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Research paper

Composite films for vaginal delivery of tenofovir disoproxil fumarate and emtricitabine

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ABSTRACT

Prevention of male-to-female HIV transmission remains a huge challenge and topical pre-exposure prophylaxis (PrEP) using microbicides may help overcoming the problem. In this work, different types of films containing the antiretroviral drugs tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC) were developed. Formulations based in poly(vinyl alcohol) and pectin were produced as single- or double-layered films. Films containing TDF/FTC or TDF/FTC-loaded Eudragit® L 100 nanoparticles (NPs) obtained by nano spray-drying were tested for physicochemical, technological and biological properties relevant to microbicide development. All systems featured organoleptic and mechanical properties considered suitable for vaginal use and potentially favoring users' acceptability. Film design (single- or double-layered, and the incorporation or not of NPs) had a greater impact on disintegration time and drug release in a simulated vaginal fluid. Upon film disintegration, pH and osmolality of the fluid remained within values considered compatible with the vaginal environment. Double-layered films significantly reduced burst effect and the overall release of both drugs as compared to fast releasing, single-layered films. The effect on delaying drug release was most noticeable when TDF/FTC-loaded NPs were incorporated into double-layered films. This last design seems particularly advantageous for the development of a coitus-independent, on-demand microbicide product. Moreover, all film types were shown potentially safe when evaluated by the MTT metabolic activity and lactate dehydrogenase release assays using HeLa and CaSki cervical cell lines. Overall, results support that proposed films may be suitable for the vaginal delivery of TDF/FTC in the context of topical PrEP.

1. Introduction

Oral pre-exposure prophylaxis (PrEP) based on tenofovir is currently recommended by the World Health Organization as an effective strategy for preventing sexual HIV transmission [1]. Once daily Truvada® (Gilead Sciences), a combination product containing 300 mg of tenofovir disoproxil fumarate (TDF) and 200 mg of emtricitabine (FTC), is the most usual regimen, being currently approved by the US Food and Drug Administration and the European Medicines Agency for the protection of individuals at high risk of infection. Oral PrEP is already available in various countries worldwide, including the United States, France, South Africa, Canada or Australia. Others are expected to follow [2]. Various clinical studies support that the use of TDF/FTC is highly effective when used consistently [3–5]. However, conflicting results from two trials suggest that protection provided by oral PrEP to women may be diminished as compared to men who have sex with men [6,7]. Poor adherence to daily regimens seems to be the main reason for these observations [8], although acceptability issues, onset of systemic effects, and differential risk perception may also play key roles [9]. One additional but highly relevant question concerns actual biological differences between the cervicovaginal and colorectal compartments that limit the drug concentration that is reached in each mucosae following oral administration of TDF/FTC. For instance, a recent pharmacokinetics/pharmacodynamics (PK/PD) modeling study found that only nearly perfect adherence to oral PrEP with TDF/FTC may be able to provide considerable protection in the female genital tract, while discontinuous use could still prevent rectal HIV transmission [10]. Data from clinical trials also seem to backup this hypothesis [11,12]. Thus,

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further insights are needed regarding the most appropriate way to pursuit PrEP strategies for women, namely by enabling higher and prolonged TDF/FTC levels at the cervicovaginal tract.

An interesting alternative to oral TDF/FTC regimens in women could be topical PrEP with vaginal microbicides [13]. These investigational products have been designed to be present in the vagina during and following intercourse and prevent male-to-female HIV transmission. The ability to generate high local drug levels while minimizing systemic exposure may be beneficial, not only for enhancing protection in the mucosa but also for avoiding side effects [14]. For example, moderate protection from infection was observed in previous clinical trials for women using a tenofovir gel [15] or a dapivirine ring [16,17], without differences being observed in terms of adverse events as compared to matching placebos. One important issue of the development of a microbicide product has to deal with the choice of dosage form. The use of vaginal rings has been previously proposed for the delivery of the TDF/FTC combination [18,19]. Despite allowing maintaining potentially protective drug levels in the vagina for several weeks to months, rings require continuous use and may not be acceptable, particularly by women engaging in erratic sexual activity. Vaginal tablets containing tenofovir (base) and FTC have also been developed as microbicides for discontinuous use [20], but this dosage form is regarded by women as less preferable [21]. Conversely, vaginal films are typically well accepted, easy and discrete to use, and suitable for the design of on-demand microbicides [22]. The technology involved in film manufacturing is well established, allowing to obtain highly reproducible, stable, inexpensive and versatile drug delivery platforms that can be scaled up utilizing conventional industrial setups. Polymers used as matrix-forming materials usually confer mucoadhesive properties to films, which are regarded as beneficial in prolonging vaginal drug retention [23]. Furthermore, the modification of the basic film design by incorporating drug-loaded nanocarriers that allow to better control release kinetics represents an appealing strategy [24]. In this study, we report on the development of different composite films, including one produced by the incorporation of drug-loaded nanoparticles (NPs), to obtain novel platforms for the vaginal delivery of TDF and FTC in the context of topical PrEP.

