where all factors operate at their extremes simultaneously. Once the films were manufactured under the experimental conditions, they were evaluated and compared to the DPV optimized HME formulation at target conditions of 0,0,0. This study allowed evaluation and confirmation of the ability to meet reproducibility and to establish consistency of the process. It demonstrated no significant changes from batch to batch (at target condition and formulation), which established the robustness of the HME for vaginal film manufacturing. Additionally, in the numerous conditions studied only screw speed had a significant effect on the film attributes (drug content, weight, thickness, puncture strength, and dissolution rate). The screw speed on the HME can affect the degree of mixing, melting temperature, and melting pressure of the process. It was hypothesized that the slow screw speed of the HME results in lower pressure at the die and therefore the formulation was pushed at a slower rate out of the die with a lower melting pressure. The slow speed of the screws created an increase of material in the zone of the die, which leads to a thicker and heavier film. At the high screw speed, the low DPV drug content was found. This could have occurred as because of the higher pressure that the screw speed applied to the polymers and may have result in an effect on the polymer structure to hold the DPV in the film. This finding demonstrated that the final film product specifications can be altered by changing the screw speed. This finding can be applied to other film manufacturing processes in order to meet

predetermined product specifications. Overall, these studies served as a process validation for the HME for reproducibility and consistency in vaginal film production.

In summary, HME manufactured DPV film was assessed and determined feasible and robust for vaginal use. DPV was successfully incorporated into a vaginal film using HME, while maintaining acceptable physicochemical characteristics and anti-HIV activity, with limited toxicity to the human tissue, TZM-bl, and the *Lactobacilli* present in the vagina. These results demonstrate that HME is a highly functional and efficient technique that could lead to lower cost and greater scalability of film manufacture. This work represents the first application of HME to the development of a vaginal antiretroviral film product.

3.6 ACKNOWLEDGMENT

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4.0 AIM 2 PART A: MULTILAYER FILM FOR CO-DELIVERY OF ANTIBIOTIC AND PROBIOTICS FOR THE TREATMENT OF BACTERIAL VAGINOSIS

4.1 INTRODUCTION

Bacterial Vaginosis (BV) is the most common vaginal infection in the female urogenital tract. In fact 15% of the sexually active population have experienced BV infection. Moreover, BV is associated with other upper urogenital tract infections, such as amniotic fluid infection, pre-term delivery, and post-partum endometritis [105]. BV can be asymptomatic or symptomatic, and both conditions are characterized by a change in the composition of the vaginal microflora. A healthy microflora is predominantly colonized by *Lactobacillus* species, but in BV, a mixture of harmful bacteria, which can include *Gardnerella vaginalis (G. vaginalis), Bacteroides* spp, *Mobiluncus* spp and *Mycoplasma hominis,* predominate [106]. Clinical symptoms associated with BV include increased vaginal pH (pH>4.5), white discharge, fishy odor, and presence of clue cells. Clue cells are vaginal epithelial cells that are covered with coccobacilli and in some cases the margin of the cells are obscure [106]. BV is also associated with increased susceptibility to human immunodeficiency virus (HIV) and increased instance of cancer [107-109]. Meta-analysis conducted by Fethers et al. reported that contraction of BV was found to be significantly associated with sexual contact with a new partner or multiple partners. Additionally,

this study reported that protected sexual activity or less frequent unprotected sexual activity can reduce the recurrence of BV infection [110].

Since BV is composed of a mixture of pathogenic vaginal bacteria and is a complex condition (symptomatic and asymptomatic), it is crucial to have robust and accurate diagnostic tools. Although, BV remains challenging to diagnose, diagnostic tools that do exist can be categorized into two main categories: laboratory-based and clinical-based. Laboratory-based diagnostics rely on culturing vaginal bacteria to determine if they are present in the patient, while clinical-based criteria rely on patient observation in the clinic.

Laboratory-based diagnostics can discern specific bacterial species; however, this tool possesses some drawbacks. The most common organism associated with BV is *G. vaginalis,* and it has been found on laboratory media in up to 94% of symptomatic patients. However, *G. vaginalis* from asymptomatic patients is also able to grow, which can lead to false diagnosis [111]. Additionally, other culture-based diagnostic tools, such as those that test for *Bacteroides* and *Peptostreptococcus,* are very expensive [112]. Beyond culturing vaginal specimens, the Nugnet score is often applied to BV diagnosis. In 1991, Nugent et al. reported a scoring system using vaginal swabs for the diagnosis of BV. The scoring system is a gold standard for BV diagnosis, and is calculated by gram staining and evaluating bacterial morphology in the vaginal specimen [106]. For example, the presence of gram-positive rods, consistent with *lactobacilli* morphology, is scored from 0 to 4, with 4 denoting a smaller amount of *Lactobacillus* species. On the other hand, small gram-variable rods, associated with G. *vaginalis* morphology, receive a score of 0-4, with 4 denoting a larger G. *vaginalis* population. Finally, curved gram-variable rods, associated with *Mobiluncus* spp, receive a score from 0-2, with 2 being a larger amount. The sum of all three scores can range from 0 to 10, with a score of 7-10

defined as BV. Today, the Nugnet score is mainly applied to clinical studies, as it is time consuming, involves many resources, and requires trained personnel for microscopic evaluation [106].

Beyond lab-based tests, which may be inconclusive and time-consuming, clinical diagnostics are important to add value to laboratory diagnoses. Amsel at el in 1983 reported the Amsel Criteria as the standard for clinical-based diagnosis of BV. This diagnosis relies on patients experiencing 3 out of 4 main symptoms of BV, which include (1) elevated vaginal pH (pH > 4.5), (2) >20% clue cells present from vaginal swabs, (3) white, milky vaginal discharge, and (4) a fishy odor [113]. In the clinic, vaginal pH can be evaluated using a pH indicator, clue cells can be detected with a light microscope, the discharge can be confirmed visually, and a fishy odor can be detected by adding 10% potassium hydroxide to a swab sample of the vaginal discharge. After assessment and confirmation of BV, an antibiotic can be prescribed to treat the condition.

Two common antimicrobial agents are currently approved by the Food and Drug Administration (FDA) for BV treatment: metronidazole and clindamycin [114]. It is known that these treatments inhibit not only the BV-associated bacteria, but also the healthy bacterial species, especially at high doses [115]. Since *G. vaginalis* grows in a resilient biofilm-like structure, a high dose of antimicrobials is needed for complete inhibition [23, 115]. Study results, conducted by Hiller et al. found that antibiotic resistance is more likely to develop with extended use of clindamycin than of metronidazole [79], and therefore, metronidazole was preferable for this project.

Metronidazole can be administered orally or topically. Metronidazole is an antimicrobial agent that inhibits microbial growth and division. The most common dosage form used for the

BV treatment is metronidazole vaginal gel, because of its high acceptability, feasibility, and low cost. Additionally, the intravaginal metronidazole gel has been found to have 80-90% efficacy [116]. However, Bradshaw et al. reported high recurrence rates of BV over the course of 12 months, following a metronidazole therapy study (e.g. Nugent score [NS] of 7–10 or 4–6 combined with 3 Amsel criteria) [117]. High recurrence rate is associated with the lack of selectivity for harmful bacteria and the disruption of the natural microflora.

The human vagina is comprised of a dynamic environment of bacterial species that play a role in maintaining a healthy vaginal tract and preventing infection. *Lactobacillus* species are the predominant bacteria within the vagina and help to maintain a low pH through the production of lactic acid. In addition, some lactobacilli can produce hydrogen peroxide which is toxic to harmful organism in the microbiota, especially those who lack of H_2O_2 scavenging enzymes, such as catalase peroxide [23]. *Lactobacillus jensenii* (*L. jensenii*) was selected as the model bacteria for this project since they produce both lactic acid and hydrogen peroxide and are known to be one of the dominant bacteria present in normal microflora. Studies have demonstrated the ability of lactobacilli to inhibit the growth of pathogens, specifically *G. vaginalis*, which is present in 99% of Bacterial Vaginosis (BV) cases. Therefore, it is important to keep the microflora dominated by *Lactobacillus* species. This can potentially be done by local application of *Lactobacillus*-containing products [118]. *Lactobacillus*-containing products are also defined as probiotics which are products containing live microorganisms that offer benefits when administered to the host [90]. Vaginal probiotics may be composed of one or multiple species of *Lactobacillus.* In order to develop a vaginal drug delivery system for probiotics, the bacteria first need to be in a viable state in order to maintain the bacterial viability and functionality over time.

The application of lactobacilli probiotics--alone or in a combination with metronidazole--is currently being investigated as a BV treatment strategy [119, 120]. However, these treatments must optimize the delivery system to prevent toxicity as well as prove effective against BV infection. One of the challenges of this co-delivery system of antibiotics and lactobacilli probiotics is the toxicity of the antibiotic to the probiotic *Lactobacillus* species [23]. To overcome this challenge, we propose a vaginal film delivery system that will contain both *L. jensenii* and metronidazole in non-intersecting, separate layers, to prevent compatibility and toxicity issues. The polymeric thin film dosage form was chosen for this project due to its preference among women and low toxicity *in vivo* [14, 36]. The production of multilayer film containing antibiotics and probiotics can be done by the utilization of co-extrusion system [121]. Co-extrusion systems are able to extrude two or more formulation to create multi-layered extrudate while maintaining complete separation between the formulations [121]. In this study, the hot melt extrusion (HME), manufacturing technique was selected for the production of the two layer vaginal film. One layer contains the antibiotic, metronidazole, and the second layer probiotic, *L. jensenii*.

4.2 MATERIALS

Metronidazole was purchased from Spectrum (Gardena, CA, US). PolyOx N10, (Polyethylene oxide, MW, 100,000) and Polyethylene glycol 4000 were purchased from Dow Chemical Company (Midland, MI, USA). Polyethylene glycol 400 and Vitamin E were purchased from Spectrum (Gardena, CA, US). Ultrapure water was obtained from an in-house MilliQ water purification system. Phosphate buffered saline 10x molecular biology grade was purchased from

Mediatech, Inc. (Manassa, VA). Columbia Sheep's Blood agar plates, Human Blood Tween Bilayer Medium and litmus milk were purchased from Becton Dickinson and Co.(Sparks, MD). The *L. crispatus* (ATCC# 33197), *L. iners* (ATCC # 55195), and *G. vaginalis* (ATCC 14018) were purchased from ATCC (Manassas, VA). The twin screw extruder was purchased from Thermo Fisher Scientific, (Tewksbury, MA). NANO 16mm twin screw HME technology was purchased by Leistritz Corporation (Somerville, NJ).

4.3 METHODS

4.3.1 Development of Combination Film

Metronidazole vaginal film was produced using a NANO 16mm twin screw HME technology made by Leistritz Corporation (Somerville, NJ). Twenty five percent of metronidazole was mixed with 39.5% (w/w) polyethylene oxide (PEO) PolyOx N80, 26.3% (w/w) polyethylene glycol (PEG) 4000, 2.6% (w/w) PEG 400, and 2.6% (w/w) vitamin E. The extrusion barrel temperature was set as: zone 1:110 °C, zone 2: 115 °C, zone 3: 115 °C and zone 4: to 120 °C. The die melt temperature was 125 °C and the HME screw speed was 185 rpm. At the end of the extrusion process, the product was collected using a system capable of rolling and cooling the film sheet extrudate.

Bacteria-loaded film was manufactured using a twin screw HME. The film forming polymer, PEO (51.4% (w/w)), was mixed with 34.2 % (w/w) PEG4000, 8.4% (w/w) PEG 400 as well as 3.2% (w/w) antioxidant Vitamin E. 3% *L. jensenii*, bacterial powder was added to the mixture and subsequently transferred to the HME hopper. The extruder was set to 60 °C and the

screw mixing speed was set to 40 rpm. After extrusion, the metronidazole film was placed at the end of the die to allow the bacteria-loaded film to be extruded directly on top of it. As the soft bacterial film extrudate was allowed to harden, the two films became fused into the final multilayer form. Once the film sheet was cooled, it was hand cut into 0.5x1 inches. Each film unit dose was then packed individually and sealed.

4.3.2 Film Biological and Physical Characterizations

4.3.2.1 Metronidazole Film Analytical Assay for Drug Detection

Refer to chapter 2, method section 2.3.3.2- Titled: Hydrophilic Molecule: Metronidazole HPLC Detecting Assay.

4.3.2.2 Bacterial Viability, Colonization Evaluation, and Uniformity Assessment

Evaluation of bacterial viability, colonization and uniformity was conducted utilizing Columbia Sheep's Blood agar plates (BA). Bacteria-loaded film combinations were dissolved in sterile PBS and serial dilutions were performed. Each of the dilutions was plated onto BA plates and incubated at 37 °C with 6% CO_2 for 48 hours. The colony forming units from each dilution were counted and reported.

4.3.2.3 Bacteria/Metronidazole Film Bioactivity

To test the bioactivity of bacterial/metronidazole combination film, *G. vaginalis* (ATCC 14018) was plated on Human Blood Tween Bilayer (HBT) plates to form a lawn. Bacteria/metronidazole film was placed in the middle of the HBT plated bacteria and plates were incubated at 37°C with 6% CO_2 for 48 hours. Post incubation, the plates were visually inspected for the formation of a

zone of inhibition and *L. jensenii* growth. Placebo films, metronidazole drug substance, and metronidazole film were prepared and tested in the same manner as the controls.

