**Universidade do Minho**
Escola de Ciências

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Dissertação de Mestrado
Mestrado em Bioquímica Aplicada

Trabalho efetuado sob a orientação do
Doutor José das Neves
Professor Doutor João Marcos

Outubro de 2017

DECLARAÇÃO

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Assinatura:

*‘Treino duro, combate fácil’*

*Sargento Saldanha*

**Para o meu avô Ramiro, que Deus o tenha, e para os pilares da minha vida**

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# RESUMO

O vírus da imunodeficiência humana (VIH) infecta milhares de pessoas todos os anos, sendo que as mulheres, habitualmente infetadas pela via de transmissão heterossexual, constituem metade dessa população. Infelizmente não existe uma cura e, como tal, a prevenção tem sido um elemento crucial na erradicação do VIH. A profilaxia de pré-exposição (PrEP) oral, comercializada como Truvada®, foi recentemente aprovada em vários países, mas a sua eficácia nas mulheres tem sido pouco satisfatória. Isto deve-se não só a fatores biológicos, mas também a problemas relacionados com a adesão a este método de prevenção. Deste modo, é relevante o desenvolvimento de alternativas profiláticas. A administração tópica dos fármacos anti retrovíricos usados no Truvada® pode ser uma opção, mas para tal é necessário que a plataforma de entrega seja adequada. Neste trabalho, o sistema de nanopartículas-em-filme é proposto como uma plataforma apropriada para a entrega simultânea de tenofovir disoproxil fumarato e emtricitabina. Para a produção das nanopartículas com fármaco foram considerados dois métodos, nomeadamente, dupla emulsão/evaporação do solvente para a produção de nanopartículas de poli(ácido lático-co-ácido glicólico) (PLGA) e spray-drying para a produção de partículas de Eudragit® L100. Embora ambas apresentassem propriedades coloidais consideradas adequadas, apenas as partículas de Eudragit® L100 (aproximadamente 687 nm) foram capazes de associar, com sucesso, ambos os fármacos (2.5% e 5.4% para a emtricitabina e para o tenofovir disoproxil fumarato, respetivamente). Filmes constituídos por álcool polivinílico e pectina foram produzidos e caracterizados em termos de propriedades organoléticas, físico-químicas e mecânicas. Posteriormente, as partículas foram incorporadas nestes filmes, através da sua dispersão manual e recorrendo a uma prensa para unir os dois filmes. O filme resultante apresentava propriedades consideradas adequadas para uso vaginal, em particular, uma libertação de fármaco durante pelo menos 48 h. Estes resultados sugerem que o sistema nanopartículas-em-filme pode ser útil para um microbicida de administração independente do coito e cuja proteção contra o HIV é prolongada. Para além disto, os filmes apresentaram ainda baixa toxicidade in vitro. De um modo geral, o sistema nanopartículas-em-filme aqui proposto parece adequado para a entrega vaginal de TDF e FTC, sendo um potencial alternativa ao uso do PrEP oral, Truvada®, na prevenção da transmissão vaginal do HIV.

PALAVRAS-CHAVE: HIV, PREVENÇÃO, MICROBICIDAS, FILMES VAGINAIS, NANOPARTÍCULAS

# ABSTRACT

The human immunodeficiency virus (HIV) infects millions of people every year. Among those, women account for half of these numbers and heterosexual transmission is the predominant mean of infection. Unfortunately, no cure is available, thus making prevention a cornerstone in the battle against HIV. Oral pre-exposure prophylaxis (PrEP) with daily Truvada® (tenofovir disoproxil fumarate/emtricitabine) has been recently approved in various countries but its efficacy in women is less than optimal, namely due to biological and adherence issues. Thus, alternatives to this preventive modality could be helpful. Topical delivery of the active drugs in Truvada® could be an option but proper selection of delivery platform is required. In this work, nanoparticles-in-film systems are proposed as suitable platforms for the combined vaginal delivery of tenofovir disoproxil fumarate and emtricitabine. Two different approaches were used initially considered for the production of drug-loaded nanoparticles, namely a double emulsion/solvent evaporation method for the production of poly(lactic-co-glycolic acid) (PLGA)-based particles and a spray-drying method for the production of Eudragit® L100-based nanoparticles. Although particles featuring acceptable colloidal properties were obtained, only Eudragit®-based particles (around 687 nm) allowed obtaining suitable association levels of both drugs (2.5% and 5.4% for emtricitabine and tenofovir disoproxil fumarate, respectively). Films based on poly(vinyl alcohol) and pectin were developed and characterized regarding organoleptic, physical-chemical and mechanical properties and considered for particle incorporation. Nanoparticles were physically dispersed in between two sheets of pre-formed film and the system consolidated by using a hydraulic press. The resulting film presented characteristics considered acceptable for vaginal use and, in particular, featured prolonged drug release for at least 48 h. These results suggest that nanoparticles-in-film may be helpful in achieving a coitus-independent microbicide product that could provide extended protection from HIV transmission. Also, films presented low in vitro cytotoxicity. Overall, proposed nanoparticles-in-film system appears to be suitable for the vaginal delivery of TDF and FTC and may be useful as an alternative to oral PrEP with Truvada® in preventing HIV vaginal transmission.

KEYWORDS: HIV, PREVENTION, MICROBICIDES, VAGINAL FILMS, NANOPARTICLES

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# LIST OF ABBREVIATIONS AND SYMBOLS

| Abbreviation | Designation |
|---|---|
| **AIDS** | Acquired Immune Deficiency Syndrome |
| **AE** | Association Efficiency |
| **ANOVA** | Analysis of Variance |
| **ARV** | Antiretroviral |
| **ATCC** | American Type Culture Collection |
| **cART** | Combination Antiretroviral Therapy |
| **EMA** | European Medicines Agency |
| **FBS** | Fetal Bovine Serum |
| **FDA** | Food and Drug Administration |
| **FTC** | Emtricitabine |
| **HAART** | Highly Active Antiretroviral Therapy |
| **HIV** | Human Immunodeficiency Virus |
| **HPLC-UV** | High Performance Liquid Chromatography – Ultraviolet |
| **MTT** | 3-(4,5,-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| **PBS** | Phosphate Buffered Saline |
| **PDI** | Polydispersion Index |
| **PEG** | Poly(ethylene glycol) |
| **PEP** | Post-exposure Prophylaxis |
| **PLGA** | Poly(lactic-co-glycolic-acid) |
| **PrEP** | Pre-exposure Prophylaxis |
| **PVA** | Polyvinyl alcohol |
| **SD** | Standard Deviation |
| **SEM** | Scanning Electron Microscopy |
| **STD** | Sexually Transmitted Disease |
| **SVF** | Simulated Vaginal Fluid |
| **TDF** | Tenofovir Disoproxil Fumarate |
| **VCF** | Vaginal Contraceptive Film |

# Chapter 1

# 1. INTRODUCTION

During the early 1980s, the first evidence of what was later to be known as acquired immune deficiency syndrome (AIDS) was reported and immediately associated with sexually active homosexual men.[1] Soon enough this relationship was proven to be fallacious, when AIDS rose to epidemic proportions and spread across the globe, amongst people from every race, economic class and sexual orientations.[2] People with AIDS would frequently develop opportunistic diseases and, in the worst case scenario, succumb to death. With millions of people being decimated by this new unknown plague, scientists needed to rush into finding its etiology. It was a couple of years later, at the Institute Pasteur, that Barré-Sinoussi, Montagnier and colleagues identified and isolated the causative agent of the disease, now known as human immunodeficiency virus (HIV).[3-4]

On the years that followed and until now, despite all effort, no cure has been developed, and thus, most efforts had to move towards the control of the infection and prevention. Treatments based on antiretroviral (ARV) drugs have been used frequently and with a relatively high rate of success, but these are also associated with often troublesome side effects. Also, ARV drug treatments are commonly allied to complicated oral pill regimens that hamper patients' adherence and may ultimately result in treatment failure and medical complications[5]. In the absence of a cure, prevention remains the best approach to abbreviate the HIV/AIDS burden. Amongst different strategies, currently Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved Truvada® (Gilead Sciences Inc., Foster City, CA, USA) is available for oral pre-exposure prophylaxis (PrEP). Truvada® comprises two antiretroviral drugs, tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC), and has been proven effective in several trials involving injectable drugs users and men who have sex with men. Still, when tested in women, satisfactory results were never obtained, probably due to the low adherence and/or biological differences.[6-11] The situation of women is aggravated by the lack of women's rights especially pronounced in developing countries. Among other things, it leads to the inability of women to protect themselves, namely for negotiating the use of condoms or even refuse sexual intercourse, making them more vulnerable to HIV transmission.[12] The aforementioned justifies, at least in part, that women account for roughly half of the infected population with the virus worldwide.[13] More important, it highlights the urgent need for alternative and effective preventive strategies that are suitable for women and

that, if needed, are able to be used without the partners' knowledge. Discrete vaginal microbicides may come as a way to meet such demands.

Different vaginal microbicide products have been developed over the last quarter of a century but none has been shown as a definitive answer to women's needs. Among other more conventional strategies, nanotechnology-based approaches for the vaginal delivery of promising ARV microbicide drugs has been proposed, often with several advantages. Thus, the goal of this work was to develop a potentially acceptable drug delivery platform for the administration of the two antiretroviral drugs currently prescribed for oral PrEP, namely TDF and FTC, by using of a nanotechnology-based strategy. Also, the combination of drug-loaded nanocarriers (nanoparticles) and film-based platforms was explored in order to obtain a potentially suitable product that could be easily used by women. In order to do so, the work was divided in different steps, starting with the manufacturing of a vaginal film platform, followed by the production of the nanoparticles loaded with the ARV drugs and their incorporation into the film.

The following book chapter was also published in the framework of the current thesis:

Cautela MP, Sarmento B, das Neves J. Antiretroviral drug-loaded nanosystems for preventing HIV transmission. In: Uskokovi V (Ed). *Nanotechnologies in Preventive and Regenerative Medicine*, 2017, Elsevier: Amsterdam, Netherlands. pp. 20-44. ISBN: 978-0-323-48063-5.

## 1.1 Structure of the thesis

The present document is organized in five chapters. The first chapter briefly provides an overview of the scope and objectives of the work, highlighting its potential importance. The second chapter addresses relevant subjects to the field of HIV/AIDS, sexual transmission and prevention, and microbicides that allow to better understand the present work, providing insights on what has been done and the novelty of the present work. The third chapter describes all the methodology used throughout the work to develop films, nanoparticles and their combination. Chapter four presents obtained results regarding the characterization and evaluation of nanoparticles, films and nanoparticles-in-films and provides critical analysis in light of what has already been reported in the literature. The fifth and final chapter includes the main conclusions of the work, as well as future perspectives.

# Chapter 2

# 2. LITERATURE REVIEW[1]

## 2.1 Epidemiology, transmission and treatment

HIV belongs to the retroviruses family and can be classified in two types, HIV-1 and HIV-2. Type 1 is the most prevalent, infectious and aggressive and is responsible for the majority of HIV infections.[14] Therefore, the present work addresses only type 1 viruses, simply referred to as HIV, unless otherwise stated.[1]

The United Nations Programme on HIV/AIDS estimates that, during 2015 alone, 2.1 million people became newly infected with HIV. During that same year, 1.1 million deaths were also reported. Furthermore, there were 36.7 million people globally living with HIV/AIDS by the end of 2015, women accounting for half of the infected population.[13] Although a slight decrease in the number of new infections has been observed over the last two decades, it appears that the 2020 goal, which aims for a reduction of new HIV infections down to 500 000 cases, is now unattainable, considering the tendency that has been observed over the last years (Figure 1).[15] Furthermore, this reduction has not been uniform in all regions of the globe, emphasizing the importance and influence of different routes of transmission and risk behaviors on the efficacy of HIV transmission.[16]

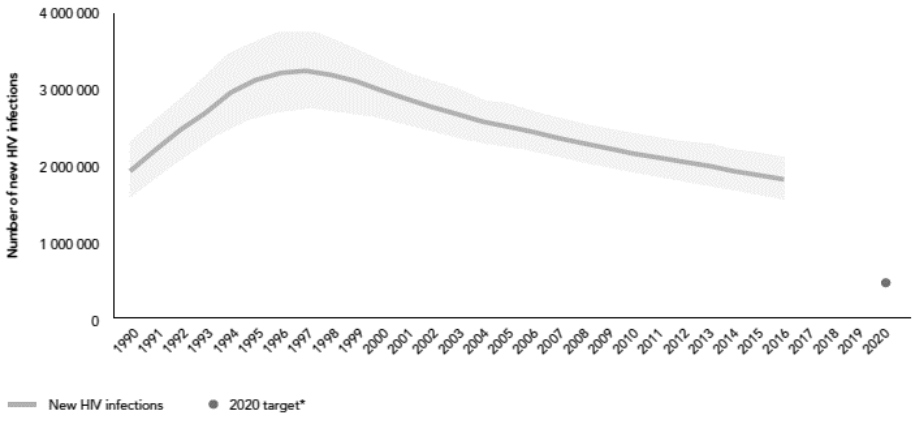


**Figure 1.** New HIV infections, all ages, global, 1990-2016 and 2020 target. Adapted from [15].

[1] This section has been partially published as '' Cautela MP, Sarmento B, das Neves J. Antiretroviral drug-loaded nanosystems for preventing HIV transmission. In: Uskokovi V (Ed). *Nanotechnologies in Preventive and Regenerative Medicine*, 2017, Elsevier: Amsterdam, Netherlands. pp. 20-44. ISBN: 978-0-323-48063-5.

Viral transmission occurs mainly in three ways: (i) from mother to child during pregnancy, labor or breastfeeding; (ii) contact with infected blood, which is particularly relevant in injectable drug users due to needle sharing; or (iii) upon sexual intercourse, namely during unprotected insertive vaginal or rectal sex.[16-17] The last one, more specifically heterosexual sexual intercourse, is acknowledged as the leading means for the spreading of HIV. Having multiple sexual partners, being in a serodiscordant relationship or exposed to sexually transmitted diseases (STDs) are behaviors known to increase the risk of sexual transmission of HIV.[16, 18-19] Also, specific population groups may be more predisposed to be infected. Women, for instance, are more vulnerable to this virus, not only because of social factors but also due to inherent biological factors, as it is the case of hormonal changes and higher prevalence of STDs.[20]

The establishment of an infection leads to the development of AIDS, which is a condition that severely affects the immune system, resulting in a high vulnerability of otherwise healthy patients to opportunistic diseases.[21] In order for the infection to occur, and by consequence of the location of its target cells, the virus needs to cross several natural barriers, starting with the cervicovaginal mucus layer. This fluid acts in a protective way by entangling the virus to a certain extent.[22-23] Alongside with this physical barrier, there are the natural antiviral molecules produced by host or microbiota (e.g. chemokines, cytokines, lactic acid, etc.), the physicochemical variations in the different fluids that cover the cervicovaginal region (in mucin content, for example) and the vaginal acidic pH.[24-25] These natural defenses may lead to viral inhibition, influence its viability, transport and retention on these barriers, or even possibly result in inactivation of HIV, respectively. However, these are not infallible and may be modulated, namely by semen, and for that reason one should not rely on them for protection.[17, 26]

Even though HIV discovery goes back to the 1980's, no cure has been found to date. Thus, the control of the worldwide epidemic falls on the hands of effective and sustainable prevention and treatment. The second is based on antiretrovirals, which are molecules known to somehow interfere with the viral cycle, either by acting on viral or host targets, ultimately hindering the infection. These anti-HIV compounds may be divided, according to their action site in those acting on HIV enzymes, namely reverse transcriptase, integrase and protease and those acting as entry inhibitors.[27] Despite first being used as monotherapy, early findings of rapid emergence of resistance to treatment motivated their use in combination, resulting in what is now called combination antiretroviral therapy (cART; formerly referred to HAART – highly

active antiretroviral therapy).[27] This approach is highly effective in diminishing viremia to very low levels (improving the patients' health condition) and reduces HIV progression to AIDS, turning a previously deadly disease into a chronic one. [19, 27-28]

Notwithstanding the positive outcomes, it is important to emphasize that treatment does not lead to cure but instead, it provides a way to reduce the free virus cargo. Thus, a life-long treatment is still required. Responding to these demands is complicated, considering the low adherence and therapy associated side effects, such as renal, hepato or mitochondrial toxicity, metabolic changes (e.g. lipodystrophy and diabetes mellitus) and immune reconstitution disease. Furthermore, the development of resistance and establishment of cellular and anatomical reservoirs (where the virus persists even through treatment) may limit the chronic use of cART. However, quick loss of viral suppression alongside with other adverse health consequences or even death may result from the withdrawal of therapy.[19, 28-29] Considering the drawbacks and limitations of the currently available treatment, and acknowledging that prevent is as important as to treat, it is of high urgency to have preventive measures that can stop or, at least, reduce the spreading of HIV.