2. Materials and methods

2.1. Materials

TDF was obtained from Kemprotec (Cumbria, UK), FTC from Sequoia Research Products (Pangbourne, UK), poly(vinyl alcohol) (PVA; 87–90% hydrolyzed, 30–70 kDa) from Sigma-Aldrich (St. Louis, MO, USA), poly(ethylene glycol) (PEG) 4600 from Union Carbide (Houston, TX, USA) and glycerin from Aliand (Mem Martins, Portugal). Eudragit® L 100 (methacrylic acid/methyl methacrylate copolymer, 1:1) and high methoxy pectin [GENU® pectin (citrus) type USP/100] were kind offers from Röhm GmbH (Darmstadt, Germany) and CP Kelco (Lille Skensved, Denmark), respectively. All other materials and solvents were of analytical grade or equivalent.

2.2. Production and characterization of drug-loaded nanoparticles

Eudragit® L 100 NPs containing both TDF and FTC (TDF/FTC-NPs) were produced by spray-drying [25]. Briefly, 0.45 g of copolymer, 30 mg of TDF and 20 mg of FTC were dissolved in 90 mL of a mixture of purified water and ethanol (50:50, *v/v*), and then sprayed using a BUCHI B-90HP Nano Spray-Dryer (Flawil, Switzerland) in closed cycle with the following setup: 5.5 μm mesh; 105–110 °C inlet temperature; 22–25 °C outlet temperature; 100 L/min gas flow rate; 20–25% pump speed; 80% spray power; and 80–90 kHz frequency. Drug-free NPs were produced using the same procedure but without including TDF and FTC in the initial Eudragit® L 100 hydroalcoholic solution. All NPs were stored at room temperature until further use. The hydrodynamic diameter, polydispersion index (PDI) and zeta potential of NPs were assessed after NP dispersion in 10 mM sodium chloride (25 μg/mL) using a ZetaSizer Nano ZS (Malvern, Worcestershire, UK) at 25 °C. The size and morphology was further analyzed by scanning electron microscopy (SEM) using a Zeiss Ultra-Plus microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany). Images were acquired using secondary electrons at 2 kV acceleration voltage and at a working distance of 2.5–4.0 mm. Diameter values were calculated from the estimated area of 300 individual NPs randomly selected from representative SEM images using the elliptical selection tool of ImageJ software (v. 1.51j8, NIH, Rockville, MD, USA). Data were fitted to a Gaussian distribution using Prism® v. 5.03 (GraphPad Software, La Jolla, CA, USA) in order to calculate mean and standard deviation (SD) values.

Drug content was determined by dissolving one milligram of NPs in two milliliters of purified water and ethanol (50:50, *v/v*), followed by TDF and FTC assay by HPLC-UV. The analytical method was performed using a Merck-Hitachi 7100 series HPLC system (Tokyo, Japan) under the following conditions: Zorbax Eclipse XDB-C18 column (3.5 μm, 3.0 × 75 mm; Agilent Technologies, Santa Clara, CA, USA); mobile phase comprising phosphate buffer 10 mM, pH 6/methanol; gradient mode (from 96:4 at 0 min to 45:55 at 2 min, 45:55 until 8 min, from 45:55 at 8 min to 96:4 at 9 min, and 96:4 until 15 min); 1 mL/min flow rate; 10 μL sample injection; and 260 nm detection for both drugs. The method was linear ($r^2 \geq 0.9998$) in the range of 3–100 μg/mL for both drugs, with retention factor values of 5.2 and 11.3 for FTC and TDF, respectively. The retention factor was calculated as the ratio of the difference between the retention time of the drug and the solvent, and the retention time of the solvent. The association efficiency, expressed as the percentage ratio between the amount of drug used and the amount recovered after spray-drying, and the drug loading (corresponding to the total amount of drug per weight of NPs) were calculated.