4.3.2.4 Detection of Lactic Acid Production

Litmus milk assay was used for the detection of lactic acid production. Bacteria/metronidazole combination film, bacteria powder (before HME process), bacteria loaded film, placebo film and as a control, *L. crispatus* (ATCC # 33197) were placed in 5 mL sterile litmus milk solution in individual vials. Each vial was placed at 37°C with 6% CO_2 for 48 hours. Litmus milk solutions were visually inspected for color change, and recorded as (+) for color change and (-) for no change. The litmus milk changed from purple to pink color with the production of lactic acid. Litmus milk contains litmus, which is a colorimetric pH indicator that appears purple at neutral pH and pink under acidic condition. It is also contained bacteria metabolized molecule including milk sugar, lactose, milk protein, and casein.

4.3.2.5 Detection of Hydrogen Peroxide Production

Hydrogen peroxide detection was determined using a Tetramethyl-benzidine (TMB) plate. Bacteria powder and bacteria/metronidazole film were tested using the same method. Bacteria-loaded films were dissolved in sterile 1x PBS and placed onto TMB plates. The plates were placed in an anaerobic box at 37°C for 48 hours. The plates were then exposed to air. The horseradish peroxidase, present in TMB plate, oxidizes TMB in the presence of hydrogen peroxide produced by the lactobacilli to form a blue pigment [23]. The presence of blue color was visually compared to positive and negative controls. The *L. crispatus* (ATCC# 33197) served as a positive control for this test, because of its ability to produce hydrogen peroxide,

while *L.iners* (ATCC # 55195) served as a negative control, due to its lack of hydrogen peroxide production.

4.3.3 Film Physical Assessment

4.3.3.1 Appearance, Thickness, Mass

Refer to chapter 2, method section 2.3.4.1- Titled: Appearance, Thickness and Mass

4.3.3.2 Moisture Content

Refer to chapter 2, method section 2.3.4.4- Titled: Moisture Content

4.3.3.3 Puncture Strength

Refer to chapter 2, method section 2.3.4.2- Titled: Puncture Strength.

4.3.3.4 Disintegration Time

Refer to chapter 2, method section 2.3.4.3- Titled: Disintegration.

4.3.3.5 Film Dissolution and Bacterial Release

A film was suspended in a mixture of sterile MilliQ water and Sheath buffer (BD Biosciences) (3:1 ratio). Samples were constantly and gently mixed throughout the experiment. 200 µL of the samples were collected at the following time intervals: 0, 15, 30, 45, 60, 75, 90, and 105 minutes and tested using flow cytometry technique. After removing each sample at each time point from the vessel, 200 µL of dissolution media was added to allow constant total volume in the system. *L. jensenii* film samples were analyzed by a LSRII (BD Biosciences) flow cytometer with the

forward scatter (FSC) and side scatter (SSC) parameters on. This method allows the separation of bacteria from the film formulation based on the size and granularity of the bacteria.

Metronidazole dissolution assay was conducted using USP apparatus IV flow-through dissolution system (SOTAX CE7 smart, Sotax Switzerland) connected to an auto sampler with 16 mL/min flow rate. The dissolution assay was conducted at 37 °C for 60 minutes. At predominant time interval, (3, 6, 10, 15, 30, 45, 60 minutes), samples were collected and analyzed using HPLC assay.

4.4 RESULTS

4.4.1 Development of Combination Film

The film formulation of the combination drug was manufactured as two separate layers. Metronidazole film was manufactured first and bacteria film was extruded afterward as the secondary film layer. The combination film was transparent, flexible, and smooth. Originally, the HME was set to 65 °C and 180 rpm for production of bacterial film. However, bacteria lost all viability during the process. To assess when bacteria were damaged, samples were acquired at each of the HME process steps (scale, mixing, screws and final product). These assessments revealed that the screw-mixing step was causing a loss in viability. Since stability data demonstrated that the bacteria are stable at 70 °C for one hour, the bacterial damage was determined to be caused by high shear of the screws. Therefore, screw speed was optimized to 40 rpm, achieving 100% bacterial viability (~10^7 CFU/mg film) at the end of the HME process.

4.4.2 Film Physical and Biological Characterizations

4.4.2.1 Film Assessment

The films were characterized as individual film (single layer film containing single entity of bacteria or metronidazole) and also as multilayer film (films containing both bacteria and metronidazole). Originally, metronidazole film was developed with 3 different film-forming polymers as the main polymer, in order to understand the effect of the polymer on the release profile of the drug. Figure 4.1 shows that PEO based film demonstrated a burst release of metronidazole with a release rate of 12.5 mg/(√t) while HPC demonstrated controlled release of metronidazole with release rate of 3.5 mg/(√t). HPMC also demonstrated a burst release (10.8 mg/(√t)) when compare to HPC based formulation but it had slightly slower release than the PEO based formulation. For this project, the PEO based formulation was selected and fully characterized for the quick release of the metronidazole to be as similar to the gel release and to treat the BV as soon as the film comes in contact with a small amount of fluid in the vagina. Metronidazole PEO based single films were found to be white, translucent, soft, and flexible. The average mass and thickness were 205.8 ± 14.2 mg and 0.2 ± 0.02 mm, respectively. Metronidazole drug content was found to be uniformly distributed (relative standard deviation < 10%) with a total drug content of 243.6 ± 7.2 [drug (µg)/film(mg)]. The moisture content in the film was less than 1%, with a puncture strength of 3.7 ± 0.2 kg/mm. The disintegration time was found to be 75.8 ± 13.6 sec and 94.8% of drug was released from the film within the first 60 minutes of the *in vitro* dissolution test. Finally, the metronidazole film was determined to remain stable over a 3 month period while maintaining antimicrobial activity.

Bacteria single layer film was visually determined to be transparent, soft, flexible, and smooth. The average mass and thickness of the film were recorded to be 133.9 ± 8.7 mg and 0.1

± 0.008 mm, respectively. Bacteria were uniformly distributed throughout the single film at a CFU of 10^7. The water content in the film was approximately 1%, which was expected for the solvent-free HME film. The quick disintegration of the film (64.2 ± 0.3 sec) and the rapid bacterial release (peak release within 30 minutes) of the film suggest that the formulation is quick dissolving. During the *in vitro* dissolution assay, the viability of the bacteria was monitored during the 90 minutes time period and showed bacteria remained consistently viable.

After each individual film was developed and manufactured, the two-layered film was manufactured and evaluated. Films were smooth, soft, white, and translucent. The bacterial uniformity and concentration (10^7 CFU) remained the same as in the single layer bacteria film (10^7 CFU) as well as in the metronidazole film. The combination film's water content was less than 1% and the puncture strength (5.5 ± 0.5 kg/mm) was also similar to the single layer film. The mass and thickness, however, were almost doubled, at 238.0 ± 47.9 mg and 0.3 ± 0.03 mm, respectively. This increased mass and thickness was expected since combination film contains two film layers.

The bacterial peak release was detected after 30 minutes, starting when the film came in contact with the dissolution media. This quick release was similar to the results observed in the single layer film. Likewise, 70% of the metronidazole was released within the first 15 minutes of the dissolution test.

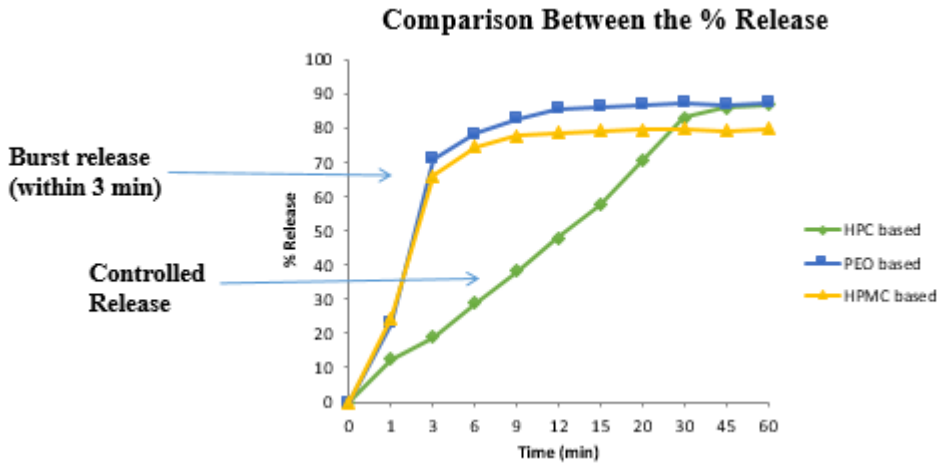


Figure 4.1: Metronidazole Drug Release from the *In Vitro* Dissolution Assay.

PEO and HPMC based film demonstrated burst release. PEO had the fastest release rate in compare to HPC and HPMC based films. The HPC based film demonstrated more control release of metronidazole with the slowest release rate when comparing to PEO and HPMC based film.

4.4.2.2 Bacterial Viability, Colonization Evaluation, and Uniformity Assessment.

Bacteria/metronidazole HME combination films were evaluated for bacterial uniformity and viability. Bacteria from the combination films were found to be uniform and viable. The bacterial CFU from the combination film was similar to the CFU found in the bacteria powder (pre HME process) and bacteria-only film (10^7 CFU). The retained viability and uniformity confirm that HME can be utilized for production of a Bacteria-loaded vaginal film.

4.4.2.3 Bacteria/Metronidazole Film Bioactivity.

The bacteria/metronidazole combination multilayer film was tested for anti-microbial activity of metronidazole and recolonization activity of the bacteria. The combination film demonstrated a 1.5cm zone of inhibition within the *G. vaginalis* lawn, comparable to the metronidazole only film zone of inhibition. The placebo film did not demonstrate any inhibition properties (refer to Figure 4.2, (A) combination film, (B) metronidazole only film, (C) placebo film). Additionally, the bacteria were released from the combination films and able to colonize within the zone of

inhibition created by the metronidazole (refer to Figure 4.2 (A) and 4.3). Therefore, the desired *in vivo* antimicrobial and probiotic effects of the combination film were confirmed *in vitro.*

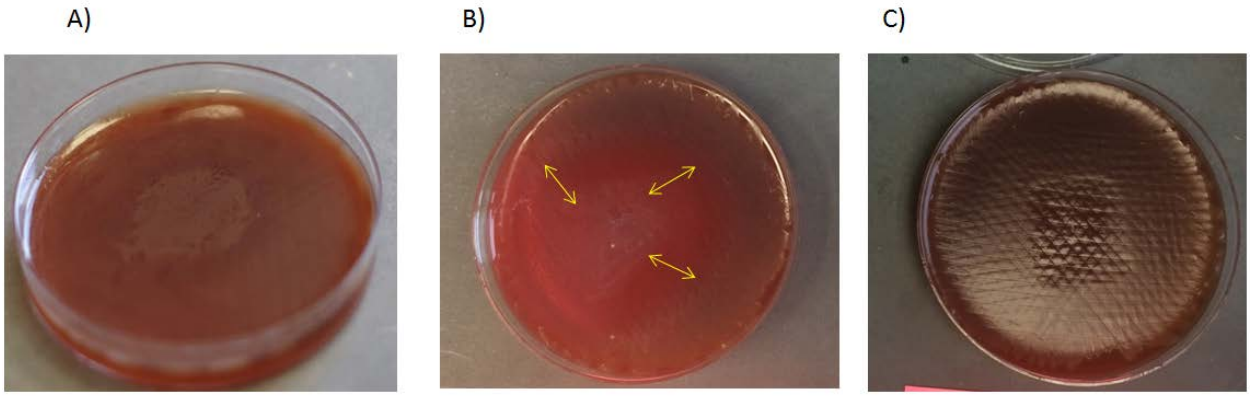


Figure 4.2: Bacteria/Metronidazole Combination Film Bioactivity Evaluations.
A) The combination film showing the metronidazole layer inhibited the growth of *G. vaginalis* in the area where the film was placed, and the *L. jensenii* layer colonialized within the zone of inhibition (see Figure 4.3 for microscope image). B) Metronidazole-only film inhibits the growth of *G. vaginalis* in the area where the film was placed. C) Placebo film did not inhibit the growth of *G. vaginalis.*

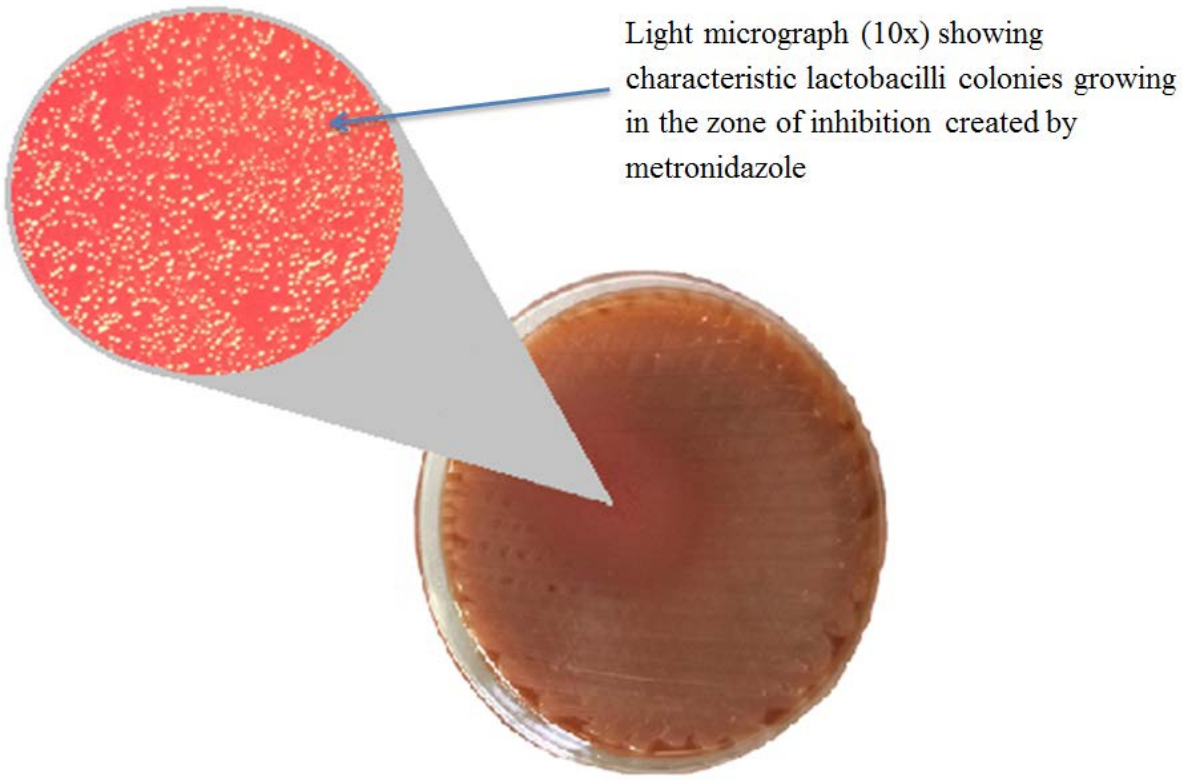


Figure 4.3: Bacteria/Metronidazole Combination Film Microscopic Image of *L. jensenii* Growth.