## 2.2 Preventive measures for sexual transmission

While several attempts of developing an effective vaccine have failed, there are preventive measures already available that offer, at least, partial protection from HIV (

Table ***1***). Although some of them show high protection rates when used in a consistent way, as in the case of condoms, implementing them at a worldwide scale may be challenging. This comes as a reflection of how socioeconomic issues, such as poverty, discrimination and lack of women's rights in some regions, are key factors to the prevention and control of the spread of this virus. It is, therefore, important to adjust the prevention strategies according to the geographic region, transmission route and behavioral risks. It is equally important to create a package of preventive strategies, since each one cannot be effective on its own.[14, 28, 30-34]

**Table 1.** Preventive measures for sexual HIV transmission, with main advantages and disadvantages.[35]

| Preventive measures | Advantages | Disadvantages |
|---|---|---|
| **Condom use** | The most effective for preventing HIV sexual transmission<br>Inexpensive and easy to use<br>Widely available | Adherence is far from perfect<br>Requires partner consent (difficult to negotiate in several settings)<br>Dependent on coitus |
| **Socio-behavioral interventions (e.g. monogamy, sexual abstinence, counseling, regular testing for infection)** | Usually require limited resources in order to be implemented<br>Not dependent on coitus | Difficult to implement, namely in large scale<br>Difficult to implement in setting prevailing gender inequality |
| **Medical male circumcision** | Relatively high protection obtained after one single surgical intervention<br>Not dependent on coitus | Requires appropriate medical facilities and specialized staff<br>Does not protect women<br>Only partially effective |
| **Testing for HIV infection** | Reduces the probability of risky sexual intercourse | May not be readily available<br>Possibility of false results |
| **Antiretroviral treatment of HIV positive individuals** | Reduces risk of transmission to uninfected partner<br>Beneficial to infected partner (significantly suppresses viral replication and reduces viral load)<br>Not dependent on coitus | Requires life-long treatment and monitoring<br>Toxicity issues may compromise success<br>Costly (still unavailable in many low resource settings) |
| **Oral PrEP** | Highly effective, particularly in men who have sex with men, if used consistently<br>Not dependent on coitus | Require near perfect adherence and long-term use<br>Contradictory results in the case of women<br>Costly<br>Toxicity issues may arise |

Along with behavioral strategies for prevention are the ones based on antiretroviral drugs. Among those, there is antiretroviral therapy, that can work both in a treatment and preventive manner. This is especially important for people enrolled in a serodiscordant relationship, since decreasing the viral load of the infected partner will also decrease the probability of infecting the seronegative partner.[28, 36] Oral post-exposure prophylaxis (PEP) and pre-exposure prophylaxis (PrEP) have earned themselves some attention, although PEP has significant limitations regarding its use. The former usually combines three different oral antiretroviral drugs, usually TDF, FTC and a protease inhibitor. The regimen is meant to be used by HIV-negative people to reduce the risk of acquiring HIV after being potentially exposed to the virus. Low adherence to PEP is thought to greatly impact its effectiveness, as suggested by different studies following non-occupational exposure, which ultimately revealed that this approach is not completely reliable.[37-39] Furthermore, this specific prophylaxis is meant to be used after a

single exposure to HIV and not routinely, significantly limiting the usage of this strategy as routine prevention.[40]

Oral PrEP, on the other hand, is designed with the intent of being used on a daily basis and as something to administer prior to being exposed to the virus. Still, the main goal is the same as in oral PEP, which is to prevent HIV infection by means of antiretroviral drugs. Oral PrEP is currently commercialized as Viread® (Gilead Sciences, Foster City, CA), which is a pill that contains TDF or, more commonly, Truvada® (Gilead Sciences, Foster City, CA), a tablet that combines, as before mentioned, the two reverse transcriptase inhibitors TDF and FTC.[41] The approval of this one-of-a-kind HIV prevention by the FDA and EMA was the landmark that stated the transition of antiretroviral therapy from treatment to prevention.

Results regarding oral PrEP effectiveness have been controversial, with some studies reporting high rates of protection, namely amongst men who have sex with men and injectable drug users, while others report the opposite for women.[6-11] Overall and similar to what was seen with oral PEP, the adherence to a daily regimen possibly compromises the efficacy of the therapy and has not been enough to decrease HIV transmission in women.[8, 11] The previous may be justified by the fact that the required continuous regimen, even when one is not exposed to HIV, is not what most patients find ideal, reporting they would prefer an intermittent regimen.[42] Despite this, some studies showed evidence of greater anti-HIV potency when using the TDF/FTC combination rather than TDF alone.[6] On the other hand, there are some concerns regarding the adverse effects known to result from the continuous administration of this pill, namely, the development of renal impairment or decrease in bone mineral density. This makes this pill possibly not be suitable for all population, as it is the case of people who are at risk of developing one of these conditions.[43] The previous, allied to the evident – and proved in several studies – lack of adherence of women to the current strategies, either by cultural reasons or others, justifies the urgent need for alternative strategies that women can commit to without needing consent or even knowledge of the partner.[20]

## 2.3 Topical PrEP as alternative to oral PrEP

Even though oral PrEP proved to be effective in certain risk groups, it has not been able to prevent transmission at a worldwide scale.[30] Microbicides are one promising alternative strategy to prevent HIV, and have been under research for the last two decades. These topical products are meant to be self-applied in the vagina of non-infected people in order to prevent

early transmission events on the vaginal mucosa. An important advantage of these therapeutic system over the conventionally used oral tablets is its topical administration.[44-45] The vaginal route offers advantages such as ease of administration and the ability to provide higher local drug bioavailability in virally exposed surfaces, thereby, potentially reducing the systemic exposure to the active molecules and the resultant side effects.[44-46]

These so called microbicides can be classified according to their mode of action in those having non-specific activity or those acting as ARV carriers. First generation microbicides comprised surfactants and other non-specific molecules that would act on the membrane of a wide range of microbes and spermatozoa. Nonoxynol-9 is the best known one, although not for good reasons.[47-48] This membrane disruptor, which had been used as spermicide for many years, was found to increase the risk of being infected by HIV when tested as a possible microbicide. Surfactants were quickly put aside as microbicides after showing no protection against the virus. Still, valuable knowledge came out of the first microbicide trials, namely regarding safety issues and clinical trial design.[48-49]

After realizing the positive outcomes of using antiretroviral drugs in a preventive fashion, researchers found themselves recycling the concept of 'microbicide', resulting in what is now recognized as topical PrEP, whose aim is to deliver antiretroviral drugs topically to the vaginal or rectal surface. Contrary to the non-specific type of action seen with the first-generation microbicides, ARV-based microbicides offer more specific activity, since these drugs are designed to affect viral or host targets involved in cell infection.[50-51]

An ideal microbicide should not only be qualified in terms of efficacy, safety and effective drug delivery, but should also be affordable and acceptable within the target population, in this case, women. Microbicide drugs may be administered in the form of gels, tablets, capsules, rings or films. Furthermore, they can be applied in a coitus-dependent manner (which means they should be administered around the time of sexual intercourse), daily or can even be designed to have a preventive effect over several days.[44, 50]

Drugs that were already used in HIV treatment and that have shown interesting properties in terms of safety profile, activity against HIV and pharmacokinetics, as well as emergence of resistance, appeared to be promising candidates for a topical approach. Although several chemoprophylactic drugs were being tested in nonclinical studies, only a few are close to or are already being assessed under clinical studies.[52-54] Tenofovir and its prodrug TDF, both alone or in combination with emtricitabine, alongside dapivirine appeared to fulfill the abovementioned requirements and have been tested for their microbicide potential.[8, 51-53, 55-57] Tenofovir was the

first drug to be tested in a phase I study for its potential application as a vaginal microbicide, and further in a phase IIb trial (the CAPRISA 004 study) as a coitus-dependent 1% TFV gel. Initial results were encouraging but further studies revealed that this gel was not able to provide protection against HIV transmission, obliging the study to be dropped for futility.[8, 52] Still, interesting conclusions were taken out of this first ARV-based microbicide attempt, namely, the importance of using tenofovir as a microbicide drug and how topical administration can lead to higher concentrations of the antiretroviral molecule in the vaginal tissue, when compared to the oral route.[52, 58-59] Thus, this TFV gel may be seen as proof of concept of microbicides and its potential in HIV prevention.

Even though the failure of this gel remains unexplained, researchers believe that the ineffectiveness may be justified by the low rates of adherence seen in the study, which are probably resulting from the inherent messiness and leakiness from the vagina that is seen with this dosage form and that can be troublesome for some users.[8, 50] Therefore, alternatives that can be more suitable and user-friendly are urgently needed.

Solid dosage forms, as in the case of vaginal rings and films, may be the answer and have been under intensive study over the last few years. These are very practical in terms of portability, storage and administration, since they do not require the use of an applicator (Figure 2).[50, 60] Furthermore, they are useful for drugs that quickly degrade in an aqueous environment. One option that has been widely studied are intravaginal rings. These drug dosage forms can be designed to release the active ingredient either in a fast or in a sustained fashion (over minutes, days or weeks), thus having the possibility of being coitus-independent.[50] The only microbicide ring that has made it to clinical trial so far is a dapivirine ring, which ended up having successful results in terms of safety issues related with long-term use. Besides that, it revealed protection rates around 30% and higher, if the ring was correctly and consistently used.[53, 56]

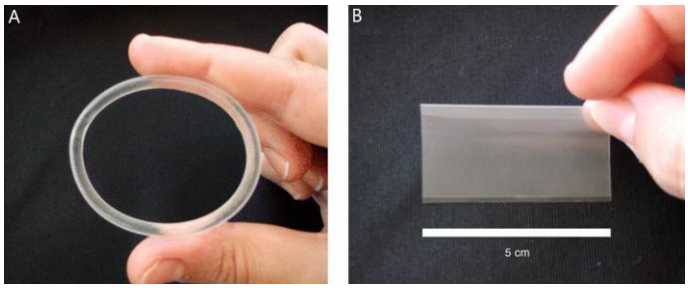


**Figure 2.** The vaginal ring.) NuvaRing® vaginal ring made from poly(ethylene-vinyl acetate) (reservoir design ring with 5.4 cm diameter and 0.4 cm cross-section) A vaginal film (VCF® Vaginal Contraceptive Film) folded in half. Adapted from reference [60] with permission from Wiley (© 2014 John Wiley & Sons, Ltd).

Despite the potential of rings, not all molecules can sustain the manufacturing process and the process itself may be too expensive, making this type of strategy not an option at times.[50]

### 2.3.1 Vaginal films as a platform to deliver antiretroviral drugs

It is becoming increasingly clearer how the effectiveness of microbicides is dependent on features such as adherence and acceptability and that the 'one size fits all' mindset is a misconception.[61-62] This means that each individual will have her own dosage form related issues and preferences, and therefore, different alternatives should be available, so that the potential female user can choose what suits her better. The design and choice of drug dosage form is of paramount importance and should not revolve solely around the active molecule stability and physical-chemical properties, but also on the target population preferences and acceptability.[61-64] This was clearly seen in the CAPRISA 004 trial, where adherence rates to the 1% TFV gel influenced the protective rates of the microbicide, which ranged from 39% to 50%, for cases in which low adherence and high adherence were reported, respectively.[52]

Notwithstanding the promising results seen with the dapivirine vaginal ring, women's economic and social environment, as well as preference, may result in low adherence to the ring which in turn has a negative impact on the microbicide effectiveness.[61] In order to overcome this, there has been an increased interest in the development of vaginal films as a topical PrEP alternative for the vaginal administration of ARV drugs.[46]

Advances in polymer science motivated the use of polymeric films for oral drug delivery,[65] and in light of results seen for this type of topical application, vaginal films started being developed.[66] Vaginal films are typically thin , flexible and soft solid strips of water soluble polymers, with a square shape and transparent or translucent appearance.[67] These are meant to dissolve or disaggregate when in contact with vaginal fluids, in order to release an active ingredient which is incorporated in its matrix.[66] This release usually occurs rapidly, thus suiting coitus dependent use, although sustained behavior may also be achieved with proper engineering. In this last case, coitus independent products may be obtained.[50] As a solid platform, they promote higher drug stability since events such as oxidation and hydrolysis are avoided. This dosage form is made out of matrix-forming polymers and plasticizers, in which the active molecule(s) are incorporated. Other excipients may also be used, namely fillers, preservatives and coloring agents.[50, 66] Overall, work during the formulation development stage of films should result in a product with, not only good organoleptic properties, but also

mechanical ones, as well as wetting and spreading properties that will ultimately influence the vaginal film performance. Formulations also need to comply with the natural physiology of the vaginal environment.[50]

Films are regarded by users as a very convenient and comfortable method for the administration of microbicide drugs, since it provides diminished leakage, with higher retention time, and low addition of fluids upon disaggregation. Furthermore, they are very discrete, easy to store and administer, due to their small size and lack of need of an applicator.[50, 66, 68-69] These last features may and should also be seen from the economic point of view. Because they are small and do not need an applicator, manufacturing becomes a lot cheaper, resulting in a cheaper and more accessible product. This is especially important in developing countries, such as those in the sub-Saharan African region, where HIV is known to be more prevalent.[69] Moreover, the manufacturing process of vaginal films is not as harsh as the one used for intravaginal rings, allowing for the integration of a broader range of ARV molecules.[50]

During the last decade, vaginal films have gained increased interest and were developed, either for contraception, bacterial or fungal treatment and, more recently, HIV prevention.[70-73] A nonoxynol-9 film, first manufactured for contraceptive purposes and already marketed as Vaginal Contraceptive Film (VCF®), was latter investigated as a potential microbicide against HIV. Similar to what was seen with the nonoxynol-9 gel, this film did not show protection against HIV and even revealed that long-term use would lead to lesions in the vaginal tract [47, 74]. Still, women reported their preference for this type of dosage form over others, namely gels and tablets, due to the features described above,[75-76] which may lead to higher acceptability and, consequently, efficacy.

Films for the delivery of anti-HIV molecules are currently on the pipeline of microbicide development. Films with the reverse transcriptase inhibitor dapivirine were assessed for their microbicide potential and revealed not only acceptable mechanical and physical-chemical characteristics, but also activity against HIV both *in vitro* and *ex vivo*.[77-78] Other ARV drugs have also been incorporated in films and tested for their anti-HIV activity, with an overall appealing aspect and positive activity against HIV.[66, 79-82] These results support the potential of this drug dosage form in the microbicides field and justify the need for developing the vaginal film technology for microbicide purposes.