2.3. Development and manufacturing of films

All films were obtained by solvent casting technique. A basic formulation and production methodology was initially established based on preliminary experiments. Briefly, 696 mg of film-forming polymer(s) (PVA and/or pectin) and 174 mg of plasticizers (PEG 4600/glycerin 1:1, *w/w*) were dissolved in 14.13 g of purified water, and the mixture poured into glass molds (76.97 cm^2). Film sheets were collected after drying at 37 °C for 48 h in a static oven (BINDER GmbH, Tuttlingen, Germany) and cut into squared fractions of different dimensions with a precision blade. Films featuring different *w/w* ratios of PVA/pectin (100:0, 75:25, 50:50, 25:75 and 0:100) were obtained and further characterized before selection of a final film prototype for drug incorporation. Three different types of composite films containing 0.27 mg/cm^2 of TDF and 0.12 mg/cm^2 of FTC were prepared, namely: (i) single-film – TDF/FTC was dissolved in the casting mixture before pouring it into molds and drying; (ii) double-film – half the amount of TDF/FTC was dissolved in the casting mixture before pouring into molds and drying, and then two film portions were bind together by using a hydraulic press (Graseby Specac, Orpington, UK) at four tons for 30 sec; and (iii) NPs-in-film – TDF/FTC-NPs were evenly dispersed on top of a portion of plain film (without drugs) and then covered by an additional layer of plain film before binding the system using a hydraulic press at two tons for 30 sec. All films were protected with aluminum foil, sealed into plastic bags and stored at room temperature until further use. Since the protective vaginal dose of TDF/FTC in humans has not been established, the rationale used in the present study was based on recent work suggesting that 30–150 μg of TDF associated to poly(lactic-*co*-glycolic acid) NPs and incorporated into a thermosensitive gel would be able to provide full protection against intravaginal challenge with HIV in humanized mice [26]. Thus, the amount of TDF in proposed films at dimensions suitable for use in mice (0.5 × 0.5 cm [27]), i.e. 67.5 μg, would be presumably enough to yield

protection. When translating to the human scenario, the dose included in a typical 5 × 5 cm film, i.e. 6.75 mg, would be in line with the amounts predicted as protective in macaques for TDF released from intravaginal rings (0.4–4 mg/day) [28]. Since no data are available for vaginal FTC, its dose was simply set in order to roughly comply with the 1.5:1 TDF/FTC ratio in Truvada®.

2.4. Characterization of films

Films were characterized as previously detailed [27,29]. Thickness was evaluated with a digital micrometer (I.C.T, Lardero, Spain). Moisture content was assessed by heating 100 mg film samples up to 100 °C over two minutes using a MX-50 moisture analyzer (A&D, Tokyo, Japan). Disintegration time was determined by immersing 1.5 × 1.5 cm film samples in one milliliter of a simulated vaginal fluid (SVF) pH 4.2 in 6-well cell culture plates maintained under 20 rpm orbital shaking at 37 °C. The SVF was adapted from Owen & Katz [30] and contained water, 0.5% (*w/v*) glucose, 0.351% (*w/v*) sodium chloride, 0.2% (*w/v*) lactic acid, 0.14% (*w/v*) potassium hydroxide, 0.1% (*w/v*) acetic acid, 0.04% (*w/v*) urea, 0.022% (*w/v*) calcium hydroxide, 0.016% (*w/v*) glycerin, and concentrated hydrochloric acid (enough amount for pH 4.2). The behavior of films in SVF was monitored visually and the time required for complete disintegration was recorded. The resulting dispersions were further recovered, homogenized and used to determine the pH and osmolality using a PHT 810 pH-Meter (ebro Electronic, Ingostadt, Germany) and a Micro-Osmometer Type 15 (Löser Messtechnik, Berlin, Germany), respectively. Mechanical properties such as puncture strength and distance at burst were evaluated by performing puncture tests. Films were placed on a support rig (HDP/FSR) mounted onto a TA.XT*plus* texture analyzer (Stable Micro Systems, Godalming, UK) and punctured by a 5 mm spherical probe (P5/S) moving at 1 mm/s. Puncture strength was determined as the ratio between the strength required for film burst and the cross sectional area of samples. The elongation of the film until breakage was considered as the distance at burst. *In vitro* drug release studies were conducted for films containing TDF and FTC. Briefly, film samples (1 × 1 cm) were immersed in one milliliter of SVF in 6-well cell culture plates kept under 100 rpm orbital shaking at 37 °C. Samples of medium (200 µL) were collected at pre-determined time points and replaced with fresh SVF, and further centrifuged at 14,000 rpm for 15 min. Supernatants were separated and analyzed for TDF and FTC using the HPLC-UV method described above. Obtained drug release profiles were compared by calculating the similarity factor (f_2) as described by Moore and Flanner [31]. Values of f_2 between 50 and 100 were considered as denoting similarity.