This figure demonstrates the zone of inhibition created by the metronidazole film in the middle of the plate, as well as a light micrograph of the *L. jensenii* taken in order to visualize the colonies. The colonies of *L. jensenii* are small, round, and cream-colored which are characteristics of *L. jensenii* colonies on blood agar.

After the bioactivity of the combination film was confirmed, the bacteria that grew within the metronidazole-induced zone of inhibition were swabbed from the HBT plate. The bacterial-swab was transferred to litmus milk and a TMB plate, for lactic acid and hydrogen peroxide evaluation, respectively. See Figure 4.4 for the schematic diagram of the experiments.

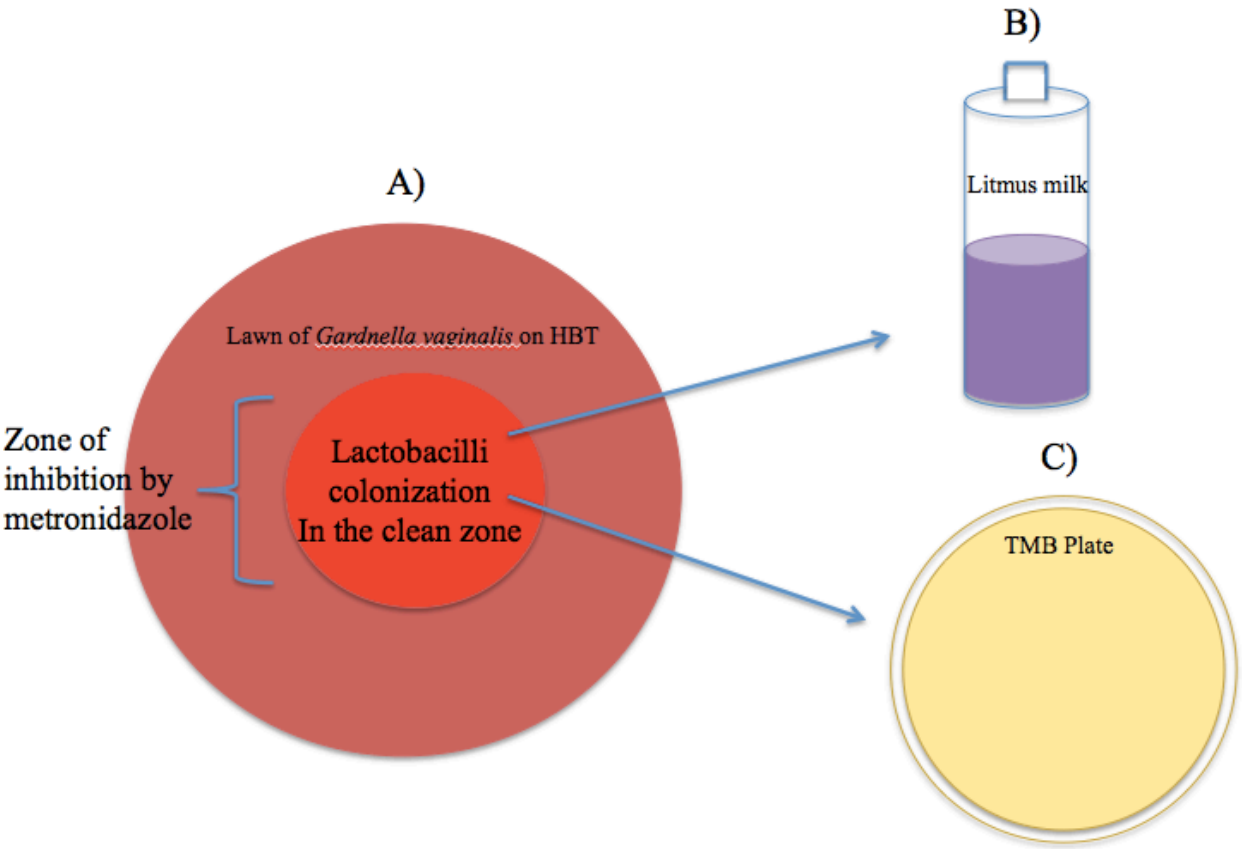


Figure 4.4: Schematic Diagram of Sample Collection from HBT Plate for the Bioactivity Evaluation of *L. jensenii* Post Extrusion Process.

A) Bacteria/metronidazole combination film was placed on HBT that was plated with a lawn of *G. vaginalis*. Plate was incubated for 48 hours at 37 °C. Metronidazole layer created a zone of inhibition of the *G. vaginalis*. *L. jensenii* grew in the cleared zone. B) *L. jensenii* from the cleared zone was transferred to the litmus milk solution for lactic acid production evaluation test. C) *L. jensenii* from the cleared zone was transferred to TMB plates for hydrogen peroxide evaluation assessment.

4.4.2.4 Detection of Lactic Acid Production

L. jensenii were swabbed from the zone of inhibition on the HBT plate after bioactivity test and placed in litmus milk (Figure 4.4). The results from the litmus milk assay (post HME process and post exposure to both metronidazole and *G. vaginalis*) showed a decrease in pH. The color of the litmus milk changed from purple to pink, which indicates lactic acid production. Both the HME combination film and the control sample (refer to Figure 4.5) were assigned (+) for color change, and the litmus milk negative control was assigned (-). This color change confirms that the

bacteria produced lactic acid after release from the film and after exposure to metronidazole and *G. vaginalis*. Since higher vaginal pH is associated with increased susceptibility to vaginal infections, such as BV, the low pH maintained by the lactic acid production post-release from the film is a fundamental requirement for a probiotic vaginal product.

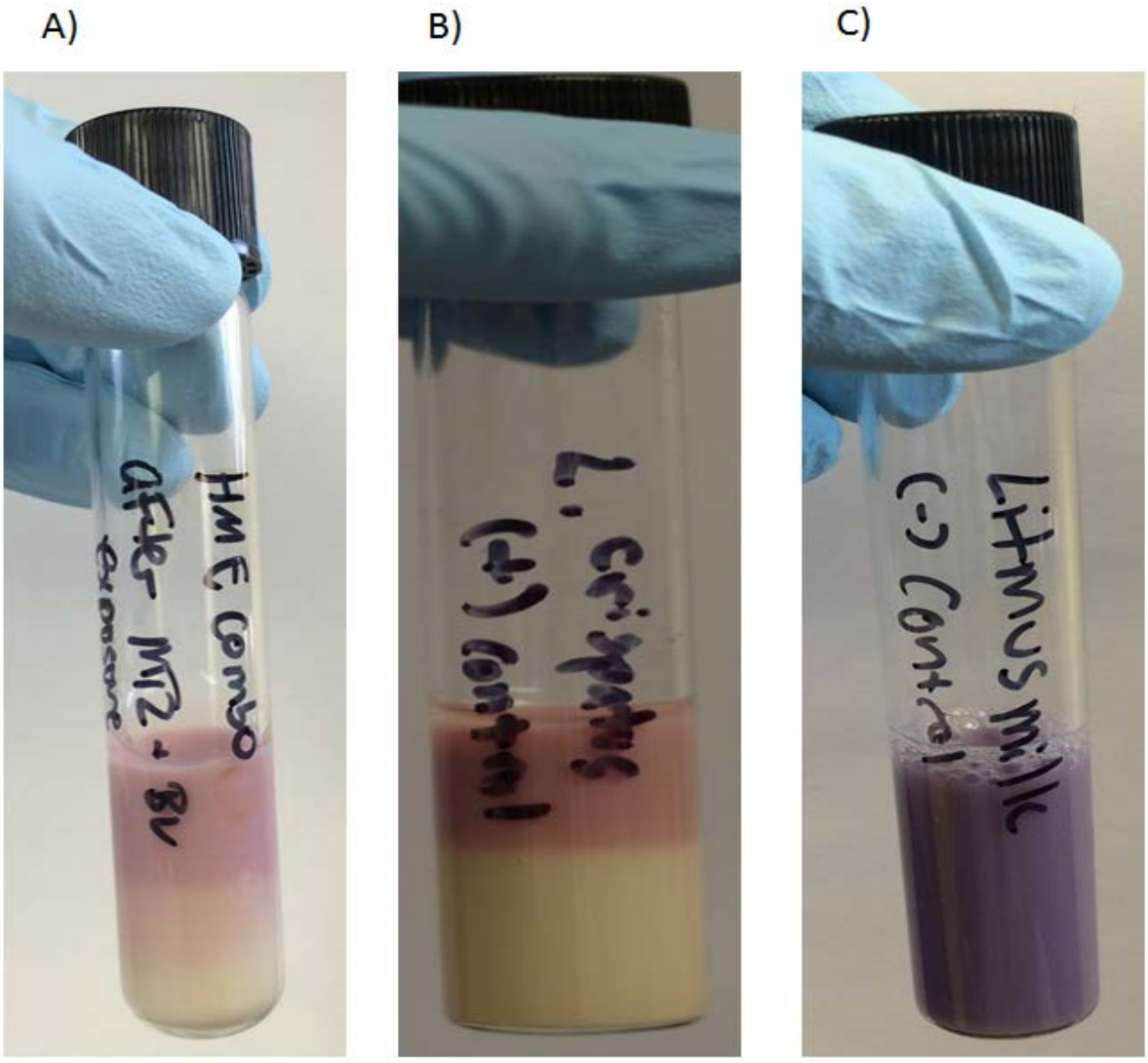


Figure 4.5: Evaluation of Lactic Acid Production by *L. jensenii* Formulated in the Combination Film.

A) *L. jensenii* from the HME combination film produced a pink litmus milk color change indicating pH reduction and bacterial lactic acid production (+) B) *L. crispatus (*ATCC# 33197) positive control (+) C) Litmus milk (negative control) showed the expected lack of color change (-).

4.4.2.5 Detection of Hydrogen Peroxide Production

Lactobacillus was collected from the zone of inhibition on the HBT plate after the bioactivity test (Figure 4.4) and spread on a TMB plate. After 48 hour incubation at 37 °C in an anaerobic box, the plates were exposed to air. Blue color was detected on the TMB plate post incubation and exposure to air. The blue color was compared to the blue color present in the zone of growth and was similar to the *L. crispatus* positive control zone. On the contrary no blue color was produced in the zone of *L. iners* growth (Figure 4.6). The results suggested that the *Lactobacillus* from the HME combination film retained the ability to produce hydrogen peroxide post extrusion and post exposure to the metronidazole.

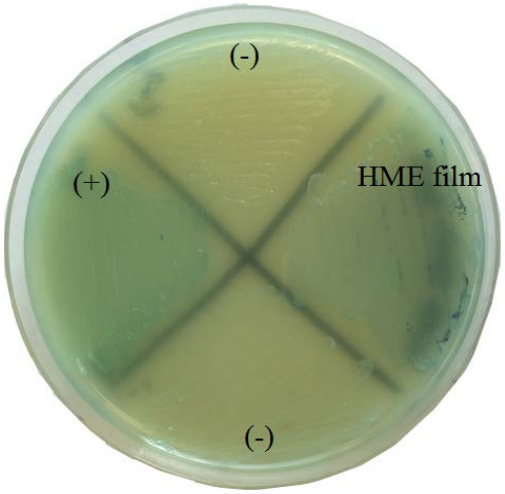


Figure 4.6: Hydrogen Peroxide Evaluation of Bacteria/Metronidazole Combination Film Using TMB Plate.

On the left: blue color indicating hydrogen peroxide production in the zone of growth of the control *L. crispatus*. On the bottom and top: negative control *L.iners* does not producing hydrogen peroxide, indicated by the lack of the blue color in the zone of growth. On the Right: the *L jensenii* from the film maintained hydrogen peroxide production post extrusion process indicated by the blue color.