### 2.3.2 Nanoparticles-in-films as topical PrEP

Despite the above described advantages of vaginal films, these too can still be further developed in order to yield enhanced drug products. One particularly strategy has been the association of films with drug nanocarriers.[26, 50] These last may be defined as systems ranging from a few nanometers up to nearly one micrometer (nanoparticles) that carry and shape the behavior of active molecules. These nanosystems are characterized by their increased surface area allowing for extensive interfacial interactions with biological systems.[26, 83] Nanocarrier systems emerged with nanotechnology development and they are now known to have very interesting advantages, particularly when considering mucosal drug delivery (such as in the case of vaginal microbicides).[26] Besides their ability to provide a more controlled release, these systems are able improve mucosa distribution and drug permeation or accumulation, as well as modulate mucoadhesion.[84] Also, they may increase the drug's circulation time while reducing its toxicity, with all this possibly leading to higher efficacy.[26, 50]

Nanoparticles, either having intrinsic activity or acting as drug carriers, have been investigated over the last few years for numerous therapeutic applications, and more recently for HIV prevention.[85] Dendrimers have been particularly relevant in the case of those possessing intrinsic activity as related to microbicides. Amongst the different developed dendrimer-based microbicides, VivaGel® (Starpharma, Australia) is probably the most relevant one and comprises the SPL7013 dendrimer formulated as a gel.[26, 50, 86-87] When assessed for its anti-HIV activity, this dendrimer revealed a good activity profile, but the same was not seen when studied for its safety, and so far, no other studies have been developed using this dendrimer-based gel for microbicide purposes.[88-90]

More recently, investigators have been focusing their attention in nanoparticles carrying ARV drugs, since they can allow to incorporate multiple drugs in the same system, among other things.[91] These nanosystems may be distinguished according to their chemical nature into liposomes, solid lipid nanoparticles, inorganic nanoparticles and polymeric nanoparticles, among others. There appears to be some preference for the polymeric nanoparticles over others, especially for those biodegradable, probably due to the fact that they present excellent *in vitro* and *in vivo* stability, besides all the above mentioned mainstream features of nanosystems.[50, 87, 92]

Amongst the different ARV drugs with potential to be associated, there has been especial interest in those already being tested in clinical trials. The results from the CAPRISA 004 trial, even though not entirely positive, motivated the use of TFV and derivatives in

nanotechnological approaches.[93-96] Solid lipid nanoparticles, as well as polymeric nanoparticles, using poly(lactic-co-glycolic-acid)(PLGA) (alone or in combination with Eudragit) and chitosan, have been used to incorporate TFV or its prodrug TDF. Overall, both polymeric and solid lipid nanoparticles were safe with rates of association efficiency ranging up to 54%.[93-98] Machado *et al*. developed TFV-loaded PLGA nanoparticles and incorporated these into polymeric films, which further revealed to be safe for *in vivo* administration.[99]

In tandem to what is being done with TFV and TDF, other ARV drugs are also being studied for their potential use in the development of nanotechnology-based microbicides, namely, dapivirine, efavirenz, saquinavir and PSC-RANTES. [100-106] In general, all particles revealed a safe *in vitro* profile. Furthermore, these studies are proof of concept of how nanoparticles may improve features such as the half maximal inhibitory concentration ($IC_{50}$) and toxicity of the associated drug, when compared to the drug alone, as well as their uptake by HIV susceptible cells.[101, 106] Other properties were also seen to improve when using nanotechnological strategies, namely, the pharmacokinetic profile and anti-HIV activity. das Neves *et al*. developed dapivirine-loaded nanoparticles that, among other things, showed a reduction in the systemic exposure when administering these particles intravaginally to mice, which can ultimately affect not only the bioavailability of the drug, but also the therapeutic dosage needed and the prevalence of side effects.[100, 102-105] IQP-0528, another reverse transcriptase inhibitor was also encapsulated in nanoparticles and these last were incorporated in a vaginal film and then evaluated for its safety and pharmacokinetic profile. It was seen that this film was not toxic and it did not affect the vaginal pH of the macaques who the film was administered to, which is important, since the pH acts as a natural virus inhibitor.[57] Regarding the pharmacokinetics, these studies revealed an improved pharmacokinetic behavior of the nanoparticles-in-film system. More recently, Cunha-Reis et al. encapsulated TFV and efavirenz into PLGA nanoparticles and further incorporated them in a vaginal film. *In vivo* studies revealed that this film is compatible with the vaginal environment. An improved pharmacokinetic profile was seen for the nanoparticles-in-film formulation when compared to the film containing the drug alone.[107]

The association of nanoparticles to films is not limited to ARV drugs and this type of strategy may also be applied in gene therapy. A PVA and λ-carrageenan vaginal film containing siRNA-loaded nanoparticles was developed by Gu and collaborators. *In vitro* studies revealed a successful intracellular delivery as well as gene knockdown. This comes as a revelation of the broad application of vaginal films for HIV prevention.[108]

It is well known how massively HIV has been infecting and killing people ever since, especially women in developing countries, and how the current treatment and prevention still have their pitfalls. Different approaches have been tested to solve this problem, amongst them, vaginal films. These have earned themselves recognition and their association to nanoparticles was found to be very interesting and advantageous. Considering the relative success of the combination of TDF and FTC in certain risk groups, the purpose of this work was to develop TDF/FTC-loaded nanoparticles, as well as incorporating them in vaginal films. These films are to be used as topical PrEP, in alternative to the current approved and recommended by the WHO and several other worldwide national authorities, oral Truvada®.

# Chapter 3

# 3. MATERIALS AND METHODS

## 3.1 Materials and cell lines

Poly(lactic-co-glycolic acid)(PLGA) (Purasorb PDLG 5004, 50/50 DL-lactide/glycolide copolymer ratio, 0.4 dl/g inherent viscosity) was kindly provided by Corbion (Gorinchem, The Netherlands). TDF was acquired from Kemprotec (Cumbria, United Kingdom) and FTC from Sequoia Research Products (Pangbourne, United Kingdom). Eudragit® L100 was acquired from Degussa (Darmstadt, Germany). Poly(vinyl alcohol) (PVA; 87–90% hydrolyzed, 30–70 kDa) and poloxamer 407 from Sigma-Aldrich (St. Louis, MO, USA). High methoxyl Pectin USP HM was kindly provided from CP Kelco (Lille Skensved, Denmark). PEG was acquired from Union Carbide (Texas, USA) and glycerin from Aliand (Mem Martins, Portugal). Methanol was acquired from Fisher Scientific (New Hampshire, USA), monobasic phosphate potassium and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were acquired from Sigma-Aldrich. All other materials and solvents were of analytical grade or equivalent. HeLa cervical epithelial cell line was acquired from ATCC (Manassas, VA, USA).

## 3.2 Cell culture conditions

HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM, Lonza, Verviers, Belgium) supplemented with 10% (v/v) fetal bovine serum (FBS, Merck Millipore, Massachusetts, USA), 100 U/mL of penicillin (Merck Millipore) and 100 µg/mL of streptomycin (Merck Millipore) at 37°C, 5% $CO_2$ and 95% humidity. Medium was changed every 2-3 days and cells sub-cultured when reaching approximately 90% confluency.

## 3.3 Development of a HPLC-UV analytic method

In order to simultaneously quantify both TDF and FTC associated to the nanoparticles, a High performance liquid chromatography with ultraviolet detection (HPLC-UV) gradient method was developed by adapting a previously described isocratic method by Machado et al.[99] Initally, an isocratic method was attempted, composed of phosphate buffer pH 6:methanol at a ratio of 60:40 or 54:46. Based on those results, a gradient method was developed. Variable ratios of phosphate buffer 10 mM pH 6:methanol, described in Table 2 were tested. All the

experiments were performed at a flow rate of 1 mL/min and 10 µL volume of injection in a Merck-Hitachi 7100 series HPLC system (Merck, NJ, USA), using a Zorbax Eclipse XDB-C18 column (3.5 µm, 3.0×75 mm; Agilent Technologies, Santa Clara, CA, USA) and UV detection at 260 nm.

**Table 2.** Composition and gradient of the mobile phase used for HPLC-UV.

| Gradient method | Time (min) | Phosphate Buffer pH 6 (%) | Methanol (%) |
|---|---|---|---|
| **A** | 0 | 96 | 4 |
| | 5 | 50 | 50 |
| | 10 | 50 | 50 |
| | 11 | 96 | 4 |
| | 15 | 96 | 4 |
| **B** | 0 | 96 | 4 |
| | 2 | 50 | 50 |
| | 10 | 50 | 50 |
| | 11 | 96 | 4 |
| | 15 | 96 | 4 |
| **C** | 0 | 96 | 4 |
| | 2 | 45 | 55 |
| | 8 | 45 | 55 |
| | 9 | 96 | 4 |
| | 15 | 96 | 4 |
| **D** | 0 | 96 | 4 |
| | 2 | 40 | 60 |
| | 7 | 40 | 60 |
| | 8 | 96 | 4 |
| | 15 | 96 | 4 |

## 3.4 Production of nanoparticles

### 3.4.1 Production of nanoparticles loaded with TDF or FTC by double emulsion

PLGA-based nanoparticles loaded with either tenofovir disoproxil fumarate (TDF) or emtricitabine (FTC) were produced by adapting a previously described double emulsion/solvent evaporation method.[107] Briefly, 8 mg of drug (TDF or FTC) were dissolved

in 0.5 mL of ultra-pure water and posteriorly mixed with 2 mL of ethyl acetate containing 40 mg of PLGA. The mixture was vortexed for 30 seconds in order to form the first emulsion (w/o) using a VWR vv3 vortex (Vila Nova de Gaia, Portugal). This emulsion was then added to 10 mL of a 1% (w/v) poloxamer 407 solution and sonicated at 70% intensity for 60 s, using a Vibra-Cell™ VC 50 (Sonics & Materials, Danbury, CT, USA). The resulting w/o/w emulsion was poured into an additional 10 mL of 1% poloxamer 407 solution and left for 5 h under 300 rpm magnetic stirring at room temperature in order for the organic solvent to evaporate. After that, the particles were washed twice with 10 mL of ultra-pure water and concentrated using Amicon Ultra-15 filter units (MWCO 100 kDa; Merck Millipore, Tullagreen, Ireland) and centrifugation at 3000 rpm in an Eppendorf™ 5810R Centrifuge (Fisher Scientific, New Hampshire, USA). The particles were stored at 4°C until characterization.

### 3.4.2 Production of nanoparticles loaded with TDF and FTC by spray drying

Eudragit® L100 nanoparticles encapsulating both TDF and FTC were produced by a spray drying method in collaboration with Prof. Alejandro Sosnik and Chen Moshe at Technion – Israel Institute of Technology, Haifa, Israel. Briefly, 0.45 g of Eudragit® L100 were dissolved in 90 mL of a water:ethanol (50:50) mixture and stirred for one hour at room temperature. Afterwards, 30 mg and 20 mg of tenofovir disoproxil fumarate and emtricitabine, respectively, were added to the Eudragit® solution and left under magnetic stirring for an additional hour. The solution was then sprayed using a BUCHI B-90 (Flawil, Switzerland) nano spray dryer (Closed cycle, Medium mesh, solution under magnetic stirring). The particles were stored at room temperature until further use.

## 3.5 Physical-chemical characterization of nanoparticles

### 3.5.1 Average size, polydispersion index, surface charge and morphology

The particles' hydrodynamic diameter, polydispersion index and zeta potential were assayed at 25°C in 10 mM NaCl using a ZetaSizer Nano ZS (Malvern, Worcestershire, UK). For the spray-dried nanoparticles, scanning electron microscopy analysis was performed to assess morphology using a Zeiss Ultra-Plus SEM (Carl Zeiss NTS GmbH, Oberkochen, Germany). Particles were placed upon carbon tape, coated with amorphous carbon and analysed

at 2-4 kV acceleration voltage, being images acquired using secondary electrons at 2.5-4 mm working distance.

### 3.5.2 Association efficiency and drug loading

The filtrates resulting from the nanoparticles washes were used to indirectly quantify the drug association efficiency (AE%) through the HPLC-UV method developed and described in subchapter 3.3. In parallel, particles were freeze-dried for 48h at -80°C using a Freeze Drier (Labconco, USA). Afterwards, the particles were dissolved in acetonitrile and left under magnetic stirring overnight. The solution was then centrifuged at 14 000 rpm using a microcentrifuge (Labnet, New Jersey, NJ, USA) for 10 min. The supernatant was used to directly quantify the association efficiency (%) using the above referenced HPLC-UV method. To calculate the association efficiency through direct and indirect method, as well as the drug loading, the following equations were used, respectively:

$$Association\ Efficiency(\%) = mass\ of\ TDF/FTC \times 100 \quad \text{(Eq. 1)}$$

$$Association\ Efficiency\ (\%) = \frac{initial\ mass\ of\ TDF/FTC - recovered\ mass\ of\ TDF/FTC}{initial\ mass\ of\ TDF/FTC} \times 100 \quad \text{(Eq. 2)}$$

$$Drug\ Loading\ (\%) = \frac{initial\ mass\ of\ TDF/FTC - recovered\ mass\ of\ TDF/FTC}{theoretical\ mass\ of\ nanoparticle} \times 100 \quad \text{(Eq. 3)}$$

For the spray-dried particles, only the direct quantification was performed. In order to do so, one milligram of particles was dissolved in two milliliters of the same water:ethanol (50:50) and left to stir overnight at room temperature, followed by HPLC-UV analysis using the above described method.

### 3.5.3 *In vitro* drug release studies of Eudragit® nanoparticles

Release studies of TDF and FTC of spray-dried Eudragit® nanoparticles were performed using two different media: a simulated vaginal fluid pH 4 and phosphate buffered saline (PBS) pH 7. The simulated vaginal fluid (SVF) was made as previously described and contained water, 0.5% glucose, 0.351% sodium chloride, 0.2% lactic acid, 0.14% potassium hydroxide, 0.1% acetic acid, 0.04% urea, 0.0222% calcium hydroxide, 0.016% glycerin. Hydrochloric acid was used to adjust the pH until 4.2. For the phosphate buffer, 240 mg of monosodium phosphate were dissolved in 100 mL of ultra-pure water and then NaOH was added to adjust the pH to 7. Initially, an amount of one milligram of particles was dissolved in 2 mL of PBS or simulated vaginal fluid and left in Eppendorf tubes for 48 h under stirring in an orbital shaker at 100 rpm and 37 °C. Tubes were collected at 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h and finally at 48 h and centrifuged for five minutes at 14 000 rpm using a microcentrifuge (Labnet, New Jersey, NJ, USA). Recovered supernatants were analyzed by HPLC-UV and the amount of drugs was quantified and used to assess the percentage of release during 48 h.

A similar study was done, but using an acetate buffer pH 4 and the same phosphate buffer saline pH 7 above described. For the acetate buffer, 120 mg of acetic acid were dissolved in 100 mL of water and then NaOH was added to adjust the pH to 4. For this study, five milligrams of particles were weighted into the film molds (described further in this chapter) as well as 15 g of one of the buffers. These molds were then covered with parafilm and placed in a Binder oven (Tuttlingen, Germany) at 37°C and without stirring. Aliquots of 500 µL were collected and replaced fresh medium at 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h and finally at 48 h and centrifuged for five minutes at 14 000 rpm using a microcentrifuge. Posteriorly, they were analyzed by HPLC-UV and the amount of drugs was quantified and used to assess the percentage of release during 48 h.

To assess the differences between the release at pH 4 and pH 7 for each drug, the $f2$ factor was used and calculated as described in the following equation:

$$f2 = 50 \times log\left\{\left[1 + \left(\frac{1}{n}\right)\sum_{t=1}^{n}(Rt - Tt)^2\right]^{-0.5} \times 100\right\} \quad \text{(Eq.4)}$$

where n is the number of time-points considered, and Rt and Tt are the percentage of drug released at each time-point (t) for pH 4 and pH 7. Drug release profiles with values of f2 between 50 and 100 were assumed to be similar.[109]

## 3.6 Manufacturing of vaginal films

Films comprising PVA, pectin, PEG and glycerin were produced using the solvent-casting technique. Briefly, 0.87 g of excipients were dissolved in ultra-pure water at 5.8% (w/w) and posteriorly poured into glass molds of 76.97 $cm^2$ and left to dry in a Binder oven (Tuttlingen, Germany) at 37°C for 48h. The mixture of excipients included 80% of PVA, pectin or a blend of both, and 20% of plasticizers (PEG and glycerin in a 50:50 ratio). Different ratios of PVA:pectin, ranging from 75:25 to 25:75, were tested in order to assess the most fitting film composition. The drying conditions (time, temperature and pressure) were also optimized. Optimized film compositions were further considered for the incorporation of nanoparticles or the free drugs. All films cut into required dimensions using and X-Acto knife and were stored at room temperature in aluminum foil and sealed plastic bags until further use.