2.5. Cytotoxicity of films

HeLa and CaSki cervical epithelial cell lines, both from ATCC (Manassas, VA, USA), were used for testing the cytotoxicity of films. Cells were maintained in Dulbecco's Modified Eagle's Medium (Lonza, Verviers, Belgium) supplemented with 10% (*v/v*) fetal bovine serum (Merck Millipore, Burlington, MA, USA), 100 U/mL penicillin (Merck Millipore) and 100 µg/mL streptomycin (Merck Millipore) under routine cell culture conditions (37 °C, 5% CO_2 and 95% RH). The cytotoxicity potential of films was determined by using the triazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich) metabolic activity assay and the lactate dehydrogenase (LDH) release assay. Experiments were performed in accordance with ISO 10993 on the biological evaluation of medical devices [32]. Briefly, cells were seeded in 96 well plates (10^4 cells/well) and maintained for 24 h under standard conditions, before incubation with film extracts for an additional 24 h. Film extracts were pre-prepared by incubating testing samples with cell culture medium at a film surface area-to-volume of medium of 1 cm^2/mL for 24 h at 37 °C. Plain medium and 2% (*v/v*) Triton X-100 in medium, treated in the same way as film extracts, were used as controls. Then, cells were washed twice with phosphate buffered saline pH 7.4 and incubated for 4 h with medium containing 0.5 mg/mL of MTT under standard conditions. Finally, the medium was discarded, the newly formed formazan derivative crystals were dissolved with 200 µL of dimethyl sulfoxide, and cell viability was determined by measuring the absorbance at 570 nm using a plate reader. Viability was calculated as the percentage of metabolic activity relative to the results obtained for plain medium. LDH levels from cell supernatants obtained after incubation with extracts were assayed using a commercial kit (Takara Bio, Shiga, Japan) according to the instructions of the manufacturer. Results were expressed as percentage cytotoxicity as compared to controls: plain medium and 2% (*v/v*) Triton X-100 in medium were considered as 0% and 100% cytotoxicity values, respectively.

2.6. Statistical analysis

One-way ANOVA with Bonferroni post-hoc test was selected for performing multiple comparisons using GraphPad Prism®. Values of $p < .05$ were considered as denoting significance. All results are presented as mean ± SD from three independent experiments.

3. Results and discussion

Despite currently regarded as highly effective, implementation of oral PrEP with TDF/FTC remains challenging due to a multitude of factors, and has been particularly difficult to establish in women. The development of a topical microbicide based on TDF/FTC may be an interesting and even complimentary prophylactic strategy allowing to rapidly achieve high and, ideally, long-lasting drug levels at the infection gateway. Moreover, animal data support that the topical administration of this drug combination can provide protection against vaginal HIV transmission [33]. TDF/FTC-loaded rings have been proposed for such purpose [18,19]; however, such type of delivery system requires continuous use and may not be well accepted by women requiring protection [34]. Conversely, on-demand microbicides, i.e. only requiring application around the time of sexual intercourse, may be preferred. Nonetheless, conventional vaginal dosage forms (e.g. gels) typically feature fast drug release and poor ability to sustain protective levels at the cervicovaginal mucosa. In order to overcome these limitations, we developed TDF/FTC-loaded polymeric films bearing different designs and tested their ability to modulate drug release *in vitro*.

The nano-encapsulation of hydrophilic drugs using conventional methods (e.g. emulsification methods) is a difficult task due to the fast migration of active compounds to the external aqueous phase [35]. To ensure extensive association of TDF and FTC, we produced drug-loaded NPs of the FDA-approved pharmaceutical excipient Eudragit® L 100 by nano spray-drying. These NPs were then incorporated into polymeric films to achieve a more controlled release. The matrix-forming polymer of NPs was also selected due to its well described safety, mucoadhesiveness and possibility to control drug release at vaginal acidic pH [36]. TDF/FTC-NPs were successfully produced and featured mean hydrodynamic diameter of 680 ± 23 nm and PDI of 0.32 ± 0.04, as determined by dynamic light scattering (DLS). Also, zeta potential was −39.1 ± 0.7 mV, in accordance with the negative charge provided by the abundant carboxylic acid groups in the side-chain of Eudragit® L 100. Taken together, size and zeta potential values suggest that produced particles may be stable once in suspension [37]. The majority of particles comprised a relatively homogeneous population, though size analysis indicated the presence of a smaller fraction of larger structures (mean hydrodynamic diameter of 5283 ± 23 nm). This was consistent with the presence of particle agglomerates due to the difficulty to disperse NPs in the dry state in aqueous medium. To gain more insight into the size and the morphology of the NPs, we visualized them by SEM and identified well individualized round-shaped, rough-surfaced NPs with mean diameter of 403 ± 179 nm as determined upon fitting to a Gaussian distribution (Fig. 1). This size distribution was in agreement