4.5 DISCUSSION AND CONCLUSION

To summarize, current approaches to the treatment of BV involve the application of antibiotics metronidazole or clindamycin. Although both antibiotic treatments can cure the BV infection, there is an associated of high BV recurrence rate weeks to month post treatment [122, 123]. Compared to clindamycin, metronidazole treatment has demonstrated less bacterial resistance, and thus metronidazole was selected for this project [79]. BV recurrence is associated with native *Lactobacillus* species depletion, and therefore, treatment with probiotic combined with the curable application of metronidazole may prevent BV recurrence. In this study, the HME process was applied to the development of a multilayer film containing one layer of metronidazole and one layer of *L. jensenii*. First, the metronidazole film was developed utilizing different film bases. The release rate of the metronidazole from the film revealed that the polymer composition can dictate the release of metronidazole and that PEO had the fastest release rate, and HPC had the slowest release rate. The metronidazole formulation selected for this project was the rapid-release PEO based formulation since it is crucial for an adequate amount of metronidazole to be present in the vagina immediately after the film insertion. The bacteria-loaded film was also designed as a quick release film since this study is a proof-of-concept study and more evaluation needs to take place regarding to the two release profiles and the dosing timing of metronidazole and *Lactobacillus*. Thus, this is the first time to develop a multilayer film that has the potential to deliver two APIs at different release rate.

The multilayer combination films were heavier and thicker in comparison to the single layer film. This was expected since the two-layer film was composed of two single layers extruded together. However, some of the characteristics of the combination film were similar to that of the single layer film. In addition, the puncture strength of the film remained similar,

which suggests the puncture strength is a function of the formulation rather than the structure and the APIs of the film. Following the development of the film formulation utilizing the HME, the bioactivity of the API was tested, since the HME process involves potentially damaging heat and shear stress and found to maintain the activity post extrusion process. In addition, the application of two APIs within the same delivery system required the evaluation of the activity of both API's post release of each other. After film manufacturing, bacteria were found to be uniform and viable within the film based on a plate count, which is crucial for the intended *in vivo* colonization of the vaginal microflora. These results suggest that the bacterial viability was not affected by exposure to metronidazole and the heat and shear from the HME process.

After assuring that the bacteria remained viable, the combination film was tested for bioactivity. The metronidazole was released from the film and created a zone of inhibition within the lawn of *G. vaginalis*, which the *L. jensenii* was able to colonize. The results confirm the ability of the probiotic to colonialize in the present of the antimicrobial agent while the antimicrobial agent inhibits the growth of the BV associated bacteria. This *in vitro* study demonstrates the applicability of multilayer film for the combination delivery of metronidazole and probiotic in one delivery system while maintaining antimicrobial activity and bacterial viability.

The *L. jensenii* was collected from the HBT plate and tested for the production of lactic acid and hydrogen peroxide. These studies demonstrated the ability of the *L. jensenii* to colonize the zone of inhibition created by metronidazole and carry out beneficial production of lactic acid and hydrogen peroxide. Lactic acid production by *Lactobacillus* in the film dosage form is a crucial requirement for successful probiotic application. This is because lactic acid maintains the low vaginal pH and helps to prevent growth of harmful pathogens in the vagina, including the

BV-associated bacteria. Additionally, hydrogen peroxide produced by *Lactobacillus* species is known to be harmful and to inhibit growth of pathogens, including BV-associated bacteria *G. vaginalis*, HIV-1, herpes simplex virus type 2 (HSV-2), and *E.coli* [124, 125]. Therefore, it is an essential function of probiotic applications for the treatment and recurrent prevention of BV. In this proof-of-concept study, only one strain of bacteria was selected. However, the film formulation can be applied to a combination of *Lactobacillus* probiotic product contains more than one bacterial species.

Overall, this study applied HME technology to the production of a multilayer probiotic/antibiotic vaginal film with the combination of metronidazole and *Lactobacillus* for BV treatment and recurrence prevention. The study demonstrated the ability to successfully deliver antibiotic and probiotic agents while maintaining the antimicrobial activity of metronidazole and the lactic acid and hydrogen peroxide production of the *L. jensenii*.

Taken together, this is the first time the HME process has been applied to the production of a multilayer film and for the application of bacterial vaginosis treatment. This study was proof-of-concept, and therefore, all applications were tested *in vitro*. Ultimately, the film will be optimized for *in vivo* studies and marketed application in hopes of improving the quality of life for women suffering from BV infection and recurrence.

4.6 ACKNOWLEDGMENT

I would like to acknowledge Kevin Stoner, for the microbiological testing support and flow cytometry training.

5.0 AIM 2 PART B: MULTILAYER FILM FOR THE CO-DELIVERY OF ANTIRETROVIRAL AND CONTRACEPTIVE AGENTS FOR THE PREVENTION OF HIV AND UNINTENDED PREGNANCY

5.1 INTRODUCTION

Human immunodeficiency virus (HIV), the virus that causes acquired immune deficiency syndrome (AIDS), is a worldwide pandemic, with an estimated 2.7 million new infections each year [126]. According to the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC), since the discovery of HIV in the 1980s, more than 60 million people have contracted HIV and 25 million people have died from HIV-related complications. As of today, it is estimated that 37 million individuals have HIV, with 24.7 million in Sub-Saharan Africa alone. Untreated infection can result in AIDS, which is characterized by a weakened immune system and associated complications, including life-threatening infections. To date, patients living with HIV are able to delay AIDS and associated complications due to better access to antiretroviral therapy. However, as the HIV infection rate continues to rise, there is no effective and available vaccine or cure. Therefore, prevention has become the main way to reduce HIV infection

HIV is a blood-borne virus that is transmitted primarily through sexual intercourse. Women are a population at high risk for acquiring the infection. HIV contraction, mainly through heterosexual intercourse, in women between the ages of 15 to 24 is rapidly increasing [127, 128]. Higher risk for contraction in women can be attributed to the vaginal anatomy. The vaginal walls

allow for extended exposure of seminal fluid, and thus potential virus, to mucosal target cells. Likewise, the vaginal epithelium can be easily damaged during intercourse resulting in abrasions or sores within the urogenital tract, which allows HIV to more easily penetrate target cells [34].

Although condom use reduces the risk of HIV infection, women—especially those from a lower socioeconomic classes or developing counties—often do not have the power to negotiate condom use by their partner [98, 99]. Therefore, developing an effective female-controlled prevention method is urgent. Microbicides are compounds that can be self-applied inside the vagina or rectum that possess properties for protection against sexually transmitted infections (STIs) including HIV. They can be formulated into several dosage forms such as gels, intravaginal rings, creams, inserts, enemas, and films. Hence, microbicides can offer a female-controlled, effective HIV-prevention mechanism.

A common issue observed in clinical trials with microbicide-based HIV prevention methods is patient adherence to the dosing regimen. Since adherence is correlated with product efficacy, products with high efficacy potential may be reported with low efficacy in clinical trials if participants do not accurately use the product. For example, in a clinical study (MTN-003, VOICE), only 23% of women adhered to a tenofovir gel regimen used in the trial, and therefore, no protection was observed [129]. In another clinical study (CAPRISA), only 50.5% adherence to tenofovir gel was reported. In this study, the efficacy of the gel was reported to be 39%. Both studies showed low adherence, and therefore, low HIV prevention potential. To increase user adherence, a prevention method that is already well established and commonly used by women can be harnessed for HIV prevention. Contraception is currently a high adherence prevention method, and thus, a combination contraception/HIV preventative microbicide can be developed to achieve dual effects against HIV transmission and unintended pregnancy.

Studies conducted in the United States showed that 99% of sexually active reproductive aged women used at least one contraceptive method. 88% of women who currently practice contraception use nonpermanent methods such as a birth control pills, injectable devices, patches, or intrauterine devices [130]. One way to address the low microbicide adherence for the prevention of HIV infection is to combine a microbicide agent with a contraceptive agent because high adherence is associated with contraception methods. Such combined prevention methods are referred to as Multipurpose Prevention Technologies (MPT) since they have dual purpose functionalities [131].

MPT products are defined to have dual functions (e.g. products that can address multiple sexual and reproductive health problems at the same time). MPTs can be targeted for the prophylaxis of both unintended pregnancy and HIV infection and improve convenience, adherence, and effectiveness while reducing costs and environmental impact [132, 133]. This way, a product that is already in high demand (contraceptive) in the target population can achieve increased uptake of a second product (microbicide).

The MPT products that are currently in development are broadly categorized as either long-acting or on-demand. Long-acting MPTs are products that can be applied less frequently in comparison to on-demand products. The long-acting MPT products that are under development include intra-vaginal rings (IVRs) and long-acting injectable products [131]. One advanced IVR MPT seeks to prevent unintended pregnancy and HIV utilizing two advanced microbicides, dapivirine (DPV) or tenofovir, in combination with the contraceptive agent levonorgestrel (LNG) [131]. The second type of MPT products, are on-demand products, which are used within the time frame of sexual activity. These products can include gels, tablets, inserts, or films. In this project, we focused on MPT products with the goal to prevent HIV and unintended pregnancy

utilizing the vaginal film as an on-demand MPT delivery platform. In order to develop the MPT films two active pharmaceutical ingredients (APIs) were selected as model molecules, DPV and LNG.

There are many challenges to achieve successful formulation of two APIs in one delivery platform. These include APIs and film stability, API release from the film, and achievement of the appropriate dosing level. In order to overcome these challenges, in this project, a hot melt extrusion (HME) manufacturing method was utilized for the development of a single layer combination film and multilayer combination film formulation for the simultaneous delivery of the two pharmaceutical agents – DPV and LNG. A single layer combination vaginal film consists of the two APIs in one film layer. A multilayer combination vaginal film contains each API in non-intersecting, separate layers that are combined into one film. DPV is a potent non-nucleoside reverse transcriptase inhibitor (NNRTI) of HIV and LNG is a synthetic female sex hormone (progesterone) that is used for the prevention of pregnancy.

LNG was chosen for use in the formulation, as it is the principle contraceptive agent that has been investigated in MPT systems. The DPV was selected because it is one of the most clinically advanced anti-HIV agents. DPV is highly potent microbicide candidate that demonstrated efficacy when was challenged post film administration [39]. Both of these agents are hydrophobic, have relatively high melting points and are heat stable.

HME is utilized for this project since the HME process does not require water, in contrast to the traditional film manufacturing process (solvent cast). Therefore, HME can eliminate the potential hurdle associated with formulation development of hydrophobic agents in an aqueous based formulation. Additionally, shear forces and high temperatures generated during the extrusion process can increase the energy state of the crystalline-structured APIs and thus the

crystalline structure is converted into the amorphous state. The new amorphous structure of the API serves to enhance dissolution, tissue permeability, and overall film stability. Furthermore, co-extrusion HME (co-HME) was evaluated. Co-extrusion manufacturing systems are capable to extrude two or more formulations to create a multi-layered film while maintaining complete separation between each layer on the extruded film [121]. The co-extrusion process adds value to the co-delivery system because it allows each drug to be modulated independently, enabling manipulation of the API release profile independently [134].

5.2 MATERIALS

Dapivirine used in these studies was provided by the International Partnership for Microbicides (IPM). Levonorgestrel was purchased from CHEMO (Chatam, NJ). Polyethylene oxide (PEO, PolyOx N80) and Polyethylene glycol (PEG) 4000 were purchased from Dow Chemical Company (Midland, MI, USA). Hydroxypropyl cellulose (HPC, Klucel EF) was purchased from Ashland, Inc (Bridgewater, NJ). PEG 400 and vitamin E were purchased from Spectrum (Gardena, CA, US). Ultrapure water was obtained in house from an in-house MilliQ water purification system. Acetonitrile (ACN), methanol, and methyl-tert-butyl ether (MTBE) were obtained from Fisher Scientific (Pittsburgh, PA).

5.3 METHODS

5.3.1 Preparation of the Vaginal Films by the HME Technique

DPV, LNG, and LNG/DPV single layer films were all manufactured using the same method. The LNG/DPV combination film, on the other hand, was manufactured in a two-step process due to unavailable access to two, twin screw HMEs for co-extrusion process. All films were manufactured utilizing a twin screw NANO 16 Leistritz extruder (Allendale, NJ). The formulations and extrusion conditions were summarized in table 5.1. In general, all excipients and APIs were blended using a bench top mixer. The powder blend was transferred to the K-trun feeder, which is connected to the twin screw extruder. The feeder was set to 250 g/h, the screws to 180 rpm, and the barrel temperature to increase from the lowest temperature (115 °C) at the feeding zone to the highest temperature (140 °C) at the die. For the multilayer LNG/DPV film, the DPV film layer was manufactured first followed by LNG film manufacturing as a secondary layer to create two layer films. The process used to develop the multilayer film required a sequential co-HME procedure. Initially, a single layer film was manufactured using the twin screw extruder. Subsequently, a second layer of film was extruded and carefully aligned on top of the initial film layer. This process yielded a multilayer film containing one layer of the DPV formulation and one layer containing the LNG formulation.

Table 5.1: Formulation Ingredients and Processing Conditions for all LNG HME film, DPV HME films, LNG/DPV Single and Multilayer Combination HME Films
(Refer to chapter 1, HME Process Description 1.3.2.2)

Component	Function
PolyOx N80	Film forming polymer
Hydroxypropyl cellulose	Film forming polymer
Polyethylene glycol 4000	Plasticizer
Polyethylene glycol 400	Plasticizer
Vitamin E acetate	Antioxidant
DPV/LNG	APIs
Zone 1	125 °C
Zone 2	130 °C
Zone 3	135 °C
Zone 4	135 °C
Die/melt temperature	140 °C
Screw speed	180 rpm

5.3.2 High Performance Liquid Chromatography (HPLC) Stability Indicating Assay

A high performance liquid chromatography (HPLC) system (Dionex Ultimate 3000, Thermo Scientific), equipped with an auto-sampler, quaternary pump, and diode array detector, was used to quantify DPV, LNG, and combination LNG/DPV in the film. To analyze the DPV film, a reversed phase chromatography method using a C18 column (acclaim 150X4.6 mm) was utilized, detected at a wavelength of 290 nm. The mobile phase consisted of (A) 0.1% trifluoroacetic acid in water (v/v) and (B) 0.1% trifluoroacetic acid in acetonitrile (v/v), at a flow rate of 1 ml/min. The mobile phase gradient was (minutes, %B): 0, 30; 6.6,45; 12.6,54.0; 13.2,80; 15,30. The LOD for this assay was 0.025µg/mL and the LOQ was 0.0825 µg/mL.