### 3.6.1 Incorporation of nanoparticles in films

A dry method was used in order to incorporate the nanoparticles in the films, namely by adding the particles to pre-formed films (obtained as described above). Particles were manually dispersed in between two squared film samples (1.5×1.5 cm) and then pressed at 180 bar for 24 h using a hydraulic press (MESTRA Talleres Mestraitua, S.L, Vizcaya, Spain). In parallel, similar samples were pressed with two tons for 30s using a hydraulic press (Graseby Specac, Orpington, UK).

Films with the free drug were also developed. To do so, two different approaches were used: one where the whole amount of TDF and FTC was dissolved in the polymeric mixture and another one were half of the amount of TDF and FTC was dissolved in the polymeric mixture. For this second approach, two squares of 1.5×1.5 cm were cut, placed on top of each other and pressed with four tons for 30s using a hydraulic press (Graseby Specac, Orpington, UK). For both approaches, the drugs were incorporated prior to film drying.

## 3.7 Characterization of films

### 3.7.1 Organoleptic properties, thickness and moisture content

Organoleptic properties were assessed through organoleptic analysis. Thickness of films was evaluated using a digital micrometer (I.C.T, Lardero, Spain) and measurements were taken

at five different points of the film, corners and center, to further check the uniformity of thickness of the film. Briefly, the film was visually divided in four equal parts, placed in between two histology blades and thickness was assessed in each square and on the center. The average of these five measurements was considered as the thickness of the film. To assess the moisture content of the films, samples of 100 mg were place in a MX-50 moisture analyzer (A&D, Tokyo, Japan), and heated until 100 ºC for two minutes. The loss of weigh was recorded and used to determine the moisture content of each film.

### 3.7.2 Disaggregation time, pH and osmolality

The disaggregation time in simulated vaginal fluid was determined by following a previously described method.[99] Film samples of 1.5×1.5 cm of the different formulations were immersed in one milliliter of freshly made simulated vaginal fluid in 6-well plates under stirring (orbital shaker, 20 rpm, 37 °C). The disaggregation of films was monitored visually and the time required for complete disaggregation was recorded. Samples of VCF® Vaginal Contraceptive Film (Apothecus, Oyster Bay, NY, USA) with the same dimensions underwent the same protocol and were used for comparison purposes. The resulting film dispersions were further recovered, homogenized and used to determine possible pH changes and fluid osmolality using a PHT 810 pH-Meter (ebro Electronic, Ingostadt, Germany) and a Micro-Osmometer Type 15 (Löser Messtechnik, Berlin, Germany), respectively.

### 3.7.3 Mechanical properties

Mechanical properties, namely, mechanical strength, puncture strength and distance at burst were assessed by performing puncture tests, as previously described, using a TA.XT plus texture analyser (Stable Micro Systems, Godalming, Surrey, UK). Samples of 1.5×1.5 cm of films were placed on a support rig (HDP/FSR) and punctured by a 5 mm spherical probe (P5/S) at a speed of 1 mm/s. Puncture strength was calculated as the ratio between the mechanical strength required to tear the film and their cross sectional area. The distance at burst, in turn, was determined by recording the distance at which the film would elongate until breaking.[99]

### 3.7.4 *In vitro* drug release studies of nanoparticles-in-film

The release of TDF and FTC from nanoparticles incorporated in films was assessed *in vitro* using a previously described protocol.[99] Briefly, samples of 1×1 cm of films with

nanoparticles were immersed in one milliliter of freshly made simulated vaginal fluid in 6-well plates under stirring (orbital shaker, 100 rpm, 37 °C). Aliquots of 200 µL were collected and replaced fresh medium at 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h and finally at 48 h and centrifuged for 15 min at 14 000 rpm using a microcentrifuge. Posteriorly, they were analyzed by HPLC-UV and the amount of drugs was quantified and used to assess the percentage of release during 48 h.

## 3.8 *In vitro* safety of nanoparticles and films

The cytotoxic potential of nanoparticles, films and its individual components to HeLa cells was assessed using the MTT colorimetric assay. The method is based on the bioreduction of the tetrazolium dye MTT by metabolically active cells.[110] Briefly, HeLa cells were seeded in 96 well plates at an initial concentration of $10^4$ cells per well and left overnight at 37 °C, 5% $CO_2$ and 95% humidity. Afterwards, the cells were incubated with increasing concentrations of nanoparticles, films or excipients previously dissolved/dispersed in medium and left for an additional 24 h in the same conditions above described. Finally, the cells were incubated with MTT solution (0.5 mg/mL in PBS pH 7.4) for 4 h at 37°C and then absorbance was read at 570/630 nm using a Synergy plate reader (BioTek, Vermont, USA). Experiments were performed in triplicate and viability results were expressed as percentage of the positive control (i.e., cells incubated with medium alone).

## 3.9 Statistical analysis

All experiments were performed in triplicate and results are presented as mean ± standard deviations (SD), unless otherwise stated. One-way or two-way analysis of variance (ANOVA) with the Bonferroni post-hoc test (GraphPadPrism software Inc., USA) was used to analyze the data. Values of $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered as denoting significance, high significance and very high significance, respectively.

# Chapter 4

# 4. RESULTS AND DISCUSSION

## 4.1 Development of a HPLC analytic method

A new method based on HPLC-UV was developed for assaying simultaneously TDF and FTC and was used throughout the characterization experiments performed throughout this work. An isocratic method was developed to start off, based on a previously described method for the quantification of tenofovir.[99] The initial mobile phase comprised 98:2 (*v:v*) phosphate buffer pH 6:methanol. Since no peak correspondent to TDF was found and considering the solubility of TDF in methanol, two different mobile phases with increased amounts of methanol were tested, namely, one with the ratio of 60:40 (*v:v*) phosphate buffer pH 6:methanol and another one with 54:46 (*v:v*) phosphate buffer pH 6:methanol (Figure 3).[111]

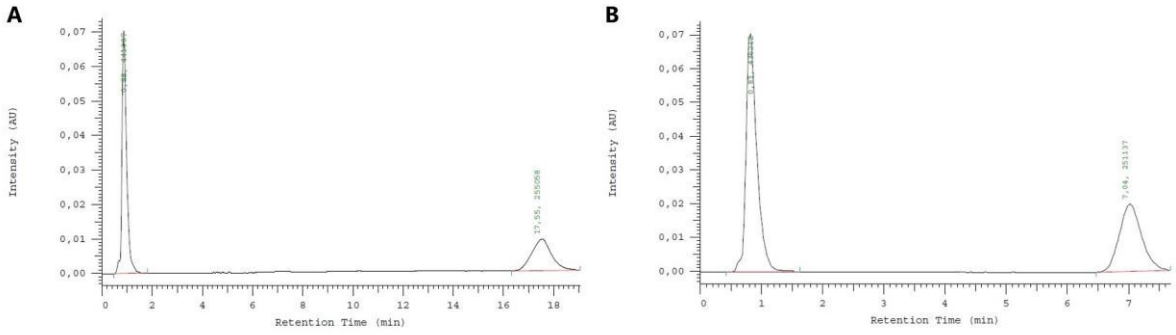


**Figure 3.** Representative chromatograms of TDF and FTC at 50 µg/mL using an isocratic method with different mobile phases, namely phosphate buffer pH 6:methanol in a ratio of (A) 60:40 (*v/v*) or (B) 54:46 (*v/v*). FTC peak at 0.88 minutes in both chromatograms and TDF peak at 17.55 and 7.04 for the first and second chromatogram, respectively.

It appears that a greater amount of organic phase leads to a faster elution of TDF (elution at around seven minutes in Figure 2B, compared to 18 minutes in Figure 3A), while FTC was clearly eluting prematurely and around the solvent retention time. Due to this lack of resolution between the solvent and FTC, a gradient method was further developed. Tested ratios of aqueous phase:organic phase varied between 96:4, 50:50, 45:55 and 40:60 (*v/v*) (Figure 4). For both drugs, the retention factor was calculated using the following equation:

$$Retention\ factor = \frac{Tsample - Tsolvent}{Tsolvent}$$

with *Tsample* and *Tsolvent* being the retention times for the sample (drug) and solvent (mobile phase), respectively. The optimal retention factor for HPLC-UV methods is usually considered to be between 2 and 10.[112] For all mobile phases, the retention factor was above 2 for both FTC and TDF.

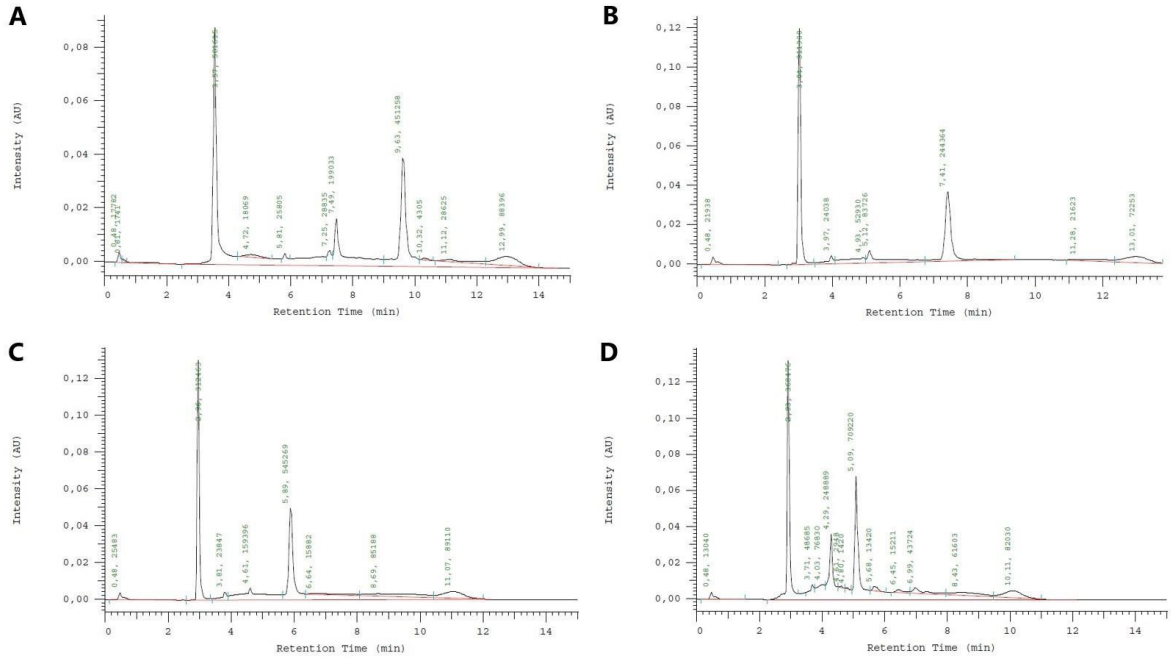


**Figure 4.** Chromatograms of TDF and FTC at 100 µg/mL using a gradient method with the following mobile phases: (A) phosphate buffer pH 6:methanol in a ratio of 96:4, 50:50 and 96:4 (*v/v*) at time 0, 5 and 11 min, respectively (B) phosphate buffer pH 6:methanol in a ratio of 96:4, 50:50 and 96:4 (*v/v*) at time 0, 2 and 11 min, respectively (C) 96:4, 45:55 and 96:4 (*v/v*) at time 0, 2 and 9 min, respectively and (D) 96:4, 40:60 and 96:4 (*v/v*) at time 0, 2 and 8, respectively. FTC peaks at 3.57, 3.04, 2.96 and 2.93 min and TDF peak at 9.63, 7.41, 5.89 and 5.09 min in chromatograms A, B, C and D, respectively.

The initial gradient method was set to mobile phase ratios of 96:4, 50:50 and 96:4 (*v/v*) phosphate buffer pH 6:methanol. Doing so, it was possible to increase the retention of FTC and separate its peak from that of the solvent. Visual inspection of the peaks suggests that these were well resolved when using this method, although a quantitative analysis was not performed (Figure 4A). Still, and in order to decrease the run time as well as the retention factor to acceptable values, the mobile phase was further modified in an attempt to elute TDF earlier. Based on the suggestion that higher percentages of methanol lead to faster elution of TDF (as evidenced in the isocratic method), percentages of up to 60% (*v/v*) of methanol were used (Figure 4C and 4D). As theorized, TDF eluted earlier with higher amounts of methanol, but an equilibrium had to be found between the time of elution of TDF and the resolution of the peaks for FTC, TDF and solvent. When using 45% of phosphate buffer and 55% of methanol (Figure

4C) the retention factor was of 11.3 for TDF and 5.2 for FTC, whereas when using 40% of phosphate buffer and 60% of methanol (Figure 4D) the retention factor was of 9.6 and 5.1 for TDF and FTC, respectively. Despite the fact that the conditions comprising the 45:55 ratio led to a retention factor value for TDF slightly above what is recommended, such elution conditions were further considered for subsequent analysis. In the case of the 40:60 ratio, an unexpected peak at 4.29 min related with gradient change could have raised difficulties in TDF peak identification in subsequent assays, particularly if the drug was present in low amounts. The chosen method was linear in the ranges of 3-100 μg/mL for TDF and FTC. Values of $r^2$ were 0.9998 and 0.9999 for TDF and FTC, respectively, clearly above what is generally recommended for analytical methods ($r^2 > 0.999$).[113]

## 4.2 Physical-chemical characterization of nanoparticles

### 4.2.1 Average diameter, polydispersion index and surface charge

TDF and FTC are two promising drugs being used as backbone of both HIV therapy and prevention.[41, 114-115] The two ARV compounds are currently being used as the standard in oral pre-exposure prophylaxis (PrEP), but not without adherence issues related with the need for daily administration.[20, 42] For this reason, researchers have been trying to create alternatives to the route and regimen of administrations, namely by considering products for coitus-dependent vaginal use. More recently, there has been a lot of interest in combining nanotechnology with vaginal administration, especially because of the widely acknowledged potential role of nanotechnology in the microbicides field. This led to several attempts to create nanosystems that could deliver these ARV drugs.[97, 116-117]

For the case of the present work, PLGA-based nanoparticles were initially developed using a double emulsion/solvent evaporation technique. PLGA was chosen due to its safety profile and suitability for vaginal delivery as well as wide application in drug delivery.[118] Poloxamer 407 was chosen as stabilizer of the emulsion during nanoparticle production. Besides this important technological role, poloxamer 407 is also known to provide a poly(ethylene glycol) (PEG) coating to nanoparticles that could be helpful in providing enhanced mucus diffusive features to the obtained nanosystems.[119] The technique used to develop the particles was a water-in-oil-in-water (w/o/w) emulsion/solvent evaporation

technique, which is one of the most conventional techniques to associate hydrophilic drugs and has been previously used to encapsulate tenofovir.[99, 107]

In parallel, Eudragit ® L100 particles were produced using a spray drying technique. This last technique was selected in order to minimize drug leakage during particle production commonly seen for hydrophilic molecules when using emulsion methods. Also, the Eudragit® family of excipients, comprising polymethacrylate-based copolymers, was chosen due to its pH dependent solubility. In particular, the L100 type copolymer is insoluble below pH 6 (as observed in the healthy vaginal environment) but dissolves at higher values, thus potentially allowing for triggered release of the drug content of the particles at alkaline pH.[120] Eudragit® L100 nanoparticles have been produced for oral drug delivery, but little work has been done regarding vaginal delivery.[121] Here, we propose the co-encapsulation of TDF and FTC in Eudragit® L100-based nanoparticles.