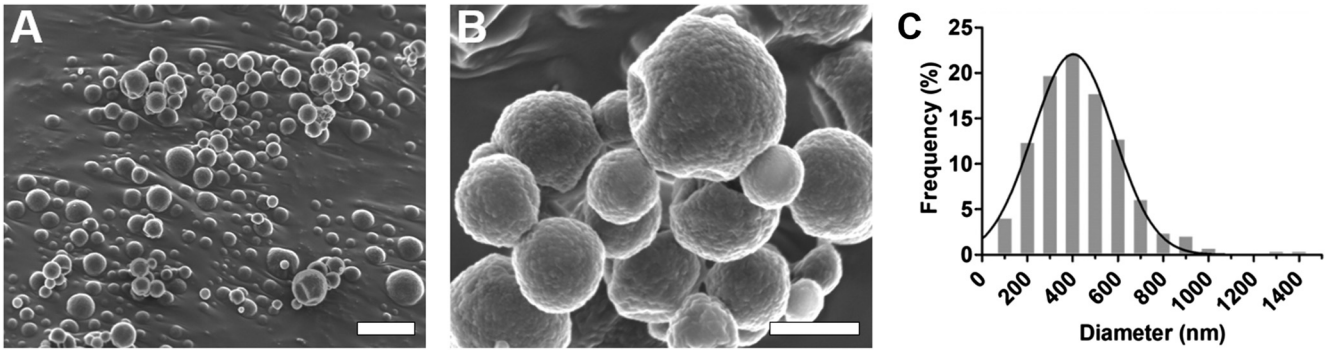

Fig. 1. Representative SEM images of TDF/FTC-NPs at the magnification of (A) 15,250× (bar = 2 μm) and (B) 100,000× (bar = 0.5 μm). (C) Particle diameter distribution as determined by SEM and corresponding Gauss fitting.

with DLS results. Apart from typical differences between techniques [38], variations in mean diameter values appear to be mostly related to the difficult dispersion of NPs in water. It is also worthwhile stressing that, as opposed to conventional production techniques, we did not use any surfactant, which could aid dispersion.

The amount of drug associated with NPs was further assessed. Values for association efficiency were determined to be 89.6% ± 9.8% and 61.3% ± 7.2% for TDF and FTC, respectively, corresponding to a total drug loading of 5.4% ± 0.9% (TDF) and 2.5% ± 0.6% (FTC). Although simultaneous loading of these drugs into nanocarriers has not been previously described, obtained results for association efficiency are at least as high as those recently reported in other studies for NPs containing TDF or FTC alone [39,40]. Lower than 100% association efficiency for NPs obtained by spray-drying may be related with losses due to sample deposition at the spray cap or because of differential molecular dragging by solvents, as similarly described in previous studies [41,42]. It is also important to point out that free drug could not be visualized by SEM, where this phenomenon is usually revealed by the presence of drug crystals [42]. The obtained TDF/FTC ratio in NPs was approximately 2.2:1, which was further considered for the production of all films.

A formulation based on PVA was initially established to produce basic polymeric films. This polymer has been commonly considered for the production of fast-dissolving vaginal films [43] and is also the main inactive ingredient in the VCF® film series available in the US market. Films including PVA as sole film-forming polymer were translucent, colorless, odorless, homogeneous, soft and flexible. PVA films also seemed to present suitable physicochemical properties for vaginal administration, as shown in Table 1, including pH and osmolality values compatible with vaginal physiology [44]. Mechanical properties such as distance at burst and puncture strength were in line with previously developed films [29], as well as the commercially available VCF® Vaginal Contraceptive Film (Apothecus, Oyster Bay, NY, USA) that was included in this study for comparison purposes. The substitution of PVA by different amounts of pectin was further tested in order to understand the effect of this last polymer on the physicochemical and technological properties of films. PVA/pectin at a ratio of 75:25 yielded translucent, yellow brownish, odorless, homogeneous, soft and flexible films. However, decreased softness and higher stiffness were apparent for equivalent weight ratio of considered polymers. Friable films were obtained at higher levels of pectin (75% and 100%), thus rendering such formulations unusable. These observations seem to be well correlated with the loss of elasticity of films, as inferred from decreasing distance at burst values, upon the incorporation of higher amounts of pectin. Also of relevance, all films featured adequate puncture strength as typically considered for pharmaceutical films [45], thus supporting that proposed systems have good resistance and pliability. Interestingly, increasing amounts of pectin from 0% to 50% substantially increased the disintegration time up to 17 min (50% pectin), which could be useful in delaying drug release. Albeit mild, significant differences were also observed for thickness, moisture content, pH and osmolality when pectin was incorporated into formulations. However, such variations are likely to be of reduced importance to the performance of films.

Films obtained using a PVA/pectin ratio of 75:25 appeared to feature suitable organoleptic, mechanical and potential to modify drug release, and were thus considered for further development of films containing TDF/FTC. Although showing delayed disintegration, the 50:50 formulation did not comply with typical organoleptic properties considered as important for users' acceptability [46]. TDF/FTC were incorporated directly into the 75:25 PVA/pectin film formulation during manufacture (single-film). Two more complex, yet feasible to produce delivery platforms were designed by compressing two preformed layers of the 75:25 ratio film. Drugs were incorporated either plain into the polymeric matrix during film manufacture (double-film) or associated to NPs and placed in-between two film layers (NPs-in-film) to produce a "sandwich" system by compression. Schematic structure and photographs of the films are presented in Fig. 2. Single-film presented similar organoleptic properties to those of the PVA:-pectin 75:25 film without drugs, thus suggesting that the incorporation of TDF/FTC had minor impact on the polymeric matrix. Conversely, films obtained by joining two individual sheets displayed substantially different characteristics, especially in terms thickness. Double-film was also less translucent, while NPs-in-film was opaque and featured some surface roughness. In both cases, films maintained their original

Table 1
Physicochemical and mechanical properties of different PVA/pectin film formulations. (*) and (§) indicate statistically significant differences ($p < 0.05$) as compared to PVA film and VCF® film, respectively. Results are presented as mean ± SD ($n = 3$).