To analyze LNG, reversed phase chromatography using a C18 column (acclaim 150X4.6 mm) was utilized, detected at a wavelength of 244 nm with LOD of 0.015 µg/mL and LOQ of

0.0495 µg/mL. The mobile phases consisted of (A) water and (B) acetonitrile, at a flow rate of 1.5 ml/min. The mobile phase gradient was (minutes, %B): 0, 40.0; 8.2, 60.0; 9.0,80.0; 16.0,60.

5.3.3 Drug Content Determination within HME Films

Film samples (LNG and DPV from the single entity film) were weighed and dissolved in 50% acetonitrile, followed by vortexing at 1000 rpm until the films were completely dissolved. These samples were then centrifuged at 10,000 rpm for 10 minutes. The supernatant of all the samples was filtered using a 0.22 µm PTFE filter to remove polymer debris. The DPV and LNG drug content present in the film was detected using an appropriately designed HPLC analysis assay, as described above. The drug concentration of the film samples was calculated by measuring the peak area of the sample and comparing it to the peak area of a calibration curve with R^2=0.999.

5.3.4 *In Vitro* Drug Release

A dissolution assay was conducted using a USP apparatus IV (SOTAX CP7 smart, Sotax, Switzerland) with a flow rate of 16 ml/min. The dissolution media was 40% acetonitrile in distilled water, which met the sink conditions for both DPV and LNG. The studies were conducted at 37 °C for 60 minutes, with sampling at predetermined time intervals. At these time intervals, a 500 µL aliquot, was transferred to be analyzed using HPLC for drug content.

5.3.5 Puncture Strength and Moisture Content

Refer to chapter 2, method section 2.3.4.2- Titled: Puncture Strength.

Refer to chapter 2, method section 2.3.4.4- Titled: Moisture Content.

5.3.6 Film Disintegration

Refer to chapter 2, method section 2.3.4.3- Titled: Disintegration.

5.3.7 *In Vitro* anti-HIV Activity and Cellular Toxicity

Anti-HIV activity testing was performed using a TZM-bl cell based assay, as previously described [36]. A 1x2 inch DPV, LNG, combination LNG/DPV, or placebo film was dissolved in 2 mL of saline and ten-fold serial dilutions were made. DPV and LNG drug substances were used as controls. The evaluation was conducted as described in chapter 2, method section 2.3.5.2- Titled: Dapivirine Film: Bioactivity and *In vitro* Cellular Toxicity.

5.3.8 Compatibility with *Lactobacillus*

Lactobacillus crispatus and *jensenii* were used for the Standard Microbicide Safety Test (SMST) of the DPV, LNG and combination LNG/DPV films as described in chapter 2, method section 2.3.5.1- Titled: Dapivirine Film: *Lactobacillus* Toxicity.

5.3.9 Stability Assessment

To test the physical stability of DPV, LNG, and combination HME films, the films were stored at 25ºC/65% relative humidity (RH) for 24 months and at 40ºC/75% RH for 6 months. The films were tested at predetermined time points. At each time point, mass, thickness, appearance,

microscopic appearance, water content, puncture strength, disintegration, drug content, and dissolution were tested. In addition, *Lactobacillus* species compatibility and *in vitro* anti-HIV activity (TZM-bl cell based model) were tested at specific periods throughout the stability study.

5.3.10 *Ex Vivo* Permeability and Toxicity Evaluation

5.3.10.1 Tissue Exposure Studies

Human ectocervical tissue samples, collected from healthy volunteers undergoing routine hysterectomy, were obtained from the University of Pittsburgh Health Sciences Tissue Bank as per approved IRB protocol PRO09110431. The tissue was prepared similarly as described in chapter 3, method section 3.3.11.1- Titled: Tissue Exposure Studies.

5.3.10.2 Tissue Processing

The tissue was processed similarly as described in chapter 3, method section 3.3.11.2- Titled: Tissue Processing.

5.3.10.3 Hematoxylin and Eosin (H&E) Staining of Human Cervical Tissue Sections

H&E staining was conducted as described in chapter 3, method section 3.3.11.3- Titled: Hematoxylin and Eosin (H&E) Staining of Human Cervical Tissue Sections.

5.3.10.4 Tissue Extraction and Ultra-High Performance Liquid Chromatography-Mass Spectrometry Analysis

After the permeability assay, tissues were homogenized (Precellys 24 Homogenizer). The homogenized tissue was then subjected to liquid extraction. DPV and LNG were extracted from

the homogenized tissue using acetonitrile, methanol, methyl-tert-butyl ether (MTBE), 25% ammonium hydroxide (NH_4OH) solution, 0.9% sodium chloride (NaCl) solution, and MilliQ water. After liquid extraction, samples were dried under nitrogen (N_2) and reconstituted in 30% acetonitrile injection solvent for analysis. After sample preparation, samples were injected into a Waters Acquity ultra-high performance liquid chromatography (UHPLC) system connected to a Thermo Quantum Access Advantage MAX triple quad mass spectrometer (with electric spray ionization source) for analysis. The method utilized a Phenomenex Hyperclone 3u BSD C8 150x4.6 mm column. The mobile phases consisted of (A) 5 mM ammonium formate (NH_4FA) buffer: ACN (40:60) and (B) 5 mM NH_4FA buffer: ACN (20:80) with a 1 mL/min flow rate. The range for the standard curve was 0.2-50 ng/mL and was determined to be linear [36].

5.4 RESULTS

5.4.1 DPV, LNG, and LNG/DPV Film Formulation by the HME Technique

DPV, LNG, and LNG/DPV combination film prototypes were composed of 37.0% (w/w) Polyox N80, 37.0% (w/w) Klucel EF, 20.0% (w/w) PEG 4000, 2.0% (w/w) PEG 400, 2.0% (w/w) vitamin E. All films were off white, translucent, flexible and smooth (see Figure 5.1).

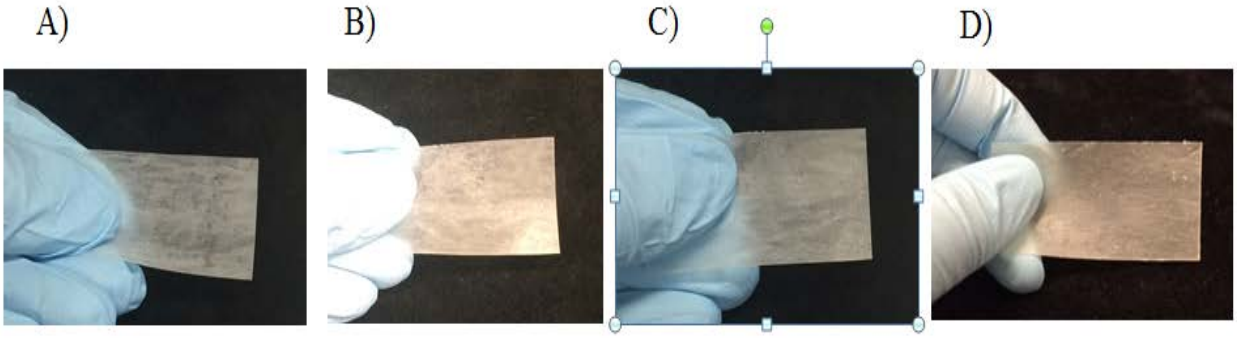


Figure 5.1: Visual Characteristics of HME Films (2''x1'').
A) LNG HME single entity vaginal film. B) DPV HME single entity vaginal film. C) HME vaginal MPT single layer film. D) HME vaginal MPT single layer film.

5.4.2 Film Characteristics

Film mass, thickness, drug content, water content, puncture strength, and disintegration were evaluated in this project, data summarized in table 5.2. The average weight and thickness of the LNG only film were 185.1 ± 11.1 mg and 160.0 ± 18.0 µm, respectively. LNG drug content was 1.7 ± 0.2 mg/film, and the water content (%) present in the film was 1.0 ± 0.1. Disintegration studies demonstrated a quick disintegration time of 82.3 ± 16.5 seconds, which indicates the quick dissolving nature of the film. The DPV only film has an average mass and thickness of 147± 10.1 mg and 158.0 ± 10.0 µm, respectively. DPV drug content was 1.3 ± 0.1 mg/film, water content was 2.0%, puncture strength of 3.4 ± 0.5 kg/mm and disintegration time of 46.6 ± 8.1 seconds. The two films were manufactured using the same HME process condition and the same formulation, and therefore, the films had similar characteristics.

The combination LNG/DPV single layer film was found to have average a mass of 163.8 ± 13.9 mg and average thickness of 156.7 ± 28.2 µm. The DPV and LNG content were found to be 1.4 ± 0.1 and 1.5 ± 0.1 mg/film, respectively. The water content remained less than 2% at 1.3 ± 0.1 (%), with a puncture strength of 3.5 ± 0.5 kg/mm and a disintegration time of 62.5 ± 7.8

seconds. The single layer film characteristics were similar to the single entity films, which indicates that the combination of the APIs in the single layer film did not have a significant effect on the film platform.

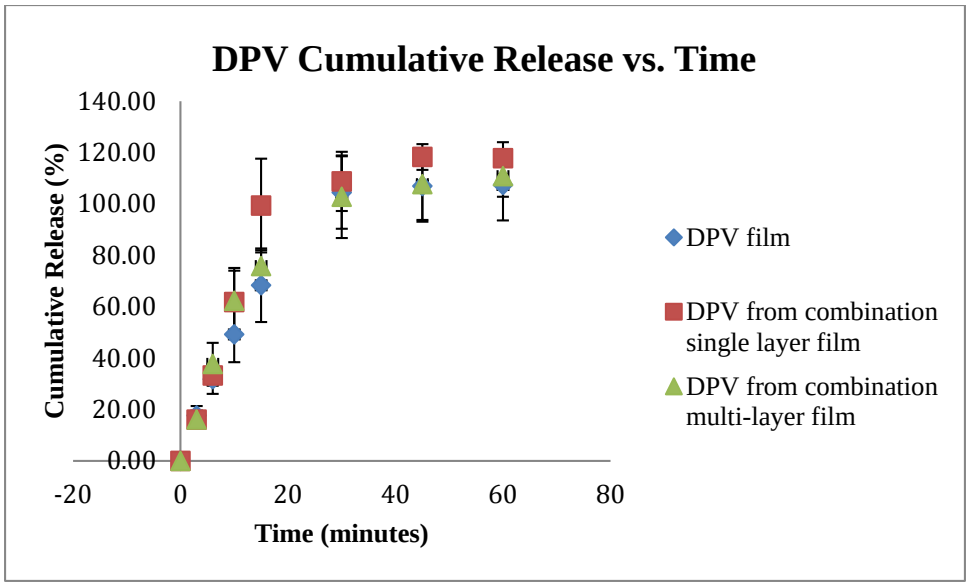
The multilayer combination film had an average mass of 335.4 ± 20.3 mg and average thickness of 338.9 ± 39.8 µm. The film mass and thickness were significant higher compared to the single layer film. This was because the film contained two layers of films in one platform. The drug content was found to be similar to the single layer film and to the single entity films (DPV 1.3 ± 0.2 mg/film and LNG 1.7 ± 0.2 mg/film). Water content and puncture strength were 1.2 ± 0.1% and 3.8 ± 0.4 kg/mm, respectively, which were similar to the single layer combination film and the single entity films. The multilayer disintegration time was not as quick as the single layer and found to be 237.2 ± 68.2 seconds. The longer disintegration time was due to the two layers of the film. The water and the probe used in this assay, need to penetrate through a much thicker film and therefore it was reported to have longer disintegration time.

Table 5.2: Summary Table for Film Characterizations

Parameter/ Film type	LNG HME film	DPV HME film	LNG/DPV single layer HME film	LNG/DPV multilayer HME film
Weight (mg)	182.9 ± 11.2	147.3 ± 10.1	163.8 ± 13.9	335.4 ± 20.3
Thickness (µm)	158.3 ± 15.9	158.0 ± 10.0	156.7 ± 28.2	338.9 ± 39.8
Drug content (mg/film)	1.7 ± 0.2	1.3 ± 0.1	DPV 1.4 ± 0.1 LNG 1.5 ± 0.1	DPV 1.3 ± 0.2 LNG 1.7 ± 0.2
Water content (%)	1.0 ± 0.1	2.0 ± 0.0	1.2 ± 0.1	1.2 ± 0.1
Puncture strength (kg/mm)	4.8 ± 0.2	3.4 ± 0.5	3.5 ± 0.5	3.8 ± 0.4
Disintegration (sec)	82.3 ± 16.5	46.6 ± 8.1	62.5 ± 7.8	237.2 ± 68.2

5.4.3 *In Vitro* Dissolution Assay

The *in vitro* dissolution assay showed that DPV and LNG were completely released from the films within 20 minutes after the films were first exposed to the dissolution media. The LNG and DPV release profiles were not affected by the presence of the other agent (Figure 5.2). This result was consistent in all 4 films: the single entity film and LNG/DPV combination film (single and multi-layer film).