Size and zeta potential of particles are two important parameters to be characterized when speaking of nanoparticles for biomedical applications. These colloidal features often determine the fate of particles inside the body and can be involved in phenomena such as mucoadhesion.[122] Similarly to HIV, the mucus layer may also entrap nanoparticles.[123] Furthermore, it may hinder the uniform distribution of particles, especially towards the underlying epithelium, as well as their retention in the vaginal tract.[119] There is not a consensual opinion on which type of particles, mucoadhesive or mucus-penetrating, are better for vaginal drug delivery, but mucus-penetrating nanosystems may present some advantages. Due to their neutral charge, such systems avoid entanglement in the mucin mesh composing mucus by preventing adhesive interactions, namely of electrostatic, hydrophobic or interpenetration nature. This, in turn, leads to a decrease in the clearance of the particles from the vagina, since they will not be in the more superficial layers of mucus (that are frequently renewed). [84, 119, 124]

Despite the great influence of the surface charge on the mucoadhesive behavior of particles, the zeta potential value should be seen not only from a surface charge point of view, since it is also related to the long-term stability of the nanoparticles.[125] Besides charge, size is also determinant in the mucus-penetrating behavior. Diameters bellow 500 nm are described as ideal to achieve mucus penetration.[84]

As seen in Table 3, both loaded and empty nanoparticles produced using the double emulsion technique presented diameter around 130 nm with a narrow size distribution as indicated by the low polydispersion index (PDI) values. It is important to notice that the

association of the drug did not apparently led to an increase on the average diameter nor changes in the zeta potential.

**Table 3.** Average diameter, polydispersion index and surface charge of PLGA-based nanoparticles produced by a double emulsion/solvent evaporation technique. Results are presented as mean ± SD (n=3).

| Formulation | Hydrodynamic diameter(nm) | PDI | Zeta potential (mV) |
|---|---|---|---|
| **TDF-loaded** | 129 ± 8 | 0.10 ± 0.03 | -2.7 ± 0.3 |
| **FTC-loaded** | 127 ± 8 | 0.11 ± 0.01 | -2.3 ± 0.3 |
| **Empty** | 131 ± 5 | 0.11 ± 0.00 | -2.9 ± 1.0 |

Produced particles presented values for diameter that will, presumably, allow them to penetrate through mucus, even if some might get stuck at 'dead ends' of the mucus channels.[84] This last phenomenon has been observed *in vitro* for particles around 100 nm but the actual impact in animal experiments was not noted.[126] More important, the zeta potential of both loaded and empty nanoparticles was nearly neutral, suggesting low ability to establish electrostatic interactions with mucin fibers. However, the dominance of a PEG corona cannot be immediately assumed since other types of adhesive interactions can still be observed, particularly due to the hydrophobic nature of PLGA. Still, previous observations from our research group (unpublished data) suggest that the presence of PEG chains when using poloxamer 407 is considerable and allows for mucus-penetrating features.[124] Overall, produced particles appear to be suitable drug delivery to the mucosal epithelium in which the first HIV-susceptible host cells are located.[91]

As for particles produced by spray drying and using Eudragit® L100 as matrix, mean diameter was around 690 nm (Table 4). The size distribution, as assessed by PDI, suggests a polydisperse mixture, which was not expected considering that the technique used is recognized to allow a better control of the diameter of particles, resulting in more uniform particle size distributions.[127]

**Table 4.** Average diameter, polydispersion index and surface charge of Eudragit® nanoparticles produced by a spray-drying technique. Results are presented as mean ± SD (n=3).

| Formulation | Hydrodynamic diameter(nm) | PDI | Zeta potential (mV) |
|---|---|---|---|
| **TDF/FTC-loaded** | 687 ± 90 | 0.55 ± 0.31 | -26.2 ± 13.8 |

These results were further supported by using scanning electron microscopy (SEM), which allowed to also assess the morphology of particles, which was spherical, as seen in Figure 5. Despite the high PDI, size analysis using SEM revealed that the majority of particles presented mean size around the values obtained by using the dynamic light scattering technique. Regarding the zeta potential of these particles, they yielded negative values that were lower than those usually described in literature. However, such results may be related to the use in other reports of PVA (uncharged polymer) as stabilizer, contrasting with the present work. PVA is able to mask the negative charge provided by carboxylic groups from Eudragit®, which are deprotonated at the pH of the assay, thus leading to zeta potential results closer to neutrality.[128]

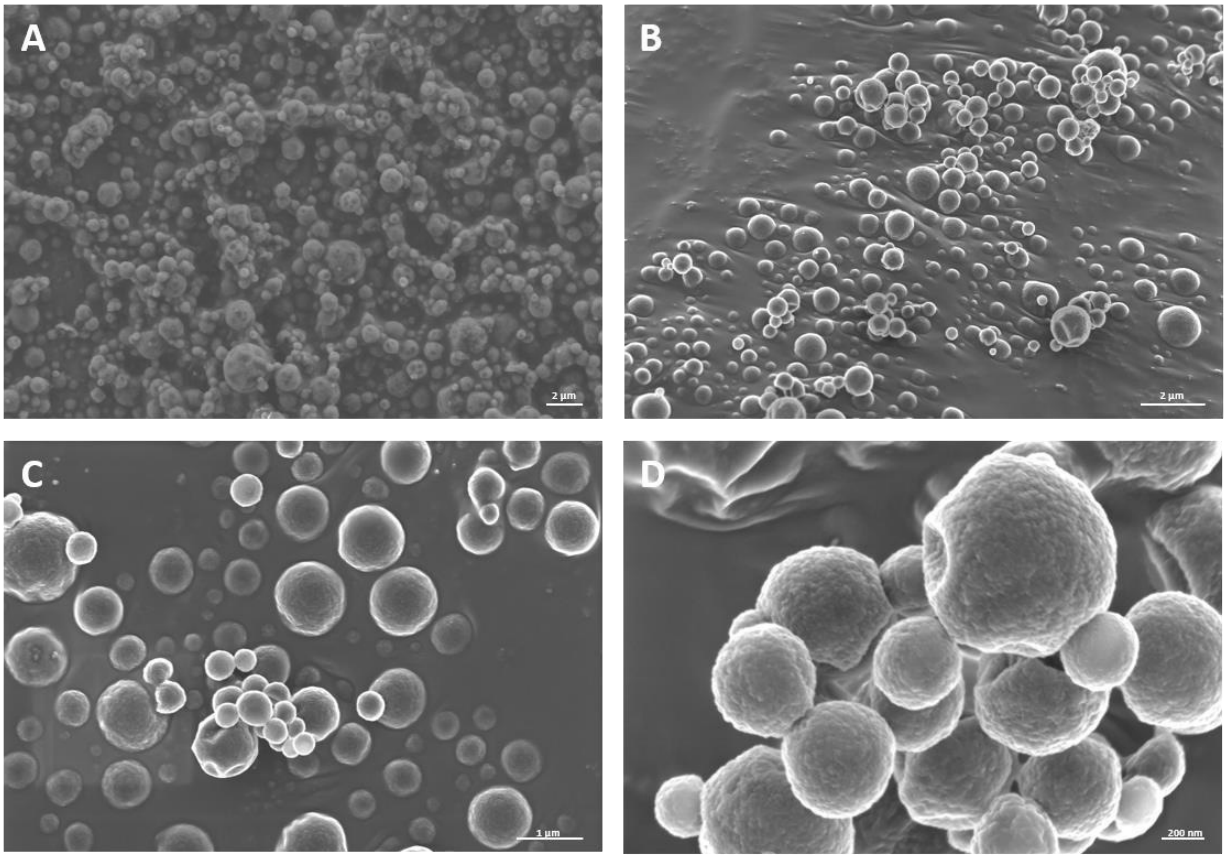


**Figure 5.** Representative scanning electron microscopy images at (A) 8650×, (B)15250×, (C) 32050× and (D) 100000× magnification of Eudragit® L100 nanoparticles.

Overall, both systems – PLGA-based and Eudragit®-based – may provide interesting properties for use in vaginal microbicide development. While PLGA-based nanoparticles may be suitable for mucus-penetration, Eudragit®-based systems may provide pH-dependent drug release.

### 4.2.2 Association efficiency and drug loading

The use of drug-loaded nanoparticles in medicine demands a careful characterization of the association efficiency of drugs to the carrier as well as loading capacity, since this will ultimately affect the amount of particles to be administered. TDF and FTC are hydrophilic drugs and, despite different approaches used (e.g., by using ion pairing agents), high association efficiency has been challenging.[97, 116-117]

When developing the formulation with PLGA, the first emulsion (that contains the drug) was more concentrated than what was described in the original protocol. This was intended to result in an increase on the drug association, since in the original protocol the association efficiency obtained for tenofovir (which is more hydrophilic than its prodrug TDF) was low.[99]

Initial quantification using an indirect method was done (Table 5). This method is based on the quantification of the drug that is not associated (recovered in the filtrates of the washes). Values of around 75% AE of TDF and FTC were obtained and much higher than the previously reported in the literature.[97, 116-117] In order to test these results, direct quantification was performed, in which the particles were destroyed and the recovered drug was quantified. The association rates seen with this method were of around 2%.

**Table 5.** Association efficiency and drug loading of TDF and FTC-loaded nanoparticles produced using a double emulsion/solvent evaporation method. Results are presented as mean ± SD (n=3).

| Formulation | Association efficiency (%) | | Drug loading (%) | |
|---|---|---|---|---|
| | Direct method | Indirect method | Direct method | Indirect method |
| **TDF-loaded** | 1.6 ± 0.4 | 73.1 ± 1.8 | 0.0 ± 0.0 | 12.8 ± 0.3 |
| **FTC-loaded** | 1.9 ± 0.6 | 74.8 ± 1.6 | 0.0 ± 0.0 | 13.0 ± 0.3 |

Differences between both methods are huge and various explanations may be pondered. For example, drugs may be retained in Amicon filters during centrifugation. Despite having a pore of 100 kDa, the filters may still oppose the passage of drugs by means of clogging with the remains of both the matrix forming polymer, in this case, PLGA and surfactant, poloxamer upon the washing steps. This ultimately may have hindered the migration of the non-encapsulated drug to the filtrated side, resulting in lower concentrations of drug in the filtrate and ultimately leading to an overestimation of the association efficiency. Another possibility has to deal with the direct adsorption of drugs to the filter. As for the direct method, destruction of the particles may have not been complete with the selected conditions and thus reduce the

amount of recovered drug. In any case, the disparity between values obtained with the indirect and direct method is extensive and further studies may be necessary in order to fully assess reason(s) for such observations. Since the direct method is considered more reliable, such results were considered as definitive and PLGA-based nanoparticles were discarded for subsequent characterization and development.

The spray drying method, on the other hand, allowed obtaining particles with association efficiency of around 90% and 61% for TDF and FTC, respectively, when using the direct quantification method (Table 6). When compared to the highest results obtained in the literature for TDF/FTC nanoparticles, namely around 50% using ion-pairing agent and an emulsion method, the hereby presented results appear to support the further development of Eudragit®-based nanoparticles.[97, 116]

Unfortunately, despite high drug loading, the ratio used in oral Truvada® (3:2 TDF:FTC) was not maintained. A new ratio of around 2.2:1 TDF:FTC was calculated for the Eudragit® nanoparticles.[41] Still, these particles were able to successfully incorporate the highest amount of drug described so far, and are expected to be able to deliver the intended dosage without requiring the administration of high amounts of particles, as previously described.[99] Thus, Eudragit® nanoparticles were considered for further development.

**Table 6.** Association efficiency and drug loading of TDF/FTC-loaded nanoparticles produced using a spray-drying method. Results are presented as mean ± SD (n=3).

| Formulation | Association efficiency (%) | | Drug loading (%) | |
|---|---|---|---|---|
| | TDF | FTC | TDF | FTC |
| **TDF/FTC-loaded** | 89.6 ± 9.8 | 61.3 ± 7.2 | 5.4 ± 0.9 | 2.5 ± 0.6 |

### 4.2.3 *In vitro* drug release studies

Eudragit® L100 is characterized for its pH dependent dissolution, as above described. Thus, particles comprising this polymer can potentially offer triggered release and be used as a long-acting microbicide. The vaginal pH in healthy women of reproductive age is acidic (around 4) but upon ejaculation can rise to values around 7.[26] Eudragit® nanoparticles are meant to dissolve only at near neutral pH, which means that particles may maintain their integrity in the vaginal tract until contact with semen (and possible exposure to HIV) triggers its dissolution and, potentially increase the release of drug content.[121]

Before performing release studies, a quick assessment of the solubility of the drugs in PBS and SVF was performed. To do so, one milligram of TDF and FTC were dissolved in two milliliters of PBS or SVF, placed onto Eppendorf tubes and left under orbital shaking (100 rpm) at 37°C. Samples were collected between 15 min and 48h and processed and assayed as described in the Materials & Methods section for drug release studies. As seen in Figure ***6***6, FTC is solubilized at both pH values after one hour. SVF, as well as PBS, are aqueous solutions, and hence, these results are consistent with the solubility described for FTC.[129] According to what has been reported in literature, TDF is supposed to be solubilized in water at the tested concentration. When at pH 4, such behavior was observed, although 100% solubilization was only reached between 24h and 48h. Paradoxically, at pH 7, the amount of solubilized TDF decreased over time. These results may be related with poor stability of TDF in PBS, which has not been described before in the literature, or simply with weighing errors associated to the small amount of drug that was used in these studies (1 mg). Since the concentrations meant to be used in the experiments within the range of TDF solubility (13.5 mg/mL), we considered that the subsequent experiments would be in sink conditions.[130]

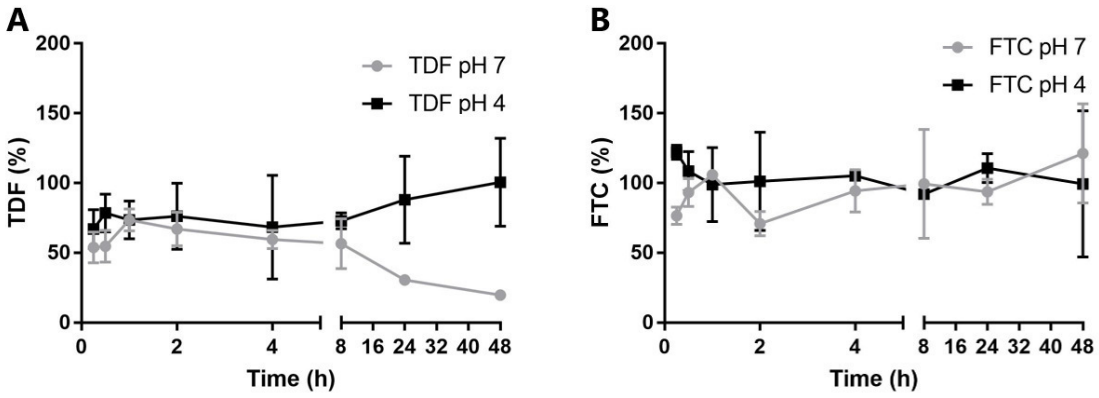


**Figure 6.** Solubility of (A) TDF and (B) FTC in both PBS pH 7 and SVF pH 4 at 37°C during 48h under orbital shaking (100 rpm). Results are presented as mean ± SD (n=3).

Posteriorly, the pH dependence of the release of these particles was assessed. *In vitro* release studies at both pH 4 and pH 7 were performed.

