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Article

Influence of Plasticizers on the pH-Dependent Drug Release and Cellular Interactions of Hydroxypropyl Methylcellulose/Zein Vaginal Anti-HIV Films Containing Tenofovir

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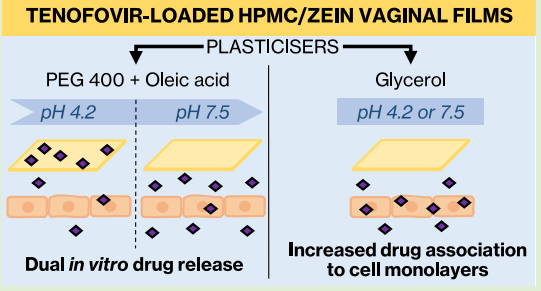
ABSTRACT: Vaginal films featuring the pH-dependent release of tenofovir (TFV) were developed for the prevention of sexual transmission of human immunodeficiency syndrome (HIV). Films based on hydroxypropyl methylcellulose and zein were prepared incorporating different plasticizers [oleic acid, lactic acid, glycerol, and polyethylene glycol 400 (PEG)] and evaluated for *in vitro* drug release in an acidic simulated vaginal fluid (pH 4.2) and a slightly alkaline mixture of simulated seminal and vaginal fluids (pH 7.5). Results revealed that optimal biphasic TFV release was possible with proper combination of plasticizers (PEG and oleic acid, 1:7 w/w) and by adjusting the plasticizer/matrix-forming material ratio. The films had similar or higher levels of TFV associated with genital epithelial cells (Ca Ski or HEC-1-A cells) but lower drug permeability compared to the free drug. These data confirm that films have the potential to achieve suitable mucosal levels of TFV with low systemic exposure. The films developed could protect women from HIV sexual transmission.



1. INTRODUCTION

Great strides have been made in recent years toward the goal of ending the human immunodeficiency syndrome (HIV)/AIDS pandemic, especially in terms of access to antiretroviral therapy and the implementation of new preventive strategies (e.g., oral pre-exposure prophylaxis).[1] However, significant gaps remain, which particularly affect more vulnerable population groups. For instance, women in sub-Saharan Africa are exposed to sexual transmission at younger ages than men and account for almost 80% of new cases in the 10−19 year-old age group.[2] Among other reasons, younger women often lack the power to negotiate the use of condoms with their male partners due to gender inequalities and cultural issues. Vaginal microbicides that can prevent early viral transmission events at the mucosal level and can be used without the consent of men represent an interesting approach to narrowing this gap.[3]

Tenofovir (TFV) is a nucleotide reverse transcriptase inhibitor that has long been a candidate for vaginal microbicide development due to its potency, prolonged half-life, and safety profile.[4] The drug was shown to be partially effective in preventing male-to-female transmission in a phase 2b clinical trial testing a TFV 1% vaginal gel.[5] Incomplete protection was explained by the lack of consistent use by women and subsequently correlated with reduced mucosal levels of the drug.[6] Adherence problems were confirmed in subsequent trials of similar TFV gels, which did not demonstrate any significant efficacy when compared to placebo products.[7,8] The coitally dependent nature of the gels requires these products to be administered close to the time of intercourse in order to sustain protective drug levels in the cervicovaginal mucosa. Alternative strategies have emerged based on coitally independent products that allow local drug levels to be sustained, with particular emphasis on rings.[9] These can be used continuously for weeks/months and release active payloads in a controlled fashion. However, clinical data on a dapivirine vaginal ring showed only mild efficacy in preventing male-to-female HIV transmission, and adherence remained an issue.[10,11] Continuous use of rings was regarded as cumbersome, particularly among younger women. The field of microbicides may therefore benefit from the development of products that can be used on demand but still ensure high drug levels in the mucosa on exposure to the virus. Formulations offering pH-dependent drug release are particularly appealing for this purpose. In particular, the well-known change in pH from acid to slightly alkaline due to the deposition of semen (the main source of HIV) in the vagina[12] could trigger an increase in drug release that boosts cervicovaginal drug levels.