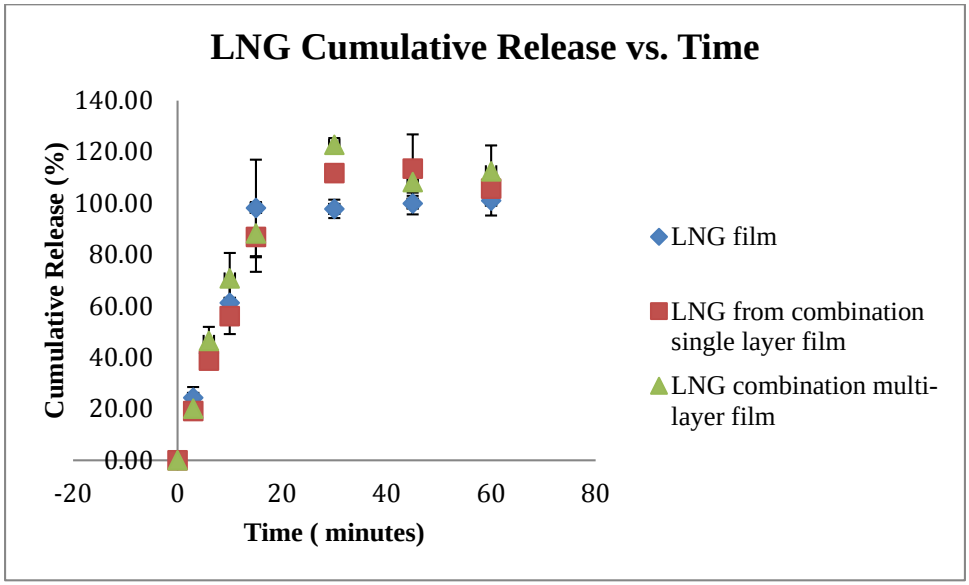


Figure 5.2: Dissolution Profile of LNG, DPV and Combination Film.

Top panel: DPV release profile from the single entity film and combination film (single and multi- layer film).

Bottom panel: The release profile of LNG from the single entity film, and combination film (single and multi- layer film).

5.4.4 *In Vitro* Cellular Anti-HIV DPV Film Activity

DPV anti-HIV activity was evaluated in the single entity film and combination film using an *in vitro* TZM-bl cell based assay. This assessment was conducted to ensure that the extrusion process, film formulation, and the presence of LNG had no impact on the antiviral potency of DPV. The DPV single entity film demonstrated similar bioactivity to the DPV drug substance; therefore, it can be concluded that the HME process did not affect the DPV bioactivity.

The DPV drug substance and the DPV single entity had a smaller IC_{50}, and found to be 2 nM (refer to Figure 5.3). The IC_{50} of DPV from the combination single layer film was found to be similar to the DPV from the multilayer combination film. However, comparing the DPV from the combination film to the IC_{50} value of the DPV single entity films and the DPV drug substance, the IC50 of the combination found to be much larger 27 nM (see figure 5.3). Since the IC_{50} of DPV in the single entity remained the same after the formulation and the HME process and the IC_{50} of the DPV in the presence of LNG was impacted. It can be suggested that the presence of LNG had an impact on the decrease of DPV bioactivity. Additionally, this *in vitro* cellular assay also showed no cellular toxicity of the HME film, placebo film, and DPV drug substance.

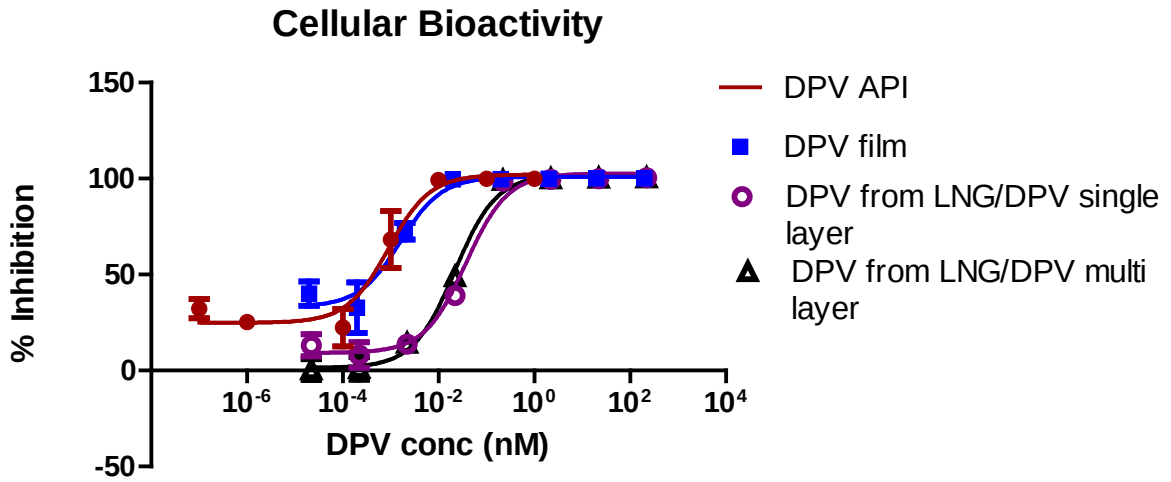


Figure 5.3: *In Vitro* Assessment of anti-HIV Activity of DPV Drug Substance, Single Entity HME Film and Combination HME Film in TZM-bl Cellular Assay.
DPV from the combination HME film maintained larger IC_{50} compared to the DPV drug substance after the formulation and extrusion process.

5.4.5 Compatibility with *Lactobacillus*

The compatibility of the film products with *Lactobacillus crispatus* and *Lactobacillus jensenii* was measured. As shown in table 5.3, the *Lactobacillus* species tested in this assay were unaffected by the exposure to the film products. To be considered "unsafe," the loss in viability must decrease by at least a log difference. Results showed that all four films were non-toxic to the *Lactobacillus* strains, and therefore, no loss of bacterial viability was observed.

Table 5.3: Evaluation of LNG and DPV Single Entity HME Films and Combination Films with *Lactobacillus*

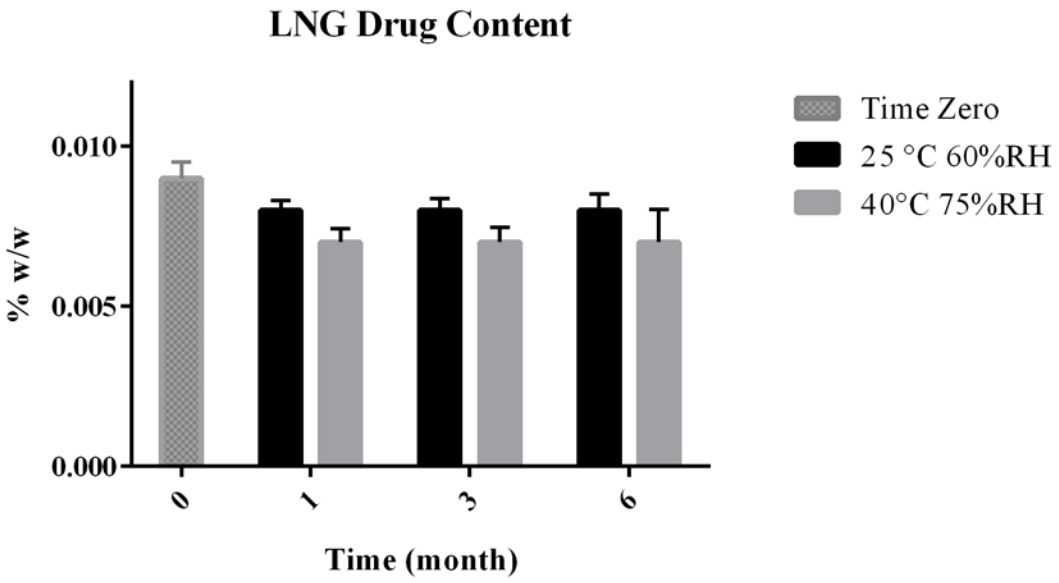
Increase in bacterial viability (CFU) represented in positive value, decrease in viability (CFU) represented in negative value. Log differences indicated product toxicity to the *Lactobacillus*.

Lactobacillus strain tested	LNG HME Film	DPV HME Film	LNG/DPV Single Layer Film	LNG/DPV Multilayer Film
L. jensenii ATCC 25258	0.106	-0.032	0.036	-0.003
L. jensenii LBP 28AB	0.021	-0.017	-0.027	0.003
L. crispatus ATCC 33197	0.078	-0.127	0.059	0.041

5.4.6 Stability Assessment

All 4 prototype films were placed on two years stability study in 25°C/ 60% relative humidity (RH) and six months in 40°C/ 75% RH. To date, six months of stability evaluations were conducted, and the films were found to be stable at both conditions. A two-way ANOVA was conducted. Time was defined as a continuous variable and condition as a nominal variable. Figure 5.4 depicts example data from the stability study. The graph demonstrates the drug content (%w/w) over time at the two different storage conditions of the stability evaluation. In general, no significant changes in film characterization or drug content were observed.

A)


LNG Drug Content
0.010
0.005
0.000
% w/w
0
1
3
6
Time (month)
Time Zero
25 °C 60%RH
40°C 75%RH

B)

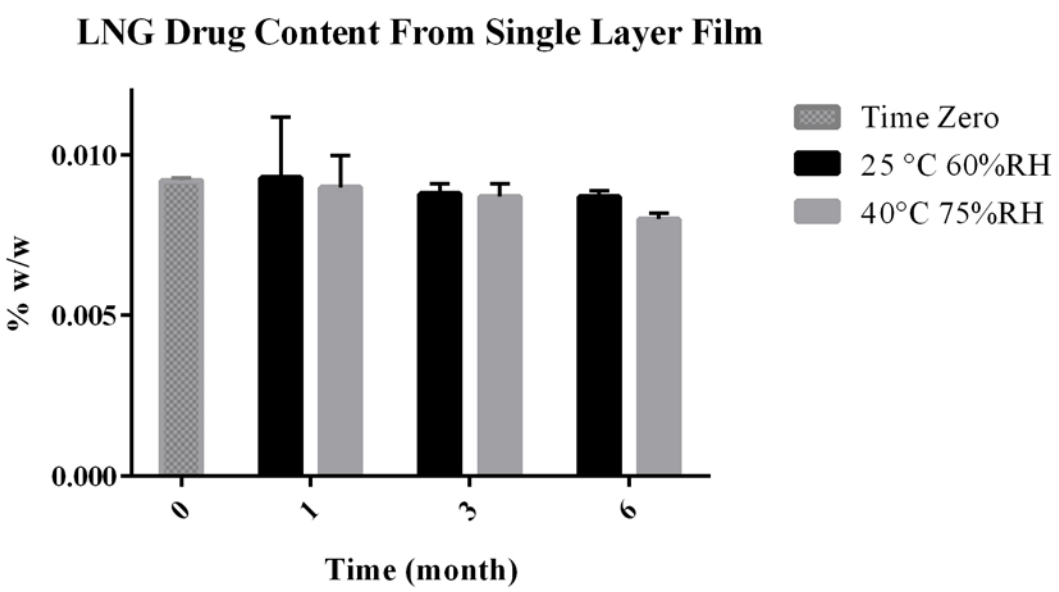

LNG Drug Content From Single Layer Film
0.010
0.005
0.000
% w/w
0
1
3
6
Time (month)
Time Zero
25 °C 60%RH
40°C 75%RH

Figure 5.4 Continued

C)

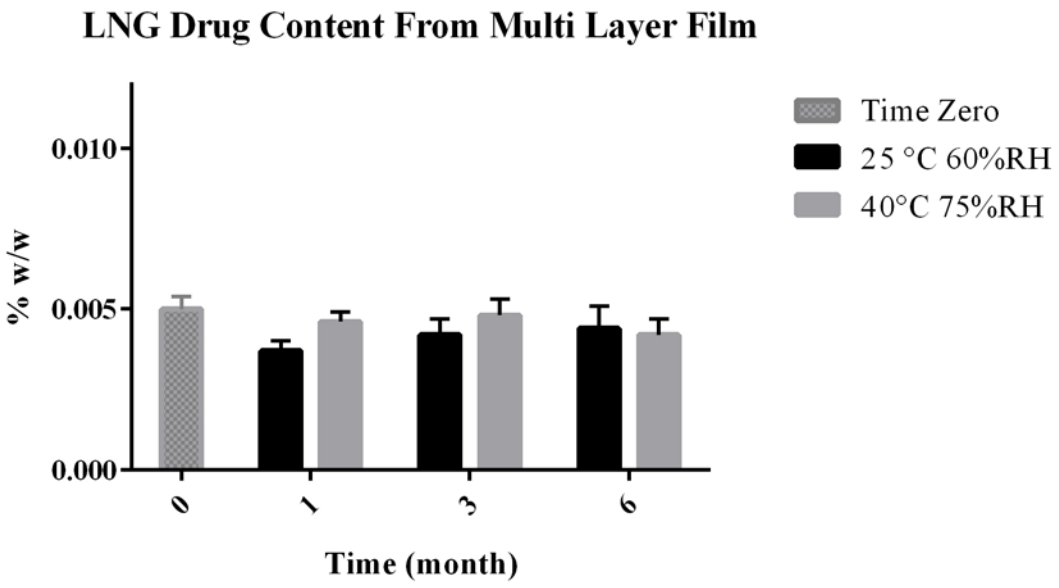


Figure 5.4: Representative Graph of the Stability Study. Drug Content of LNG from LNG HME Film, and Combination Film.