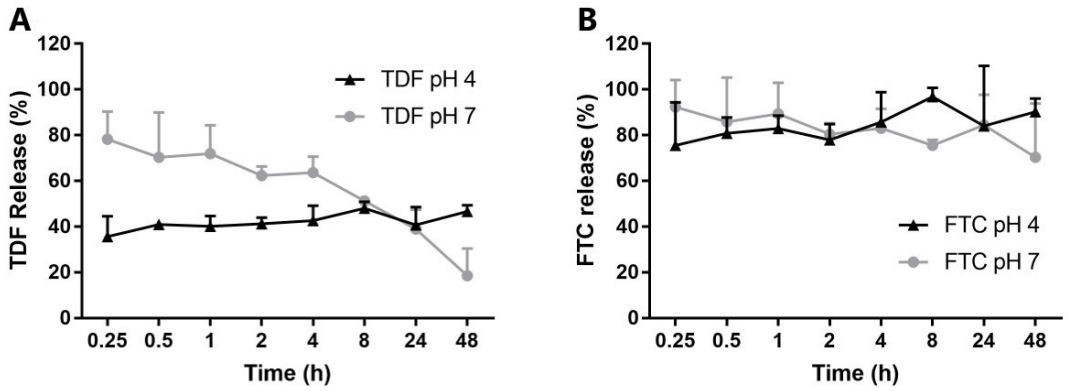


**Figure 7.** *In vitro* release of (A) TDF and (B) FTC from particles in both PBS pH 7 and SVF pH 4 at 100 rpm and 37°C. Results are presented as mean ± SD (n=3).

Data in Figure 7 appear to support that the release of TDF is dependent on pH. This was further confirmed when calculating the value for $f2$, whose value was of 29. Similarly, FTC revealed to have also a marginally pH dependent release, as revealed by its $f2$ value of 45.[109] Still, the release of FTC was of almost 100% on the first 15 min in either pH, while for TDF, at pH 4, the release was around half of that released when at pH 7, as expected.[120] One way to explain this is the possibility of FTC to be adsorbed to the surface, while TDF could be entrapped in the particles, causing a fast and almost complete release of FTC in the initial minutes. In any case, the extensive drug release at early time points seems to suggest that release was mostly related with the fast diffusion of the drugs from the polymeric matrix rather than dependent on Eudragit® dissolution. The amounts of recovered TDF, strangely, were seen to decrease over time. Again, this could be simply associated with weighing associated error, as seen in the previous experiment. However, stability issues may also be implicated in such observations.

In order to evaluate the possibility of incorporating these particles during the production of films by solvent casting, a modified *in vitro* release was performed in order to simulate the basic conditions observed during the drying step. The particles were weighed into the film molds and placed under the film drying conditions (37 °C and static conditions) with either PBS at pH 7 or acetate buffer at pH 4. This last medium, rather than SVF, was used as a putative solution to be used for film production.

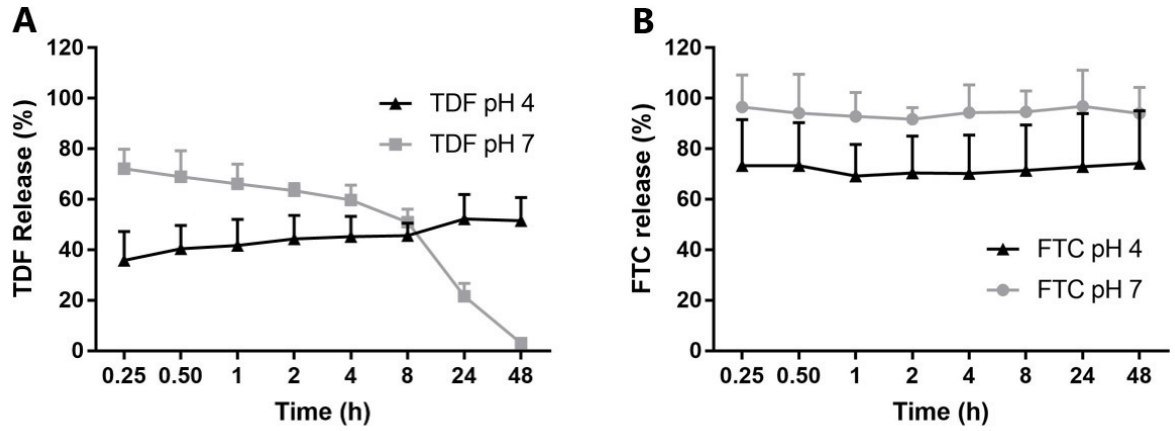


**Figure 8.** *In vitro* release profile at 37°C of (A) TDF and (B) FTC from particles in PBS pH 7 and acetate buffer pH 4 at 37°C and static conditions. Results are presented as mean ± SD (n=3).

As seen in Figure 8, and similarly to what was seen in previous experiment (performed under shaking), the release of FTC was almost immediate, with no considerable influence of pH conditions ($f2$=32). TDF release also revealed to be greatly influenced by pH, with a release of under 50% when at pH 4 and a $f2$ of 27.[131] Overall, these results indicate that incorporating the particles in the solution prior to film drying does not seem feasible, since FTC is almost totally released in the first 15 minutes (even at pH 4). TDF content, once again, was seen to decrease over time when in PBS. This further increase our suspicions that stability may be impaired in PBS. Since TDF is a prodrug that undergoes phosphorylation in order to be activated, there might be some interaction between the phosphates of the buffer and the drug, thus interfering with the HPLC-UV assay.[132]

In order to verify this, a rapid assessment of the stability of TDF in SFV pH 4, PBS pH 7 and ultra-pure water was conducted. To do so, one milligram of TDF was dissolved in 15 mL of media and assayed immediately or following orbital shaking (100 rpm) at 37ºC for two weeks. The amount of TDF was assayed at time zero and again after 15 days, using the HPLC-UV method above described. Obtained results are presented in Figure 9.

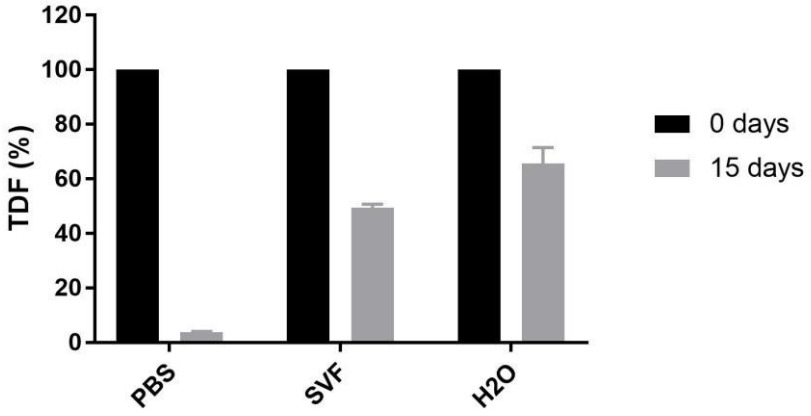


**Figure 9.** Quantification of the amount of TDF in PBS, SVF and water at day 0 and after 15 days at 37 ºC under stirring. Results are presented as mean ± SD (n=3).

The reduction of the TDF peak was evident in all conditions, resulting in a decreased amount of TDF assayed, which could be due to hydrolysis. However, the amount of remaining TDF in the case of PBS was near zero, which reinforces the idea that the presence of phosphates, or at least the difference in pH, may account for the paradoxical results observed during drug release studies. Thus, further investigation is required in the future to understand how such issues impact on the results of drug release studies and the overall stability of TDF in aqueous systems.

## 4.3 Manufacturing and characterization of films

### 4.3.1 Establishment of manufacturing conditions of films

The work with films started by the development and evaluation of production processes and formulations that could lead to the selection of a suitable platform for future incorporation of drugs or nanoparticles. The two main techniques for the production of medical films are solvent-casting and hot-melt extrusion.[133] The former was selected mainly due to ability to operate at low temperatures, as well as for the minimal requirements for specialized equipment.

Solvent-casting is a practical technique that has been used to develop vaginal films and deliver different ARV drugs.[134] It uses cheap resources (for example, water as solvent), and thus can lead to products that would be affordable even in low income countries.[135] Solvent-casting can be used to produce from single to thousands of films, either individually or continuously, and transposition from the laboratory to the industrial scale is considered feasible.[133] Even though this technique is widely used, it requires the manufacturing and drying process to be fully optimized to attain films with adequate characteristics.[69] Before performing any additional tests, the drying time, temperature and pressure, as well as formulation were optimized. Considering that PVA, PEG and glycerin are approved by FDA for mucosal use and are preferred for producing vaginal films, films comprising solely PVA (80%) and the plasticizers(10% each) were initially prepared and used to optimize the drying conditions.[136] In fact, the establishment of such setting is seen as quite important parameter, since the residual water content of films ultimately affects their organoleptic properties. Four drying conditions were considered, namely by using: (i) vacuum oven; (i) oven at 37 °C; (iii) fume hood; and (iv) open air (in the bench). For the last two, as well as for the vacuum oven, the temperature was not controlled. Drying time was initially set at 24 h for all conditions.

The choice of optimal drying conditions was based on both the organoleptic properties of the resulting films as well as the easiness to perform the technique while attaining reproducible results. Even though the evaluation of organoleptic properties can be considered quite subjective, it provides a quick and effective method to assess if the resulting films are fitting or not, since the perception of end users of these properties is known to be highly correlated to adherence and effectiveness of microbicides.[61-62]

Films dried under vacuum conditions were dried for 24 h (Table 7). Unfortunately, the resulting films presented poor organoleptic properties, with air bubbles spread all through the film. Furthermore, they were harsh at touch and non-flexible, with low ability to be folded by hand without creating permanent crease. As for the other conditions, 24 h were not sufficient to yield film formation, rather a gel like material.

**Table 7.** Organoleptic properties of films dried at different conditions: vacuum at room temperature, oven at 37 °C, fume hood and laboratory bench.

| Formulation | Drying conditions | Drying time | Organoleptic properties |
|---|---|---|---|
| **80% PVA + 10% PEG + 10% Glycerin** | Vacuum | 24 h | Transparent, colorless, odorless, homogeneous, frequent air bubbles, hard and non-flexible |
| | Oven at 37 °C | | ND |
| | Fume Hood | | ND |
| | Lab bench | | ND |

ND: Not determined due to the fact that films were not obtained after 24h.

The organoleptic properties above described for the vacuum oven drying conditions are not acceptable for a product of vaginal use, and for that reason, such setting was no longer considered an option.[79]

Production of suitable films was further pursued, this time only testing the following drying conditions: oven at 37°C, fume hood and laboratory bench (again, without temperature control) for 48 h. Distinct films were obtained after 48 h, presenting similar properties in terms of appearance (Table 8), expect for touch feel. Those dried at 37 °C appeared to be softer and more flexible, thus more appealing to touch.

**Table 8.** Organoleptic properties of films dried at different conditions: oven at 37 °C, fume hood and laboratory bench without temperature control.

| Formulation | Drying conditions | Drying time | Organoleptic properties |
|---|---|---|---|
| **80% PVA + 10% PEG + 10% Glycerin** | Oven at 37 °C | 48 h | Transparent, colorless, odorless, homogeneous, very soft and flexible |
| | Fume Hood | | Transparent, colorless, odorless, homogeneous, soft and flexible |
| | Lab bench | | Transparent, colorless, odorless, homogeneous, soft and flexible |

Such features may be due to the relatively high humidity conditions created inside the oven that resulted in higher residual water content of films. Considering these last results, we decided to move forward using the oven at 37 °C during 48 h for the drying process. It should also be noted that temperature control of the chosen condition is likely to be an important feature for future control of the drying step.

### 4.3.2 Development and characterization of PVA/pectin film formulations

Further development of the film formulation followed the establishment of the drying process. Apart from residual water, all formulations were set to include 80% of film forming polymers (PVA and/or pectin), 10% PEG and 10% glycerin. Ratios of 100:0, 75:25, 50:50 and 0:100 of PVA:pectin were tested. Pectin was added to the formulation in order to test its effects on the features of the obtained films. This last polymer is well known for its biodegradability, safety, viscosity enhancing properties and film forming ability, having a wide range of applications in food and pharmaceutical industry. Moreover, pectin is water soluble (which facilitates the incorporation in the previously established PVA-based film formulation), easily processed and widely available in nature, thus making it an accessible and cheap material.[137-138]

Different brands of pectin are available in the market according to specific uses; noteworthy, the grade of pectin used in this work complies with the requirements of the United States Pharmacopeia for use as excipient. However, formulations including pectin at 100% and 75% of the total film-forming polymer content yield films with poor organoleptic properties (Table 9), and this, were excluded from further evaluation. Apart from their opaque features, films with higher content of pectin were brittle. Overall, increasing amounts of pectin resulted in loss of suitable organoleptic properties as features by films containing only PVA as film-

forming polymer. All films containing pectin featured brownish color typical from this polymer.[139]

**Table 9.** Organoleptic properties, physical-chemical and mechanical characterization of different film formulations using PVA and pectin as film-forming polymers. VCF® was used for comparison. [a] and [b] indicate $p<0.05$ when compared to VCF® and to PVA film, respectively. Results are presented as mean ± SD (n=3).

| Film-forming polymer(s) | Organoleptic properties | Mass/area (mg/cm²) | Thickness (µm) | Moisture content (%) | Distance at burst (mm) | Burst strength (g) | Puncture Strength (g/mm²) | Disaggregation time (min) | pH | Osmolality (mOsm/kg) |
|---|---|---|---|---|---|---|---|---|---|---|
| **PVA** | Transparent, colorless, odorless, homogeneous, very soft and flexible | 9.1 ± 0.0 | 145 ± 9[a] | 5.0 ± 0.9[a] | 8.1 ± 2.2 | 3691.2 ± 522.5 | 188.3 ± 26.7 | 3 ± 0 | 4.5 ± 0.0 | 332 ± 10[a] |
| **PVA:pectin 75:25** | Semi-transparent, yellow brownish colored, odorless, homogeneous, very soft and flexible | 10.4 ± 2.3 | 226 ± 8[a,b] | 7.5 ± 1.6[a] | 5.7 ± 1.0 | 2554.6 ± 985.9 | 130.3 ± 50.3 | 9 ± 1[a,b] | 4.2 ± 0.1[a] | 304 ± 8[a,b] |
| **PVA:pectin 50:50** | Semi-transparent, yellow brownish colored, odorless, homogeneous, not very soft nor flexible | 10.0 ± 1.5 | 169 ± 2[a,b] | 8.7 ± 0.6[a,b] | 1.7 ± 0.3[a,b] | 2420.2 ± 955.5 | 123.5 ± 48.7 | 17 ± 2[a,b] | 4.1 ± 0.1[a,b] | 300 ± 4[a,b] |
| **VCF®** | Transparent, colorless, odorless, homogeneous, very soft and flexible | 9.4 ± 0.3 | 82 ± 0 | 13.1 ± 0.2 | 6.8 ± 0.7 | 2263.4 ± 172.4 | 115.5 ± 8.8 | 2 ± 0 | 4.3 ± 0.1 | 255 ± 4 |

Formulations with 100:0, 75:25 and 50:50 PVA:pectin ratios underwent different characterization assays in order to assess parameters such as thickness, moisture content, disaggregation time, osmolality and mechanical properties (Table 9). For all formulations, the mass per area was similar and around 10 mg/cm$^2$, which was lower than it would be expected (11.4 mg/cm$^2$), even if not significantly. The variance of this parameter between the different films and between the expected ratio and the experimental ratio may be due to weighing errors as well as possible losses during the preparation of the solution. Another possibility has to do with the solution that is seen to adhere to the borders of the mold, which are discarded afterwards. Both 75:25 and 50:50 PVA:pectin films were homogeneous and easy to peel off the glass mold. The 75:25 PVA:pectin films presented better organoleptic properties, namely, softness and flexibility, as well as a lighter and clearer appearance (Figure 10), which are features that have been reported as preferred in a study evolving women with different ethnical background.[139]

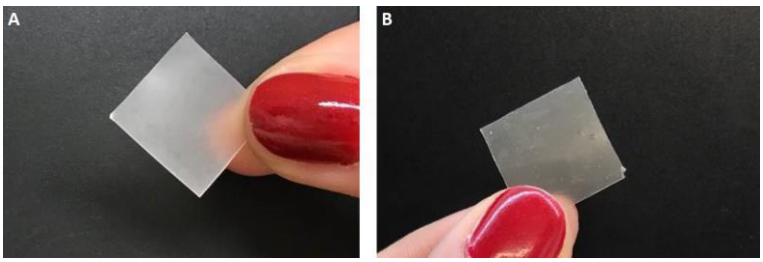


**Figure 10.** Representative images of 1.5×1.5 cm film samples with a ratio of (A) 75:25 and (B) 50:50 of PVA:pectin.

The thickness for PVA/pectin blend films was higher than the one measured for VCF®, but within the range reported for other vaginal film formulations.[80] More important, this increased thickness was not seen to affect the organoleptic properties of the resulting films, namely, the softness or flexibility. The mechanical characterization of films, as assessed using a texturometer, further provided valuable information regarding the properties of films (Table 9). The distance at burst, which is related to the flexibility and elasticity of the film, was decreased by increasing the content of pectin. Films with a ratio of 75:25 PVA:pectin had properties similar to the commercialized VCF® and above what was previously described in the literature for vaginal films.[99] Films with a 50:50 ratio, on the other hand, had limited elasticity (as apparent during the assessment of organoleptic properties), with a value of distance at burst of less than 2 mm. Burst strength also appeared to decrease with increasing content of pectin, although no statistical significance was noted. Overall, values of this last

parameter for produced films were similar to those of the VCF®. The puncture strength, which can be translated as the resistance and pliability of films, was around 103.3 g/mm$^2$ and 123.5 g/mm$^2$ for the 75:25 and 50:50 PVA:pectin film, respectively. These values are well above the minimum value (around 8 g/mm$^2$) considered enough for the film to maintain its integrity during manufacturing and application.[140] A trend to decreased puncture strength was noted with the incorporation of pectin but still not statistically significant, namely when comparing to VCF®.