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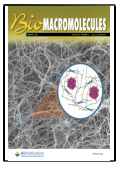

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Table 1. Composition of the Prepared Films[a]

film	HPMC (H)	zein (Z)	PEG	glycerol (G)	lactic acid (LA)	oleic acid (OA)	TFV
HZ-PEG	100	500	240				30
HZ-G	100	500		240			30
HZ-LA	100	500			240		30
HZ-OA	100	500				240	30
HZ-G/LA	100	500		120	120		30
HZ-G/OA	100	500		120		120	30
HZ-PEG/LA	100	500	120		120		30
HZ-PEG/OA	100	500	120			120	30
HZ-PEG1/OA7	100	500	30			210	30
HZ-PEG1/OA3	100	500	60			180	30
HZ-PEG3/OA1	100	500	180			60	30
HZ-PEG1/OA7-20	100	500	15			105	30
HZ-PEG1/OA7-60	100	500	45			315	30
HZ-PEG1/OA7-80	100	500	60			420	30

[a]The amount of each ingredient is given in mg per film.

These systems are often based on polymers with differential solubility depending on the pH of the media.[13−15]

Vaginal films are a particularly interesting dosage form for the development of vaginal microbicides.[16] These systems usually comprise thin polymeric sheets and combine the advantages of solid and semisolid vaginal dosage forms. For example, films avoid the leakage and messiness associated with gels, while presenting the typically excellent stability of rings and tablets.[17] Films are also easy and cheap to manufacture on an industrial scale and do not require an applicator for administration.[18] Vaginal films based on mixtures of hydroxypropyl methylcellulose (HPMC) and zein have been previously developed for sustained drug release.[19] Specifically, films produced at an HPMC/zein ratio of 1:5 and plasticized with 40% polyethylene glycol 400 (PEG) were found to sustain the release of TFV in a simulated vaginal fluid (SVF; pH 4.2) for at least 120 h. Although these materials are widely used to obtain pharmaceuticals and are especially useful in the manufacture of sustained release dosage forms,[20−23] HPMC and zein do not offer any significant changes in the pH range of interest for "smart" microbicides. The aim of this work was therefore to modify HPMC/zein films to allow the release of TFV in a pH-dependent biphasic fashion. Different common neutral (glycerol and PEG) and acidic (lactic and oleic acid) film plasticizers were screened, and their ability to confer differential erosion/drug release features to films at acidic and slightly alkaline pH was tested. Optimized films were further assessed for cytotoxicity, mucosal permeability, and retention of TFV using relevant cell-based models for vaginal drug delivery.

2. MATERIALS AND METHODS

2.1. Materials. HPMC (Methocel K 100 M) was kindly provided by Colorcon Ltd (Kent, UK). Zein, oleic acid, PEG 400 (Kollisolv PEG E 400) rat tail collagen type I, and resazurin were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-(+)-Lactic acid and glycerol were acquired from Panreac (Barcelona, Spain). TFV was supplied by Carbosynth Limited (Berkshire, UK). McCoy's 5A medium was acquired from Alfagene (Carcavelos, Portugal), RPMI 1640 medium and Hank's balanced salt solution (HBSS) from Gibco (Thermo Fisher Scientific, Waltham, MA, USA), and Dulbecco's modified Eagle medium (DMEM) from Lonza (Verviers, Belgium). Penicillin, streptomycin, and foetal bovine serum were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA). Methanol and all other reagents were of analytical grade and used without further purification. Demineralized water was used in all cases.

2.2. Preparation of Films. Films were obtained by means of a previously described solvent-casting method.[19] The matrix-forming materials (100 mg of HPMC and 500 mg of zein) and drug were placed in individual silicone templates with a diameter of 43 mm, 10 mL of a methanol/water solution of plasticizers was added, and the mixture was gently stirred until the dissolution/suspension of the polymer and protein. The templates were maintained at room temperature until the solvent had completely evaporated.

The different film compositions were screened in a phased manner. The films were first prepared by including a single plasticizer (glycerol, PEG, lactic acid, or oleic acid) and tested for their properties. Neutral plasticizers (glycerol and PEG) were then combined with acid plasticizers (lactic and oleic acid) in a 1:1 (w/w) ratio to evaluate possible synergies. The best plasticizer combination (PEG and oleic acid) was further tested in different ratios (1:7, 1:3, and 3:1, w/w) and in different ratios of matrix-forming materials and plasticizer (5:1, 5:2, 5:3, and 5:4, w/w). TFV was incorporated in films at a fixed amount of 30 mg to match the dose previously tested in the CAPRISA 004 clinical trial.[5] A summary of the film formulations tested is presented in Table 1.