No changes were observed in drug content (%w/w) over time determined by measuring LNG using HPLC assay during predetermined time points A) LNG drug content from the LNG HME single entity film. No effect of time (F= 0.1981), condition (F=0.1929), or time*condition interaction (F=0.7890). B) LNG drug content from the LNG HME single layer MPT film. No effect of time (F= 0.0137), condition (F=0.5497), or time*condition interaction (F=0.3202). C) LNG drug content from the LNG HME multilayer MPT film. No effect of time (F= 0.2106), condition (F=0.1036), or time*condition interaction (F=0.2551).

5.4.7 *Ex Vivo* Permeability and Toxicity Evaluation

Excised human ectocervical tissue was exposed to the single entity films (LNG and DPV) as well as the combination films (multi and single layer films). The results confirmed that DPV was present in the tissue after the exposure to the films (table 5.4). A two-way ANOVA was conducted. The film type was defined as a nominal variable and amount of DPV in the tissue as a continuous variable. The amount of DPV in the tissue treated with the single layer combination film was not significant different compared to the DPV present in the tissue treated with DPV

single entity film. On the other hand, the amount of DPV present in the tissue treated with the multilayered film was significantly (p=0.0283) lower when compared to the DPV HME single entity film. This can be attributed to the large amounts of polymers present in the multilayer film samples (table 5.4).

Table 5.4: Amount of DPV in the Excised Human Tissue after 6 Hours Exposure Test

	DPV HME film	DPV single layer film	DPV multilayer film
Amount of DPV found in the tissue (µg/g)	5.818 ± 2.717	3.804 ± 2.667	1.389 ± 0.953*

Two-way ANOVA was conducted. The DPV present in the tissue treated with the multilayer film was significant lower (p= 0.0283) when compared to the single entity DPV film and single layer combination film. *$p \leq 0.05$ to be considered significant.

5.4.8 DPV HME Film Safety Evaluation on Human Excised Tissue

Evaluations were conducted to determine if the combination LNG/DPV HME films caused morphological changes on the epithelial layer of the vaginal wall post-exposure to the films. The epithelial layer of all exposed tissues remained intact and maintained structural integrity (see Figure 5.5). No morphologic changes were detected after the exposure of the HME films the tissue, when compared to the pre-exposed tissue.

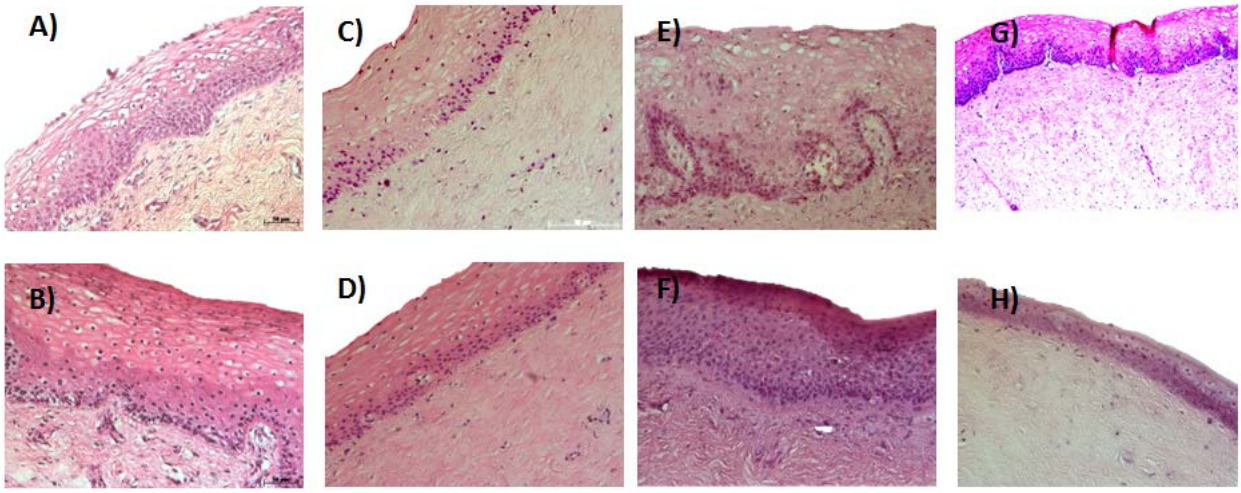


Figure 5.5: Representative Images of H&E Staining of Epithelium Pre- and Post-Exposure to the MPT HME Films.

H&E stained tissue pre- and post- 6 hours exposure to the HME films. (Images were taken using 10X and 20X magnification) tissue images of A) Pre-exposed tissue to the DPV HME film treatment, B) Tissue exposed to DPV HME film, C) Pre-exposed tissue to the LNG HME film, D) Tissue exposed to LNG HME film, E) Pre-exposed tissue to the LNG/DPV single layer HME film, F) Tissue exposed to LNG/DPV single layer HME film, G) Pre-exposed tissue to the LNG/DPV multi-layer HME film, H) Tissue exposed to LNG/DPV multi-layer HME film. No significant changes in the tissue morphology were observed post exposure to the films compared to the pre- exposed tissue.

5.5 DISCUSSION AND CONCLUSION

The goal of this project was to explore the potential of HME for the development of a multi-purpose film providing both contraception and HIV prevention. This project evaluated the HME extrusion process as optional manufacturing for MPT product. One of the major advantages of the HME is its ability for continuous manufacturing. This has implications and is desirable for all pharmaceutical products. Regulatory bodies, such as the Food and Drug Administration (FDA), encourage drug manufacturers to invest in process monitoring during manufacturing of products.

As a continuous process, HME fits perfectly within this initiative since the system can be monitored during ongoing production. Another advantage offered by the HME is the ability to produce a two- layered film, with each layer containing a single entity API that can be released at a specific rate.

LNG single entity HME film, DPV single entity HME film, and LNG/DPV combination HME films were developed. The developed films were characterized for physicochemical properties, bioactivity, and toxicity. Physicochemical characteristics included weight and thickness of the films, the drug content, water content, puncture strength, disintegration and dissolution of the film. The single layer HME films had a similar mass (mg) and thickness (µm), and the multi-layer HME film mass and thickness was nearly doubled, given that it is an overlay of two single entity films. The target-loading dose for DPV was 1.25mg, the amount of drug administered in a single dose in the clinically-advanced DPV vaginal gel [39, 102]. The loading dose for LNG was matched to DPV vaginal film loading dose. Currently, there is no target administration dose defined for efficacy from topically administered LNG [131]. Therefore, a study needs to be conducted to determine the effective dosing for topical contraceptive administration. Given the lack of available information, dosing was extrapolated from the reported amount delivered from currently investigated MPT [7].

The low water content (%) and the puncture strength (Kg/mm) were similar across all HME film developed in this project. The low water content serves primarily to enhance film stability and handling. Additionally, the puncture strength of the films was similar amongst all films. This suggests that the puncture strength of the films were primarily a function of the polymeric base used and not based on individual API properties. Furthermore, the disintegration of the single layer combination film was similar for all single entity films (DPV and LNG only

films and the single layer combination film). During disintegration the probe applies pressure onto the film in the presence of small amount of water. The slower disintegration time of the multi-layer film results as a function of the slower hydration and penetration of the water within the polymeric matrix. Therefore, the force applied by the probe took longer to break through this multilayer film composed of two layers of polymeric sheet. Rapid release of DPV and LNG was confirmed by the dissolution profile obtained in *in vitro* release testing. The rapid and complete release of the APIs from the film is crucial since the rapid release allows the APIs to reach into vaginal environment within minutes post film insertion, for a fast therapeutic effect. Additionally, the release of the two APIs occurred with no drug-drug interaction post release of the film. This is important for this product since both DPV and LNG must be fully released from the film for the simultaneous protection against HIV and unintended pregnancy, without each interfering in release of the other.

The DPV anti-HIV activity was determined using the TZM-bl cellular assay to confirm that the formulation, HME process, and presence of LNG did not impact the antiviral activity of DPV. The DPV single entity HME film showed similar anti-HIV activity to the API drug substance alone, and was reported to be 2nM. This indicates that the formulation and the HME process did not impact the DPV activity. Additionally the IC_{50} reported in this section is similar to previous reports (DPV IC_{50} of 2.2nM) [135].

The LNG single entity HME film and LNG drug substance showed inhibitory activity against HIV-1_{Bal} at high concentrations in this cellular model. The LNG inhibitory effect in this model is likely attribute to the progesterone-driven anti-proliferative effect observed in epithelial cell line such as TZM-bl line [136]. However, in previously published results it was reported that LNG had no direct activity against HIV-1 when using PM-1 $CD4^+$, which do not express

progesterone receptor. In the combination film study, the DPV activity demonstrated decrease in potency when LNG was present. This phenomenon could also be due to the anti-proliferation effect of LNG on this cell model. Since HIV replication depends on replication of its target cell, and LNG inhibits proliferation of epithelial derived cells, such as TZM-bl, it is expected that HIV activity would decrease. Therefore, the apparent decrease in DPV activity demonstrated in this model is likely due to LNG's effect on the TZM-bl cell line. Since TZM-bl cells infected with HIV-1_{Bal} were used as control cells, and the anti-HIV activity of LNG and DPV were measured as a decrease in luciferase signal from controls, this experimental model did not control for LNG's anti-proliferative effect on the TZM-bl cells. Therefore, the observed decrease in DPV potency is likely due to the limitations of the assay. Furthermore, it was reported by Evans et al. that when using PM-1 $CD4^+$ or human PBMC, which do not express progesterone receptor, for the evaluation of LNG-DPV drug-drug interaction, LNG did not alter the anti-viral activity of DPV against HIV-1 [136].

Ex vivo tissue permeability and toxicity evaluations were conducted. In this assessment, the DPV found in the tissue confirmed that the LNG present in the combination films did not impact the DPV permeability. The DPV concentrations per mg of tissue was found to be comparable to that of the clinically-advanced DPV ring (680 ng/gm (cervix)) [39, 137]. This is a promising result since the DPV ring is a well-established delivery system currently seeking to be licensed.

This is the first time an MPT film product has been developed, and therefore, toxicity evaluation of the HME film product was crucial to this study. All films-LNG HME film, DPV HME film, and LNG/DPV single and multi-layer film- were found to be compatible with the common *Lactobacillus* present in the vagina (*L. jensenii and L. crispatus*) utilizing Standard

Microbicide Safety Test (SMST). The cellular toxicity assay, which utilized a TZM-bl cell model, demonstrated film safety. Furthermore, product exposure studies utilizing human excised ectocervical tissue showed no gross morphological changes post 6 hours exposure period to the film. These safety assessments suggest that the LNG HME film, DPV HME film, and the combination films may be safe to the microenvironment and epithelium of the vagina.

Overall, HME was utilized to manufacture LNG/DPV single and multi-layer films. Films were evaluated for physicochemical, biological, and safety characteristics. The films maintained acceptable characteristics while demonstrating bioactivity and low toxicity. This project is a milestone in the development of a MPT product and the incorporation of DPV and LNG for the simultaneous prevention of HIV transmission and unintended pregnancy using a film platform. It can be concluded that the HME can be utilized for the development of single and multilayer films containing two small hydrophobic molecules with different bioactivities and pharmaceutical indications.

5.6 ACKNOWLEDGMENT

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Rohan’s lab members: Phillip Graebing at the University for developing the DPV LNG analytical assay utilizing LC/MS, Dr. Sravan Patel and Kunal Jhunjhunwala for assisting in the large scale manufacturing batches, Michael De Miranda and Taryn Serman for assisting with tissue staining and sectioning,

6.0 OVERALL USE OF HOT MELT EXTRUSION FOR THE MANUFACTURE OF VAGINAL FILMS, MAJOR FINDINGS, LIMITATIONS AND FUTURE DIRECTIONS.

6.1 INTRODUCTION

The vagina provides an effective route for local and systemic delivery of drug products and can accommodate many different delivery systems. The vaginal route is traditionally used for the delivery of pharmacologically active agents intended for the treatment of vaginal infections, contraception, and hormonal intervention [2, 4]. Factors such as its large surface area and the rich blood supply of the vaginal mucosa, make the vagina an optimal route for drug delivery [1]. Vaginal drug administration is noninvasive, can avoid first pass metabolism and limits side effects that can be caused by systematic exposure [1, 2].

There are several delivery systems that are currently on the market including gels, intra-vaginal rings, douches, inserts, creams, suppositories, and films. While all of these dosage forms provide effective vaginal delivery, films are small volume solid dosage forms, which offer many benefits over other traditional dosage forms. Advantages of films include their discreet nature, minimal product leakage during use, no requirement for an applicator, low product volume with minimal impact on the innate factors in the vagina, rapid drug release, and minimal packaging, all which make it a very desirable dosage form [28]. Manufacturing techniques and formulation strategies can both impact final product attributes and overall functionality of this highly

advantageous dosage form. Traditionally, vaginal films are manufactured utilizing solvent casting method. However, there is a potential use of hot melt extrusion (HME) for the manufacturing of vaginal films since this technology has been used in the manufacture of polymeric based products such as medical devices.

The major research goal of this dissertation work was to understand the potential of hot melt extrusion for the manufacture of films, which are capable of delivering biomolecules, small molecules, and their combinations.