Interestingly, the amount of pectin seems to be important in influencing the disaggregation time of films (Table 9). Significant differences were observed when comparing films with PVA:pectin ratios of 75:25 and 50:50, as well as films without pectin. This may be related to the thickening properties of pectin, which may allow forming a viscous sheet around the film upon contact with water and thus further oppose complete wetting of the polymeric film matrix and delay disaggregation.[137-138] Overall, this appears to indicate that it might be possible to modulate the disaggregation of films by varying the amount of pectin used, allowing to obtain quick-dissolving or long-acting vaginal films, as desired. Still, considering how pectin affects the organoleptic properties of the films, there is a need to balance the total amount of pectin required for delaying disaggregation.

The moisture content increased with the presence of pectin. The residual water content is an important parameter for both organoleptic properties (avoids brittleness and harshness to touch), as well as technical issues, namely, storage and stability (higher levels may lead to microorganism proliferation and/or drug hydrolysis.[66, 70] Moisture content values of produced films were lower than those for VCF®, but similar to others described in the literature with similar composition.[66, 70] In terms of stability and storage, the reported lower residual water content may be advantageous, although other issues (namely the presence of preservative or stabilizers) also need to be considered. Moreover, the presence of pectin appeared to increase residual water, which may be due to the hygroscopic nature of this polymer.[141]

The pH and osmolality of the solution resulting from the disaggregation of films in SVF was also measured. These parameters are crucial when evaluation the safety of vaginal products and should comply with normal physiological values.[142-143] The osmolality of PVA:pectin appeared to be significantly higher than those observed for the VCF® and for the SVF alone (255 ± 4 and 203 ± 6 mOsm/Kg, respectively). These results are likely due to obvious differences in composition, namely in the content plasticizers. Still, from a biological point of view, osmolality values for all films were within physiological values (around 300 mOsm/Kg)

and well below the maximum threshold for safety recommended by the World Health Organization for sex lubricants (1200 mOsm/ Kg).[144] Moreover, it appears that all films not induce considerable changes to the original pH of the SVF (4.2) and all results were within the normal physiological range of the vagina (3.5-4.5).[60] This is especially important, considering the role that the pH of the vaginal environment has in protecting women against HIV infection.[26] Despite minor differences, it seems clear that the incorporation of pectin induced a decrease in the final pH of SVF after film disaggregation. Such observation may well be related to the acidic features of pectin (pH=3.8 for a 1% solution, according to data from manufacturer) due to the presence of free carboxylic acid groups in its structure.

Upon overall analysis of the data obtained for the different films, the 75:25 PVA:pectin films were considered for further work and incorporation of drugs and/or nanoparticles. These films possess suitable mechanical properties that allow withstanding handing and application in the vagina. Furthermore, 75:25 PVA:pectin films present organoleptic properties that may fit typical preferences of potential microbicide user.[139]

### 4.3.3 Manufacturing and characterization of nanoparticles-in-film

The incorporation of particles in films was accomplished by using pre-formed 75:25 PVA/pectin films. The selection of a "dry procedure" relates to the possibility that nanoparticles extensively release their drug content during the drying step of film production (as inferred from data presented in Figure 8, if incorporated before casting into molds. Thus, two pre-formed film samples of 1.5×1.5 cm were pressed against each other after manually placing five milligrams of particles in between. The previous amount of particles corresponds to 0.27 mg/cm$^2$ of TDF and 0.12 mg/cm$^2$ of FTC. These doses were calculated based on previous reports for other vaginal delivery systems containing TDF and intended for protection from HIV transmission. In particular, Herold, Keiser and colleagues determined that daily doses of TDF within the range of 0.1-4 mg (as released from vaginal rings) are presumably sufficient for avoiding infection.[145-146] If considering the standard dimensions of 5×5 cm for the hereby-proposed films, as typically used for vaginal drug delivery, a dose of 6.8 mg and 3.1 for TDF and FTC, respectively, would be achieved. Thus, the previous TDF dose is roughly within the range proposed for achieving protection. Since no previous experience exists for the vaginal delivery of FTC, the dose for this drug was established solely based on the ratio obtained for Eudragit® L100 nanoparticles (2.2:1), which is close to the one for Truvada® oral tablets (1.5:1).

Films containing free TDF and FTC were also obtained for comparison purposes. To produce these last films, two different approaches were used: (i) the free drugs were dissolved in the excipient mixture before casting and drying or (ii) half of the amount of TDF and FTC was dissolved in the excipient mixture before casting and drying and then pressing two samples against each other after drying (by using the same method that was used to produce the films with nanoparticles). All films were characterized in terms of organoleptic, physical chemical and mechanical properties.

The use of pressure to form double-layered films affected the organoleptic properties (Figure 11). Surface smoothness decreased and films became opaque. This was especially pronounced for the films containing particles, but also observed for double-layered films with free drugs and, to a lesser extent, double-layered films with drugs or particles. Apart from the obvious effect of using two layers of film, the behavior to light passage was probably the consequence of the non-homogenous distribution of the particles resulting from their manual placement and distribution between the films. This could be abbreviated upon automation and continuous production of the films. Also, the amount of particles considered for incorporation was considerably high. A solution for this would be to produce larger films with the same amount of particles, so that they would be more evenly distributed throughout the films. For the films with drug that were pressed, the major impact of pressure was on the aesthetic of the film, namely, the transparency and color. As for single-layered films containing free drugs, similar organoleptic features were noted as compared to plain 75:25 PVA:pectin films. As expected, double-layered films and the incorporation of particles/drugs clearly modified the mass/area and thickness of final films (Table 10). Thickness of double-layered films either containing particles or free drugs increased substantially but still less than two-fold. Compressure due to the applied pressure is likely accountable for these results.

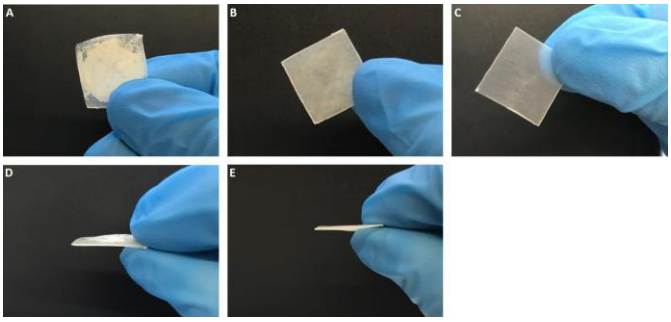


**Figure 11.** Representative images of different films. (A) Nanoparticles-in-film (two layers of 75:25 PVA:pectin films with particles in between); (B) Double-layered films with free drugs (two layers of 75:25 PVA:pectin films with drug in powder in between); and (C) Single-layered film with drugs (one layer of 75:25 PVA:pectin film with drugs dissolved before casting into molds). (D) and (E) are side views from double-layered films in (A) and (B), respectively.

**Table 10.** Organoleptic properties, physical-chemical and mechanical properties of different film formulations using PVA and pectin as matrix. (*) indicate $p<0.05$ when compared to the single-layered empty film. Results are presented as mean ± SD (n=3).

| Formulation | Organoleptic properties | Mass/area ($mg/cm^2$) | Thickness (µm) | Distance at burst (mm) | Burst strength (g) | Puncture Strength ($g/mm^2$) | Disaggregation time (min) | pH |
|---|---|---|---|---|---|---|---|---|
| **Single-layered empty film** | Semi-transparent, yellow brownish colored, odorless, homogeneous, very soft and flexible | 10.4 ± 2.3 | 226 ± 8 | 5.7 ± 1.0 | 2554.6 ± 985.9 | 130.3 ± 50.3 | 9 ± 1 | 4.2 ± 0.1 |
| **Nanoparticles-in-film** | Opaque, yellow brownish colored, odorless, heterogeneous, harsh and flexible | 57.1 ± 2.5* | 400 ± 10* | 6.0 ± 0.5 | 4303.9 ± 1183.1 | 219.6 ± 60.4 | > 2880* | 4.0 ± 0.0 |
| **Double-layered film with free drugs** | Semi-transparent, yellow brownish, odorless, heterogeneous, soft and flexible | 46.5 ± 3.1* | 337 ± 4* | ND | >5000* | ND | 30 ± 0* | 4.0 ± 0.0 |
| **Single-layered film with free drugs** | Semi-transparent, colorless, odorless, heterogeneous, soft and flexible | 26.0 ± 0.4* | 202 ± 4* | 7.0 ± 1.2 | 3019.1 ± 642.1 | 154.0 ± 32.8 | 8 ± 0 | 4.2 ± 0.0 |

ND: Non determined due to the equipment's inability to puncture with weigh higher than 5000 g.

The use of two film layers and incorporation of particles seemed to have an effect on the mechanical properties of the films (Table 10). Double-layered films featured higher burst strength and puncture strength, although not statistically significant, as compared to single-layered ones. The same was apparent for single-layered films with drugs. For the double-layered films with drugs it was not possible to quantify neither the distance at burst nor the puncture strength because of the maximum capacity of the texturometer (5000 g).

All films presented puncture strength values above the minimum value (around 8 g/mm$^2$) considered enough for the film to maintain its integrity during manufacturing and application.[140] The elasticity of nanoparticles-in-film formulation (6.0) was similar to that of single-layered empty film (5.7), which did not appear to be the case when evaluating and comparing the organoleptic properties of the previous referred formulations. Overall, it seems that the clear differences in organoleptic features between single-layered and double-layered films did not result, in most cases, in differences in mechanical properties. This is especially important because it means that double-layered films may still be considered adequate for application in the development of a vaginal product.

The transition from single-layered to double-layered films affected the disaggregation behavior. This was especially noticeable for the nanoparticles-in-film formulation, for which the disaggregation time was more than 48 h. This might have been related with the fact that Eudragit® L100 is insoluble at acidic pH, such as in the case of SVF, and thus allows retaining the structure of the double-layered film for a prolonged amount of time. Although requiring further investigation, such behavior may be interesting when pursuing the development of long-acting microbicides that would be coitus-independent regarding administration. This type of products have been reported as preferable amongst women.[42] In order to better assess this possibility, drug release studies of loaded-nanoparticles in films were conducted.

### 4.3.4 *In vitro* drug release studies of PVA/pectin films

TDF and FTC release in SVF at 37 °C was assessed during 48 h in order to predict the behavior of these films when in the vaginal environment. Results are presented in Figure 12. Overall, double-layered films were the ones presenting lower amounts of both TDF and FTC release. In particular, films containing nanoparticles released the least amount of drugs. A similar effect was previously observed for another ARV drug (efavirenz) when associated to PLGA nanoparticles-in-films.[99] The delay in both TDF and FTC release may be related with the combined and sequential release from Eudragit®-based nanoparticles and the film matrix.

Values of $f2$ for double-layered films containing either nanoparticles or free drugs were 35 and 40 for TDF and FTC, respectively, thus indicating differences between formulations. When comparing release profile of nanoparticles-in-film with and single layer films containing free drugs, $f2$ values indicated even higher differences ($f2$ of 24 and 18 for TDF and FTC, respectively).

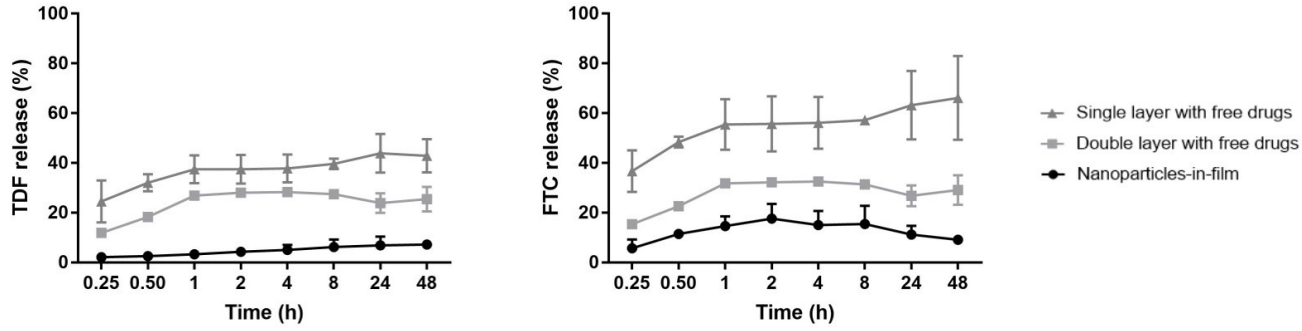


**Figure 12**. *In vitro* release profile at 37°C of (A) TDF and (B) FTC from nanoparticles incorporated in PVA/pectin films in SVF pH 4 and under orbital shaking (100 rpm). Results are presented as mean ± SD (n=3).

As noted above, the incorporation of the nanoparticles in the polymeric film matrix appeared to have a pronounced effect on the releasing rate. For both drugs, less than 20% of the total content was released until 48 h. Although not tested, it seems admissible to consider that higher amounts of TDF and FTC could be released as triggered by an increase in pH (as seen upon ejaculation) due to the solubility profile of Eudragit® L100. Such behavior may be considered as beneficial in order to release more drug when most needed, that is, at the time of viral exposure.[99] Still, one should analyze the presented results with caution due to the possible poor stability of the drugs in SVF, as noted before.

A particular explanation for the slow release observed for nanoparticles-in-film as to deal with the disaggregation time. Considering that the complete disaggregation time of the system was beyond 48 h, release results appear to be logic. In the case of films containing free drugs, release rates were not so markedly different from nanoparticles-in-films as could be inferred from disaggregation time values. Apart from obvious differences attributable the single-layered or double-layered films, it is possible that film excipients could have interacted with the drugs, not allowing for their complete solubilization or detection by HPLC-UV.