2.3. Technological Characterization of Films. *2.3.1. Drug Release.* *In vitro* drug release tests were performed in SVF (pH 4.2)[24] and in a mixture of SVF with a simulated seminal fluid (SSF)[25] in a ratio of 1:4 (v/v).[26] The SVF/SSF mixture had a final pH of 7.5. Testing was carried out following a previously described methodology.[19] Films were submerged in 80 mL of preheated release medium in screw-capped borosilicate glass bottles and then immediately placed in a shaking water bath at 37 °C and 15 rpm. Aliquots (5 mL) were collected periodically and replaced with fresh medium, and the amount of TFV was quantified by UV spectroscopy (Evolution 60S spectrophotometer, Thermo Scientific, Waltham, MA, USA) at 260 nm. Each film was tested in triplicate for both media.

Release profiles were compared using a model-independent index, namely, the similarity factor (f_2), calculated as follows

$$f_2 = 50 \log\left\{\left[1 + \left(\frac{1}{n}\right)\sum_{j=1}^{n} |R_j - T_j|^2\right]^{-0.5} \times 100\right\} \quad (1)$$

where n is the number of time points included, R_j is the drug release percentage for the reference formulation, and T_j is the drug release percentage for the test formulation. When $f_2 < 50$, there are differences between the profiles.[27]

2.3.2. Swelling Behavior. The swelling and erosion processes that films undergo on immersion in media were evaluated as described by Ruiz-Caro and Veiga-Ochoa.[28] Briefly, films were placed on stainless-steel discs and immersed in medium inside a beaker placed in a shaking water bath at 37 °C and 15 rpm. Samples were weighed

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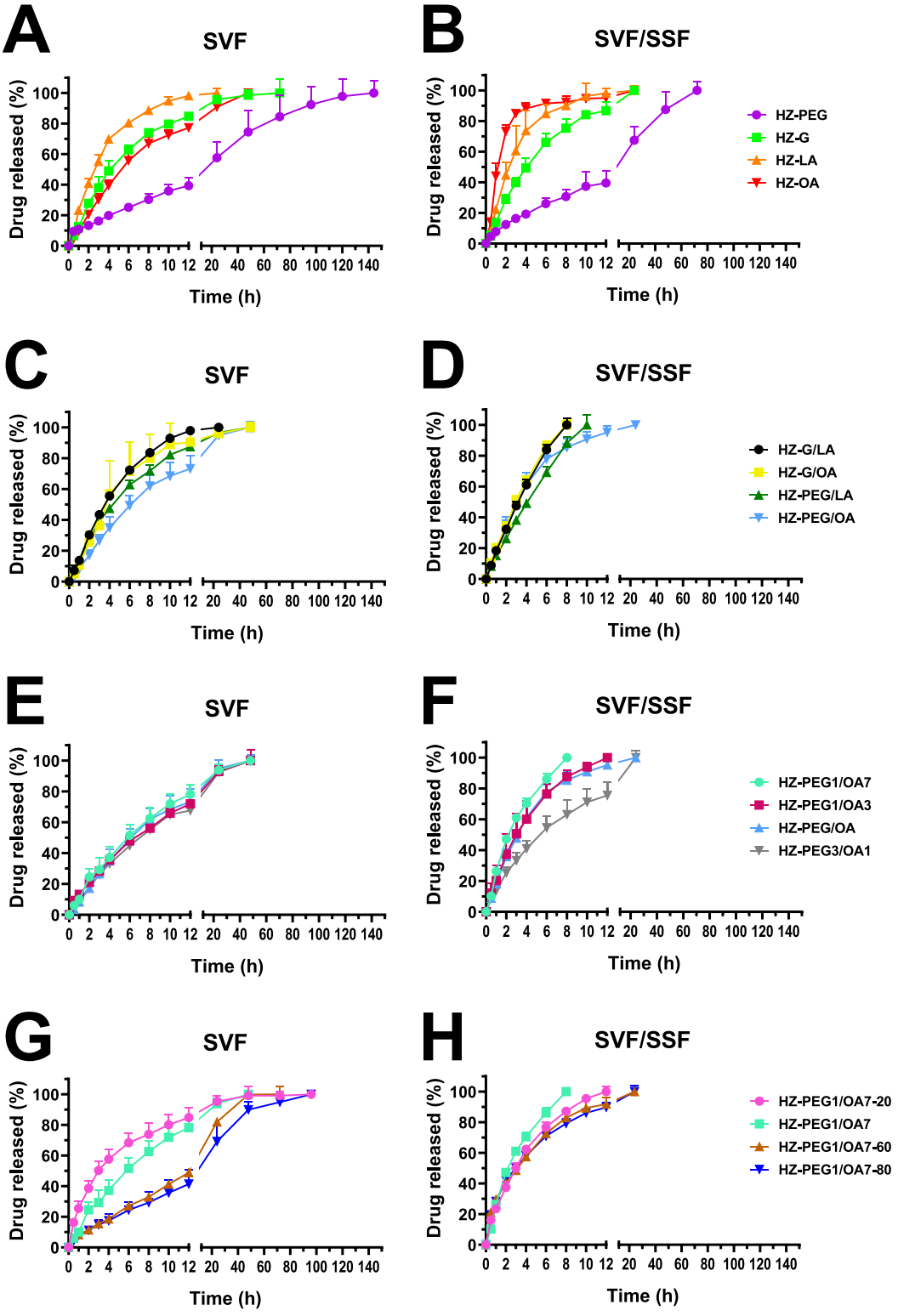