The research conducted in this work was divided into two main sections (Aims 1 and 2) that were divided into two parts (A and B). The goal of Aim 1 was to select a range of model pharmaceutical APIs which would demonstrate the feasibility of the HME manufacturing process. The objective of Aim 1 part A (chapter 2) was to formulate individual APIs, which were selected based on their physicochemical properties and pharmaceutical functionalities, into a film dosage form manufactured using the HME. The second part of Aim 1, part B (chapter 3), was to understand if this process is capable of producing films that were comparable to clinically advanced films manufactured using the traditional solvent cast technique. The second main part of this dissertation work, Aim 2, was a proof-of-concept study for multilayer film manufacture using the HME. In part A, (chapter 4) a multilayer film containing a probiotic and antibiotic was developed. The film was a physical barrier to keep these two incompatible active agents separate, as probiotic agents would lose activity if interaction occurred. Other main studies in this aim (chapter 5) focused on the development of a film delivery system, which allowed separate release profiles of two compounds with different pharmaceutical activity. Overall, these studies aim to clearly demonstrate the practicality and promise of HME in vaginal film product design and manufacturing.

6.2 SUMMARY OF MAJOR FINDINGS

Within the scope of this dissertation work, HME was evaluated for its potential as an alternative manufacturing technique for vaginal films containing a variety of APIs. This technique was evaluated for its ability to accommodate pharmaceuticals for local vaginal delivery, its proficiency in produce comparable polymeric film to current film products in development and its capacity to manufacture novel multilayer delivery systems.

Through the successful formulation development of APIs, which differed based on size, hydrophobicity, and pharmaceutical target, HME proved to be an effective manufacturing technique. After film manufacturing, films were characterized for physical (mass and thickness evaluation, puncture strength and disintegration assessment), chemical (drug content and uniformity) and biological evaluations (bioactivity, structural, binding evaluation and viability). All HME films were soft and flexible as the end product extrudate. These studies confirmed that acceptable physical attributes of the films were achieved using the HME for manufacturing process. The drug content and uniformity evaluation established that the film matrix selected allows the uniformity of the extruded film and the drug levels fit within the target specification of the loading dose for the film. This supports the research goal that the HME can be utilized to produce vaginal films with desirable target dosing level and drug uniformity in extruded vaginal films. The extruded film had followed a first order release profile, which is typical for quick release SC vaginal films, thus proving that the HME did not alter the release of the API from the film matrix. These data further support the hypothesis that HME can be used as alternative manufacturing technique and produce a comparable film to that of the SC films.

In order to prove that the extrusion process did not alter the activity of the active agent, the bioactivity of each API was evaluated in assays relevant to specific therapeutic purpose.

Efficacy of the formulated dapvirine in a TZM-bl cellular model (IC_{50}), cellular toxicity in this same model and impact on innate *Lactobacillus* species in a SMST assay were not altered through the formulation and extrusion process. These results demonstrated the functionality of HME as a manufacturing process while capable to retain the safety and the anti-HIV activity of dapivirine HME films in cellular models.

The HME metronidazole film was able to accommodate drug levels needed to achieve therapeutic efficacy and demonstrated effective antibiotic activity through a comparable inhibition profile to the metronidazole drug substance. These results prove that the HME can be used for the manufacturing of a hydrophilic molecule and produce films with large loading dose while maintaining activity.

The large molecule selected for this study was the protein griffithsin (GRFT). Due to the wide range of process parameters that can be selected using HME, this heat sensitive molecule could be extruded using the non-traditional setting of low heat and sheer. GRFT evaluation confirmed intact protein structure, purity and size, which led to the maintenance of binding ability to gp120 (HIV envelop protein). It can be concluded from this study that the HME can be used to manufacture a vaginal film containing a large molecule protein such as GRFT, while maintain its tertiary structure, therapeutic binding pocket and therefore overall efficacy.

Lactobacillus species, which represents one of the most complex and challenging species to formulate, was selected for this project. Initially the bacteria HME formulation yielded no bacteria viability post extrusion process. However, after reducing the heat and the shear of the extrusion process, acceptable bacterial-loaded films were developed. The *Lactobacillus* were found to be viable and able to produce hydrogen peroxide and lactic acid post extrusion process. A novel dissolution assay was developed to study the release profile of bacteria from the film

utilizing the flow cytometry technique. Using this assay, the bacteria were separated from the film formulation based on the size and granularity of the bacteria. The results from this chapter demonstrated the ability to utilize the HME for the development of film containing very sensitive and complex molecule for vaginal film delivery.

Overall, in this chapter a variety of APIs were selected to evaluate the versatility of the HME as manufacturing process for vaginal films. The resultant film characteristics confirmed the applicability of the HME to the manufacturing of the vaginal films since films demonstrated acceptable physicochemical characteristics and desired bioactivities. This chapter established the functionality and versatility of HME for the production of vaginal film. This manufacturing process has the promise to open the possibilities of world-wide global implication for wide spread application of vaginal film

In chapter 3, a comparison evaluation between the solvent cast (SC) DPV vaginal film and the HME DPV vaginal film was conducted. Studies from this chapter demonstrated that the HME DPV film had similar physicochemical characteristics as the SC film in terms of safety (TZM-bl cellular assay, SMST assay), bioactivity (TZM-bl cellular assay), permeability (human ectocervical excised tissue) and physiochemical properties (mass and thickness, puncture strength, water content, dissolution). Additionally, HME process evaluation was conducted applying quality by design approach. The process analysis revealed that the HME is a robust method for vaginal film manufacturing while alteration of the screw speed can be utilized for modifications of the final film attributes. These results demonstrate that HME is a highly functional and equivalent technique for the manufacture of film containing a clinically advanced drug candidate, DPV.

In the second part of this dissertation work (chapters 4 and 5) HME application was utilized to manufacture multilayer vaginal films for the delivery of two APIs. In chapter 4, a vaginal film containing both the *Lactobacillus jensenii* and metronidazole in separate layers was developed. Active agents were successfully incorporated into and released from films while they retained bioactivity, which was measured through two different microbiological based assays.

The results from this study confirmed the successful formulation of combination therapy for diseases such as bacterial vaginosis while maintaining the antimicrobial and the probiotic functionality. Additionally, this is the first proof-of-concept study for co-delivery system utilizing the multilayer films as a delivery platform.

In chapter 5, the application of HME was used to develop and manufacture combination films (single and multilayer) for the simultaneous delivery of two pharmaceutical agents – dapivirine (DPV) and levonorgestrel (LNG). LNG single entity HME film, DPV single entity HME film, and LNG/DPV combination HME films were successfully manufactured and these compounds retained activity while avoiding interaction within the film, during release and permeability evaluation. The anti-HIV activity of DPV was assessed in the present and absent of LNG using TZM-bl cellular assay. The DPV single entity HME film showed similar anti-HIV activity to the API drug substance alone. This is an indication that the formulation and the HME process did not alter the DPV activity. However, the DPV potency decreased in the presence of LNG indicating that the LNG had inhibitory effect on DPV potency. However, these data highlighted a limitation of the TZM-bl cellular model as LNG impacted cellular proliferation in this model. Furthermore, all films were found safe during the *Lactobacillus* compatibility study, TZM-bl cellular safety assay and tissue exposure study. These findings support the use of the HME as a manufacturing technique for the production of safe films. The work conducted in this

chapter is pioneering work in the development of multilayer films for the simultaneous prevention of HIV transmission and unintended pregnancy in one single, highly acceptable, on demand dosage form. From this work it can be concluded that the HME can be utilized for the development of multilayer films containing two small molecules with different therapeutic applications.

6.3 CONTRIBUTION TO THE FIELD

In this dissertation work the HME process was evaluated for the application of vaginal film manufacturing. The addition of the HME manufacturing process to the existing SC can broaden production options for films and also provide a means of manufacture for multilayer films for multiple therapeutic applications. As one of the most common manufacturing techniques in the food, plastic, and rubber industries, HME is widely distributed worldwide. This can open the potential for local manufacturing in countries with limited resources and decrease the need for the importation or exportation of highly efficient pharmaceutical products. As learned through the formulation of numerous films, HME film development requires less time than solvent cast film development since neither the API nor the other excipients require solubilization in order to be formulated into the film dosage form. Low solvent use in the HME production can eliminate the drying time required in the SC production. This allows for a smaller manufacturing footprint, and faster film production as well.

The novel application of HME to the field of vaginal pharmaceutics is beneficial not only thought the conservation of resources, but also through the ease of manufacturing scalability. Transition from small bench scale manufacturing to large batch manufacturing has proven to be a

hurdle in pharmaceutical development and manufacturing, especially in solvent cast film manufacture. The HME scale-up is simple compared to the SC as it is a dry process where no fluid dynamic is involved. There are minimal differences between most HMEs regardless of their use for small or large scale manufacturing, which allows seamless transitions during scale-up.

HME was validated as a manufacturing technique for the production of single-layer vaginal films for delivery of a range of individual APIs. This work also represents the first time, to our knowledge, that the HME process has been applied for the production of a multilayer vaginal film. The successful development of several single layer films containing variety of individual APIs provides support for further development of films for a wide range of therapeutic applications. This work proves that the HME can be used for the development of highly versatile vaginal films that can incorporate ranging loading doses, stabilize APIs regardless of chemical properties and deliver two agents simultaneously or in sequence. The knowledge developed from this dissertation work, can be applied to any vaginal or oral film delivery such for the prevention of sexual transmitted disease, prevention of pre-term birth and cancer.

Incorporation of bacteria into HME films show that heat and shear sensitive molecules, while challenging to formulate, are still viable upon extrusion. Conceptually, the extrusion of a highly sensitive microorganism can be applied for future formulation studies involving highly sensitive and complex molecules like various probiotics in order to promote vaginal health. Additionally, in this work, a large molecule protein was successfully incorporated into the vaginal film. This shows that the HME can be utilized for the manufacturing films containing large molecules, which can be applied to other large molecule formulation such as the development of antibody containing films for the prevention of HIV.

Multilayer film development in this dissertation work provided significant evidence for vaginal drug delivery strategies in which the release profile of each active agent needs to be tailored based on therapeutic target and prevent possible interaction as needed. The potential applications of this combination strategy are immense and highly promising. This technology can be applied to the field of bacterial infections, in which one agent would eradicate the infection and a delivery of a subsequent agent would promote the restoration of the natural vaginal environment. Multiple vaginal targeting is also needed in HIV prevention approaches where local vaginal targets range from lumen to epithelial tissue layers. Additionally, the two layers film can help with the prevention of drug-drug interactions, while enhancing user acceptability since it can consolidate multi-product usage.

6.4 LIMITATIONS

While this work is extremely promising for the implementation of HME for vaginal film manufacture, it is important to recognize that with any drug delivery strategy and production development there are limitations that need to be considered.

Process limitations specific to HME include a limited polymer selection for vaginal films, which can withstand the conditions of extrusion and can provide acceptable tactile properties. Polymer selection is critical in the production of polymeric film since the selected polymer must be amenable to the process technology. There are only a few available polymers that can be extruded at relatively lower temperatures (50 °C-180 °C) to maintain API stability.

Additionally, the heat and shear involved in the HME process can limit the APIs which can be manufacturing using this process and, or, the need for additional preparation steps, such

as API coating or preservation prior to the HME processing. The process also involves a high volume of waste and therefore, during the development work it requires large amounts of APIs and raw materials. Some APIs are very expensive to manufacture, thus may not be suitable for HME research and development work.

There are no standardized evaluation assays for HME vaginal film characteristics and therefore, acceptable characteristics in this work were subjective to our lab's film target specification profile.

Specific experimental limitations were found mainly in the bacterial film development and tissue assays. While the vagina is comprised of multiple *Lactobacillus* species only one bacterial species was used in the probiotic film development. Further studies with various bacterial species would strengthen evidence supporting the successful vaginal probiotic film. Another limitation found in this work is the use of TZM-bl for the evaluation of DPV anti-HIV activity in the presence of LNG. Since LNG can inhibit the proliferation of this cell line, it can affect the true results of the DPV potency in the film and therefore, a non-epithelial based cellular model should be used. Last, the flow cytometry used in these studies for the evaluation of bacteria release from the film cannot distinguish between viable bacteria to non-viable bacteria and therefore, during the study bacterial samples must to be collected for viability evaluation.

6.5 PROPOSED FUTURE DIRECTIONS

A few future studies were identified from this dissertation work. The studies will help to further understand the application of HME to the vaginal film and contribute to the pharmacological evaluation of these vaginal films.

1) The vaginal microflora is composed of multiple beneficial stains of *Lactobacillus* species. They are the predominant bacteria within the vagina and help to maintain a low pH through the production of lactic acid. In addition, some of the *Lactobacillus* species can produce hydrogen peroxide and together with the low pH can prevent growth of harmful pathogens in the microflora. It would be beneficial to develop an assay that can quantify the amount of the lactic acid and hydrogen peroxide production in order to develop the appropriate bacteria composition and concentration per film.
2) There is no animal model that has the exact bacteria species as the human microflora and therefore it might be difficult to assess the toxicity and efficacy of the combination antibiotic and probiotic film in current animal models. It would be beneficial to develop a humanized animal model by recolonizing the human bacteria and study the film efficacy and toxicity.
3) Since the TZM-bl cell assay limited the true evaluation of DPV anti-HIV activity in the present of LNG, an immune based cell model not expressing the progesterone receptor (e.g. PM-1 CD4+ T cell lines) should be studied.
4) The LNG DPV combinations were evaluated *in vitro* for human tissue exposure. Further large animal (macaque) pharmacokinetic studies should take place to evaluate the functionality of the film, efficacy, and toxicity. The DPV pharmaceutical target is in the tissue, and the LNG pharmaceutical target is in the blood and studies need to be

conducted to test localization of these compounds to their target sites. Data from the *in vivo* study should be compared to the *in vitro* evaluation for further model analysis.

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