## 4.4 *In vitro* safety of nanoparticles and films

The documented general safety of the excipients chosen for the production of films was confirmed prior to starting formulations efforts by using a MTT assay. For PVA, PEG and glycerin, concentrations ranging from 0.2 to 200 mg/mL were tested. In the case of pectin, only the maximum concentration of 2 mg/mL was able to be used since, at higher levels, the viscosity of the medium did not allow proceeding with the experiments. As seen in Figure 13, pectin was apparently safe up to the highest concentration tested. Viabilities values of 70% and over 70% are usually regarded as denoting low cytotoxicity potential.[147] These results are in accordance to what was expected, since pectin is generally recognized as safe (GRAS) by the FDA and has been widely and safely used in food and medicines.[137, 148] PVA, PEG and glycerin were above the 70% threshold up to concentrations of 20 mg/mL.

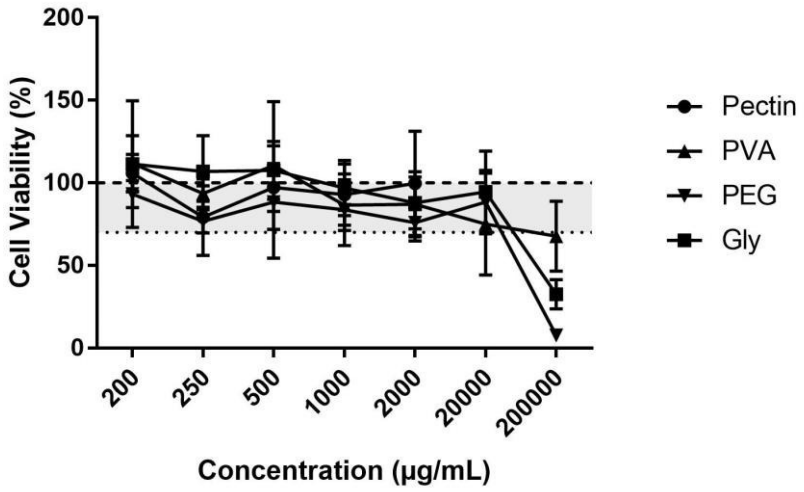


**Figure 13.** Viability of HeLa cells upon exposure to pectin, PVA, PEG and glycerine (Gly). Triton-X at 1% (v/v) and cells treated with medium only were used as negative and positive control, respectively. The area between 70% and 100% viability is highlighted in grey. Results are presented as mean ± SD (n=3).

PVA is biodegradable and widely used in pharmaceutical and biomedical applications, with no little associated toxicity issues.[149] The same stands for PEG, which is regularly used with pharmaceutical applications, namely, in drug delivery. This is consequence of, among other things, the acknowledged safety profile of this polyalcohol.[150-151] The FDA also grants glycerin with GRAS status, and thus, toxicity for this excipient is not expected. Still, decreased viability was observed at the highest concentration tested for PVA, PEG and glycerin. Such effects are likely to be related to the sharp increase in the osmolality of the medium caused by PEG and glycerin, which induces non-specific cytotoxicity. Moreover, other changes to the

properties of the cell culture medium, namely in viscosity or pH (confirmed by a change in color), are presumed to be the causes of decreased viability. In any case, the maximum tested concentration is unlikely to be observed *in vivo* for any of the tested excipients.

Thus, all excipients may be overall regarded as safe and suitable for use in vaginal film formulation.

The effect of TDF/FTC-loaded Eudragit® nanoparticles in HeLa cells viability was further assessed at concentrations corresponding to the range of 0.01 to 1000 µM (1000 µM = 635 µg/mL de TDF) when considering the content in TDF. For comparison purposes, each drug individually and in combination were also tested. Data presented in Figure 14 supports that FTC appears to be safe up to at least 1200 µM as inferred by viability values above 70%.[147] These results are in accordance with a previous report indicating half-maximal cytotoxicity ($CC_{50}$) values of FTC above 100 µM.[152] For TDF, viability sharply decreased at 100 µM. A previous study using peripheral blood mononuclear cells indicated that TDF features $CC_{50}$ values over 1000 µM; however, concentrations higher than 250 µM were seen to be toxic when HEI-OC1 cells were used.[153-154]. Thus, higher susceptibility of HeLa cells to TDF may justify our findings.

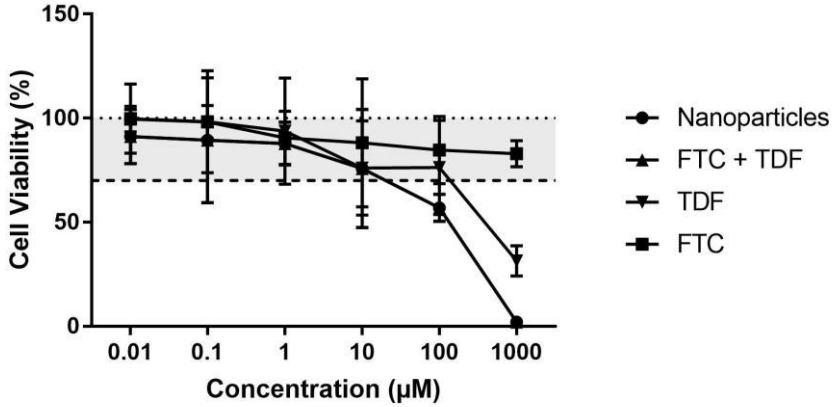


**Figure 14**. Viability of HeLa cells upon exposure to TDF/FTC-loaded nanoparticles. Triton-X at 1% (v/v) and cells treated with medium only were used as negative and positive control, respectively. Concentrations are expressed as TDF content except in the case of FTC alone. For FTC alone, concentrations were 0.012, 0.12, 1.2, 12, 120 and 1200 µM. The area between 70% and 100% viability is highlighted in grey. Results are presented as mean ± SD (n=3).

As for nanoparticles, as well as for the combined TDF with FTC in solution, the concentrations expressed in the graph are of TDF, in the way that FTC was present in crescent concentrations of 0.012, 0.12, 1.2, 12, 120 and 1200 µM (1200 µM = 302 µg/mL).

The combination of TDF and FTC in solution and in the nanoparticles appeared to be non-toxic until at least 10 µM but with maximum toxicity at 1000 µM (0.346 mg and 34.90 mg,

respectively, of particles in three milliliters of medium). The toxicity at higher concentrations of particles may however be related to the interference with the properties of medium (namely in pH as evidence by changes in color) or a physical barrier effect caused by the high content of solid material in direct contact with cells. Therefore, viability results do not necessarily reflect toxicity and should be analyzed with caution. Also, Eudragit® is regarded as a safe material but this could not be confirmed, as in the case of excipients used in films, since the co-polymer does not solubilizes properly in cell culture medium.[120]

It has been reported that the combination of TDF and FTC is expected to have a safety profile similar to the individual components, which is in accordance with the data in Figure 14. However, the combination of TDF with FTC appears to result in higher cytotoxicity than the individual drugs.[155] Still, TDF was not expected to have toxicity at such low concentrations and thus, the toxicity seen for the combination of TDF with FTC may result from this low tolerability of cells to TDF and not to FTC (since it did not interfere with the cell viability even at the highest concentration), as previously reported.[154]

The films with TDF/FTC-loaded nanoparticles were assessed for their effect on HeLa cell viability. Film extracts were prepared in accordance with ISO 10993-12 and the results are presented in Figure 15.[156] Results demonstrated that, in the concentration range tested, films seem to present reduced cytotoxicity potential, with only marginally lower values than 70% viability being observed at the highest concentration.[147]

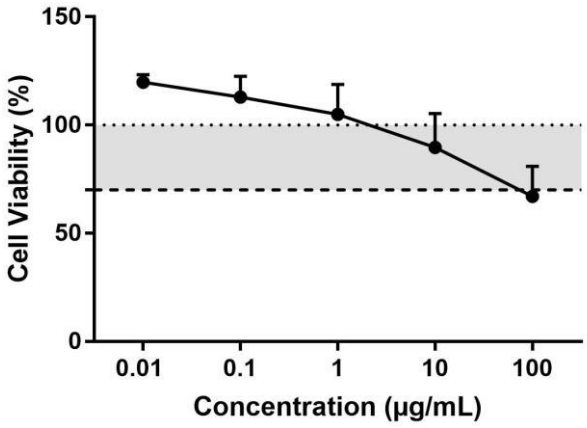


**Figure 15.** Viability of HeLa cells upon exposure to films containing TDF/FTC-loaded Eudragit® L100 nanoparticles. Triton-X at 1% (v/v) and cells treated with medium only were used as negative and positive control, respectively. The area between 70% and 100% viability is highlighted in grey. Results are presented as mean ± SD (n=3).

Increasing concentrations of nanoparticles-in-film seem to have led to a progressive decrease in cell viability. It was not possible to assess the cytotoxicity potential of the empty films and compare it to the nanoparticles-in-film due to lack of samples. Overall, data suggests that proposed PVA/pectin films with TDF/FTC-loaded Eudragit® L100 nanoparticles may be considered safe for vaginal use, although additional *in vitro* and *in vivo* testing is required.

# Chapter 5

# 5. CONCLUSIONS AND FUTURE WORK

The main goal of this work was to develop a polymeric film incorporating TDF/FTC-loaded nanoparticles to be used as vaginal microbicide for HIV prevention. In particular, the film should have organoleptic properties that could please potential users, while presenting adequate drug release and safety profiles.

Initially, we successfully developed a HPLC-UV method for the simultaneous quantification of TDF and FTC, to be used throughout the work at different stages of the development process of nanoparticles and films. The method was shown to be versatile and applicable to all intended purposes. Next, two different approaches were tested for the production of TDF/FTC-loaded nanoparticles, namely by using a double emulsion/solvent evaporation method for obtaining PLGA-based nanoparticles, or a spray-drying method for obtaining Eudragit®-based nanoparticles. Although the first strategy resulted in particles with colloidal features regarded as beneficial for vaginal drug delivery, inconsistent drug association rendered the obtained delivery systems as unsuitable for further development. In contrast, the spray-drying method allowed the efficient incorporation of considerable amounts of both TDF and FTC (5.4% and 2.5% drug loading, respectively) into particles. Eudragit®-based nanoparticles possessed mean size and zeta potential properties considered suitable for drug delivery, even though featuring some polydispersion. Moreover, the particles were able to rapidly and almost completely release FTC *in vitro* at two relevant pH values (namely 4 and 7) for microbicide development. As for TDF, the release seemed to have been affected by the variation in pH, although issues with the stability of drug, particularly in PBS, impaired clearer conclusions. Thus, further investigation into this matter is required. Overall, drug release results appear to backup that Eudragit®-based nanoparticles can indeed release both TDF and FTC upon contact with aqueous media, even if the anticipated pH-dependent drug release profiles were not observable. Apart from the stability issues in the case of TDF, it seems that the high aqueous solubility of the drugs is more relevant in determining their release than the effect of the differential solubility profile of Eudragit® L100 used to obtained particles.

The work proceeded with the study of different film formulations and their production by solvent-casting technique. The appropriate manufacturing parameters (namely, temperature, pressure and time for film drying) were selected based on various preliminary studies in order to obtain reproducible polymeric platforms with organoleptic properties regarded as appropriate for vaginal films. Upon establishment of the best conditions for film manufacturing, the effect

of different ratios of PVA:pectin as matrix-forming polymers were studied in the properties of films. In particular, the amount of pectin incorporated had considerable impact on the ability to form films: while at relatively lower levels this polymer was shown advantageous for modulating the disaggregation time of films, its presence above 50% impaired the formation of usable films. Selection of 75:25 PVA:pectin ratio allowed obtaining a film base featuring delayed disaggregation, which could be useful for sustaining drug/nanoparticle release and ultimately allow to obtain coitus-independent products. This film base also possessed physical-chemical, mechanical and organoleptic properties regarded as suitable for the development of a vaginal product, and was considered for further incorporation of drugs or nanoparticles.

The incorporation of Eudragit®-based nanoparticles into a film platform was performed by manually placing particles in between two individual films and further applying pressure. Avoidance of "wet" methods was for preventing extensive drug release from particles as could be easily predicted. Although "sandwich"-type films were successfully obtained, there were some problems related with the homogeneity of powder distribution, a fact that could be abbreviated upon scaling-up by using dedicated equipment. Relevantly, the main features of nanoparticles-in-film were considerably different from those of PVA:pectin film bases but still within values that could be regarded as appropriate for vaginal use. The disaggregation time of nanoparticles-in-films was considerably prolonged, while mechanical properties such as elasticity and pliability were increased. On the downside, the organoleptic properties of nanoparticles-in-film was not as ideal as plain films. However, drug release from the nanoparticles-in-film formulation appeared to have been sustained up to at least 48 h. Even if these results still need to be confirmed (namely due to the identified stability issues of TDF in media), it seems that the proposed nanoparticles-in-film may provide an interesting system for obtaining microbicide products featuring prolonged efficacy. Finally, nanoparticles-in-film seem to possess low cytotoxicity thus advocating for the potential safety of the proposed system.

Overall, the proposed nanoparticles-in-film system appears to be suitable for the combined vaginal delivery of TDF and FTC. This drug delivery platform may potentially allow obtaining sustained protective drug levels at the vagina and thus constitute an alternative or even complementary strategy to the established oral PrEP with Truvada® for protecting against sexual transmission of HIV to women.

Although the main objective of the project was achieved, additional efforts are still required, not only to address some weaknesses of the work performed but also to further pursuit

the establishment of nanoparticles-in-film as a putative microbicide product. For instance, it would be of interest to validate the HPLC-UV method so that it could be used in future works with TDF and FTC. One particular aspect in which a validated HPLC-UV method could be useful is the question of the stability of TDF and even FTC. Considering the inconsistency of results for the association efficiency of PLGA-based nanoparticles, this parameter should be assessed again. For example, using harsher conditions that assured the destruction of particles could be helpful. Also, revisiting the formulation of PLGA-based nanoparticles could be helpful for increasing drug association, namely by introducing agents that could originate complexes or reticulate TDF and FTC. Regarding the Eudragit®-based nanoparticles, modification and optimization of the production protocol could be considered in order to obtain the 3:2 TDF:FTC ratio present in Truvada®, apart from improving colloidal features such as by reducing polydispersion. Regarding film formulation, it would also be worthy to expand the study on the influence of pectin on the properties of films, namely, moisture content and disaggregation time, as this could be of use in future development of microbicide products. Testing intermediate ratios of PVA:pectin could be helpful in fine tuning the properties of films.

The incorporation of nanoparticles in the films was, arguably, the greatest challenge during the present work. The high solubility of TDF and FTC in aqueous systems makes nearly impossible to use water during the incorporation process. Choosing a "dry" method was inevitable due to the time and resources available for this work. Still, the use of dedicated equipment or optimization of the compression step during production could help improving the properties of nanoparticles-in-films. Also, direct spray-drying of nanoparticles onto preformed films could be an inventive alternative to the compression of particles in between two pieces of film. Regarding the final properties of nanoparticles-in-film, it would be useful to expand the studies performed during the project. This should also include testing different conditions (*e.g.*, drug release in the presence of semen) and other type of control films (*e.g.*, double layered empty films as well as double layered with free drug incorporated in the same way as nanoparticles). In particular, features such as *in vitro* drug release and cytotoxicity will likely be essential to the goal of the films, namely to safely deliver TDF and FTC in a sustained fashion in the vaginal environment. Other properties that were not considered in the present project but would be of interest include the mucoadhesive behavior of films and their ability to release nanoparticles. Apart from being able to modify drug release, nanosystems may contribute to preventing HIV by interacting directly with the mucosa at different levels.

Finally, and thinking about subsequent stages of development, *in vitro* and *in vivo* studies including the characterization of pharmacokinetics, efficacy and safety of nanoparticles-in-film would be relevant. Scaling-up studies would also be of interest in order to produce nanoparticles-in-film at the industrial level. Furthermore, acceptability studies among women with ethnically and culturally diverse backgrounds would be informative in transposing nanoparticles-in-film into clinical trials and, eventually, market scenarios.

# Chapter 6

# 6. REFERENCES


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