Film formulations	Mass/area (mg/cm^2)	Thickness (μm)	Moisture content (%)	Distance at burst (mm)	Puncture Strength (g/mm^2)	Disintegration time (min)	pH	Osmolality (mOsm/kg)
PVA	9.1 ± 0.0	145 ± 9§	5.0 ± 0.9§	8.1 ± 2.2	188 ± 27	3 ± 0	4.5 ± 0.0	332 ± 10§
PVA/pectin (75:25)	10.4 ± 2.3	226 ± 8*,§	7.5 ± 1.6§	5.7 ± 1.0	130 ± 50	9 ± 1*,§	4.2 ± 0.1*	304 ± 8*,§
PVA/pectin (50:50)	10.0 ± 1.5	169 ± 2*,§	8.7 ± 0.6*,§	1.7 ± 0.3*,§	124 ± 49	17 ± 2*,§	4.1 ± 0.1*,§	300 ± 4*,§
VCF® film	9.4 ± 0.3	82 ± 0	13.1 ± 0.2	6.8 ± 0.7	116 ± 9	2 ± 0	4.3 ± 0.1	255 ± 4

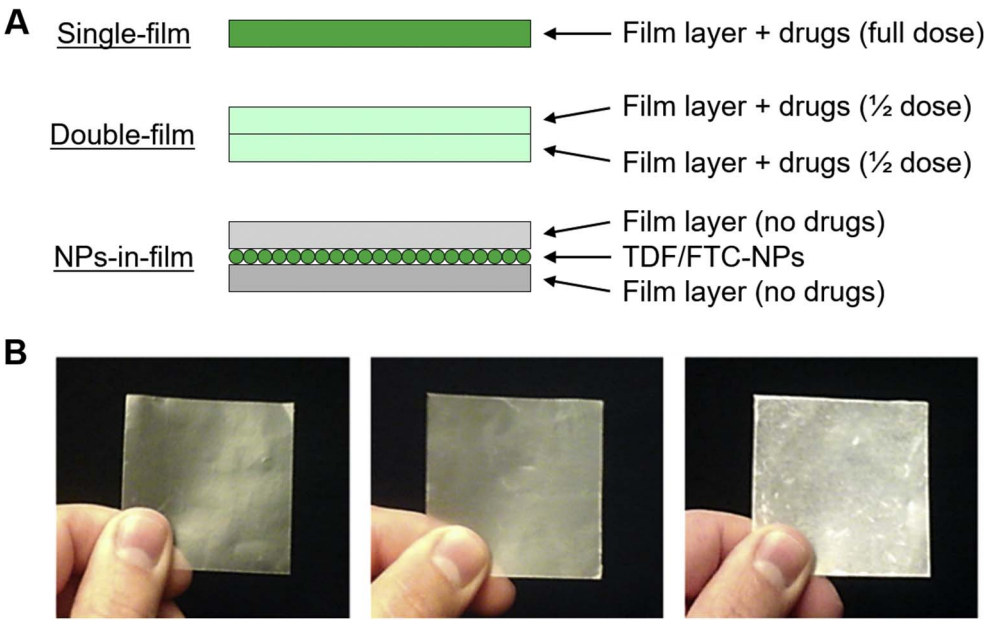

Fig. 2. Depiction of (A) the sideway schematic structure of proposed films, and (B) the top view photographs of 5 × 5 cm films (left to right: single-film, double-film and NPs-in-film).

flexibility.

The proposed TDF/FTC-loaded films were further characterized for their physicochemical and mechanical properties (Table 2). Values for mass/area and thickness, as expected, were higher in the case of systems containing two layers of film. Mechanical properties for the single-film did not differ from those of the film without drugs, indicating no impact due to the incorporation of TDF/FTC, but were dramatically changed in the case of the double-film. The probe coupled to the movable harm of the equipment was unable to puncture this last system, thus impairing the assessment of distance at burst and puncture strength. This behavior evidenced a substantial increase in elasticity when the two film layers were joined together by compression. Curiously, the NPs-in-film displayed similar results to the single-film, suggesting that the inclusion of an intermediate layer of NPs countered the increase in elasticity. Maintenance of mechanical properties may be particularly relevant in keeping suitable organoleptic features for vaginal use. A major change was observed for disintegration time: NPs-in-film did not lose their macroscopic structure up to 8 h. The pH dependent solubility profile of Eudragit® L 100 (only soluble at pH above 6) is possibly involved in such behavior and may be an interesting feature for controlling drug release in the vaginal tract that is usually acidic. Generally, variations in pH and osmolality values for all three TDF/FTC-loaded films were mild and likely without any relevant impact on film performance, namely regarding safety [47].

Next, TDF/FTC-loaded films were evaluated for drug release in order to understand how different structures impacted this critical parameter. Results shown in Fig. 3 highlight the substantial differences between tested films, as further reinforce by the analysis of f_2 values (Table 3). Nearly all drug content was released in SVF (pH 4.2) for single-film within the first hour, in line with its fast disintegration behavior. Conversely, double-film featured an initial burst release of around 50% and 60% of FTC and TDF, respectively, followed by a plateau. Fast release may be largely accounted to the drug present at

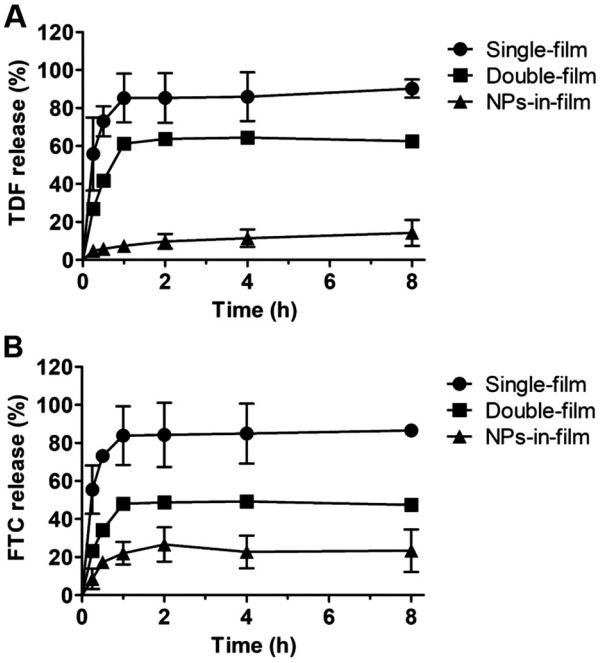

Fig. 3. Release profile of (A) TDF and (B) FTC from different films in SVF (pH 4.2). Results are presented as mean ± SD (n = 3).

Table 2
Physicochemical and mechanical properties of single-film, double-film and NPs-in-film. (*) indicates statistically significant differences ($p < 0.05$) from PVA/pectin (75:25) films without TDF/FTC presented in Table 1. Results are presented as mean ± SD (n = 3).

Film formulations	Mass/area (mg/cm^2)	Thickness (μm)	Moisture content (%)	Distance at burst (mm)	Puncture Strength (g/mm^2)	Disintegration time (min)	pH	Osmolality (mOsm/kg)
Single-film	10.7 ± 1.3	202 ± 4*	7.2 ± 0.8	7.0 ± 1.2	154.0 ± 32.8	8 ± 1	4.0 ± 0.1	319 ± 5*
Double-film	20.2 ± 3.1*	337 ± 4*	7.7 ± 2.0	NA	> 255*	30 ± 1*	4.0 ± 0.1	346 ± 14*
NP-in-film	26.8 ± 4.1*	400 ± 10*	6.5 ± 2.2	6.0 ± 0.5	219.6 ± 60.4	> 480*	4.2 ± 0.1	355 ± 5*

NA: not available.

Table 3
Values of f_2 comparing drug release profiles from different films.

	TDF	FTC
Single-film vs. Double-film	29	22
Single-film vs. NPs-in-film	7	12
Double-film vs. NPs-in-film	17	33

the wide and readily accessible surface of films. The most dramatic change in drug release profile was observed for NPs-in-film. In this case, burst release was decreased and a slow release profile obtained up to 8 h. Even at the last analyzed time point, only around 15–30% of both drugs was released. Drug release profiles from NPs alone were also assessed (Supplementary Material, Fig. S1). Around 40% and 70% of TDF and FTC, respectively, were released within a few minutes, reaching a plateau that lasted at least up to 8 h. These results indicate that a fraction of both drugs is exposed at the surface of NPs and is quickly released upon contact with aqueous media. This also increases NP porosity thus favoring more drug dissolution and release [42]. Conversely, the amount of drug that remains in the inner NP matrix is released at a slower rate owing to the low solubility of Eudragit® L 100 pH under acidic conditions. Overall, these data suggest that the combination of drug-loaded NPs and the sandwich-like film is an interesting approach to sustain the release of both TDF and FTC. Although scale-up production may be challenging, the proposed film poses an interesting alternative for the incorporation of NPs into such delivery platforms, namely in cases in which drug payloads would be quickly released upon contact with aqueous media [24]. Another relevant issue is related to the relatively low amount of drugs released from NPs-in-film. A simple and fast way to circumvent this issue would be to include part of the total amount of drugs directly into the film matrix. This would tentatively result in a burst release that was able to ensure early prophylactic concentrations at the vagina, while sustained release from the NPs could maintain drug levels for several hours. However, one should keep in mind the limitations of *in vitro* release assays that cannot mimic important physiological events, particularly occurring during sexual intercourse (e.g. increase in fluid volume, intense shear stress, pH changes due to semen) [48], that could promote further drug release. TDF and FTC released under rest may allow providing basal protective levels at the vagina, while additional release triggered by sexual activity could augment drug levels at the time when contact with HIV can occur. Still, these assumptions need experimental confirmation, namely *in vivo*.

Cell toxicity of films was further addressed in order to substantiate their potential safety. This last topic remains crucial in the development of microbicide formulations in order to abbreviate detrimental effects at the mucosal level that can lead to increased susceptibility to viral transmission [49]. Two complementary tests were used to evaluate possible cytotoxicity by different mechanisms. MTT reduction and LDH release assays are able to assess mitochondrial activity and integrity of the cell membrane, respectively. HeLa and CaSki cervical cell lines were selected as models due to their relevance to vaginal microbicide evaluation and ability to predict/abbreviate safety issues further on during animal and human studies [50–52]. Cytotoxicity/viability results are presented in Fig. 4. Films generally presented low toxicity potential, as assessed by both MTT and LDH assays, contrasting with the commercial VCF® film containing nonoxynol-9. This last spermicide is well recognized for its deleterious effects to mucosae and ability to increase HIV transmission [53]. Significant differences from negative controls were observed for films containing TDF and FTC but viability values were well above the 70% threshold defined by ISO 10993 [32]. Matching drug-free films were also evaluated and evidenced no signs of cytotoxicity thus suggesting that the drugs, not films, could be responsible for observed differences. The concentration of TDF and FTC for drug-loaded film extracts was around 220 μM and 243 μM, respectively. Half-maximal cytotoxicity (CC_{50}) values were determined as 364 μM, > 1,200 μM and 97 μM for TDF, FTC and TDF/FTC combination (2.2:1, *w/w*), respectively, by the MTT test for HeLa cells (Supplementary Material, Fig. S2). Therefore, mild reduction of viability for single-film, double-film and NPs-in-film is likely associated with the presence of drugs rather than film platforms.

4. Conclusions

TDF/FTC presents potential to be used for topical PrEP of the sexual transmission of HIV. In this work, we proposed novel PVA/pectin-based films for the vaginal delivery of the previous drug combination and conducted *in vitro* evaluation regarding technological, physicochemical and biological features, as relevant to microbicide development. In particular, drug release profiles were able to be modified by using films presenting different compositions. All films were further shown potentially safe. Although additional testing is required, namely *in vitro* permeability and *in vivo* PK and safety, developed films may be useful in the development of a vaginal microbicide product that could be acceptable to users and provide prolonged protection from viral infection.

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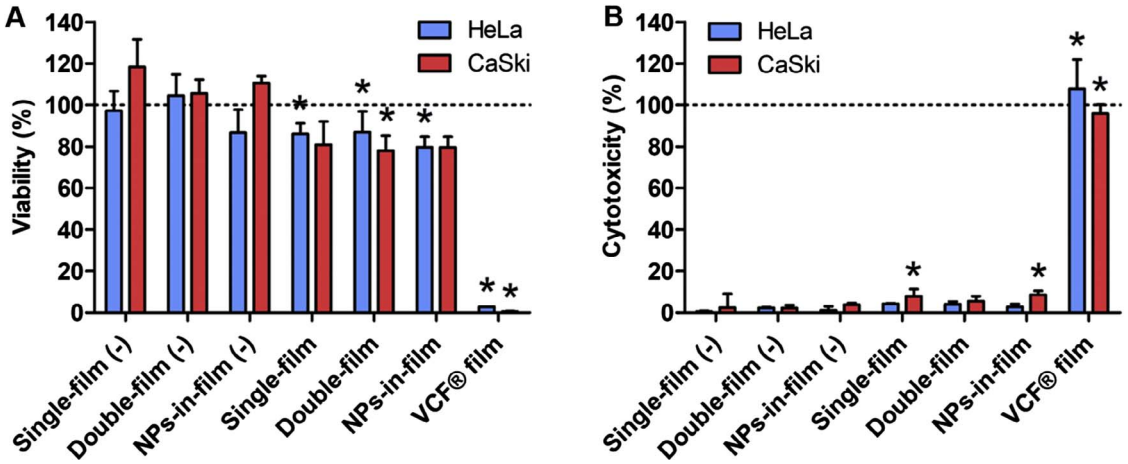

Fig. 4. Viability and cytotoxicity results for different films using HeLa or CaSki cell lines as determined by (A) MTT metabolic activity and (B) LDH release assays, respectively. (-) denotes films without drugs, and (*) indicates statistically significant differences ($p < 0.05$) as compared to the negative control (cells incubated with medium only). Results are presented as mean ± SD ($n = 3$).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ejpb.2018.02.001.

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