Figure 1. Drug release profiles of films prepared with a single plasticizer in SVF (A) and SVF/SSF (B); a combination of plasticizers in SVF (C) and SVF/SSF (D); a combination of different ratios of PEG and oleic acid in SVF (E) and SVF/SSF (F); and a combination of PEG and oleic acid with different ratios of matrix-forming materials/plasticizers in SVF (G) and SVF/SSF (H). Values for films prepared with PEG or glycerol as single plasticizers in SVF are adapted from previous published studies.[19] Results are presented as mean ± SD (n = 3).

periodically to quantify the swelling ratio (SR) percentage. A positive SR implies the capture of medium, while negative values indicate the erosion of films. Experiments were performed in triplicate for each film in each medium.

2.3.3. Mucoadhesion. The adhesion of the films to the vaginal mucosa was evaluated with an *ex vivo* test.[29] Calf vaginal mucosa was obtained from a local slaughterhouse. Tissue samples were fixed to an 8.5 × 5 cm stainless-steel plate on the serosal side with cyanoacrylate adhesive. The film was pressed on the mucosal side for 30 s using a weight of 500 g. The whole system (plate + mucosa + film) was then completely submerged in SVF at an angle of 60° and placed in an orbital shaking water bath at 37 °C and 15 rpm. The time taken for a

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film to detach from the mucosa was monitored visually and defined as the mucoadhesion time. Films were tested in duplicate.

2.3.4. Mechanical Properties. The mechanical properties of films were evaluated by means of a TA.XT*plus* Texture analyzer. The methodology is described in the Supporting Information (S1. Supplementary methods).

2.3.5. Microscopic Morphology. The surface morphology of the films was assessed by scanning electron microscopy. The methodology is given in the Supporting Information (S1. Supplementary methods).

2.4. Cell Studies with Films. *2.4.1. Cytotoxicity.* The *in vitro* toxicity of raw materials and film extracts to human genital cell lines was determined by the resazurin metabolism assay. HeLa cervical, Ca Ski cervical, and HEC-1-A endometrial cell lines were obtained from ATCC (Manassas, VA, USA). HeLa, Ca Ski, and HEC-1-A cells were maintained in DMEM, RPMI 1640, and McCoy's 5A media, respectively, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 0.1 μg/mL streptomycin. Media were refreshed every 2−3 days and cells were kept under standard conditions (37 °C, 5% CO_2, and 95% humidity).

Raw materials were tested after dissolution/dispersion in media. Film extracts were prepared by immersing samples in cell culture medium at a volume ratio of film surface-to-medium of 1 cm^2/mL in accordance with the ISO 10993-5:2009 standard (Biological evaluation of medical devices—Part 5: Tests for *in vitro* cytotoxicity). Film samples were extracted after 24 h at 37 °C and 100 rpm, and the resulting extracts were collected and tested without dilution (100%) or diluted to 50 and 10% with the medium.[30]

Cytotoxicity was determined using cells preseeded for 24 h under standard conditions in 96-well plates at 5000 cells/well. Film extracts or solutions/suspensions of raw materials in culture media at concentrations in the range of 0.001−10 mg/mL were added and the cells were further incubated for 24 h. The cells were then washed twice with phosphate-buffered saline (pH 7.4) and incubated with 10 μg/mL resazurin in medium for 4 h under standard conditions. Fluorescence readings were taken at an Ex/Em of 530/590 nm and the viability was calculated as the control percentage (cells incubated only with medium). Concentration-dependent viability data were fitted using a log-logistic regression in order to calculate half-maximal cytotoxic concentration (CC_{50}) values. Experiments were performed in triplicate.

2.4.2. In Vitro Permeability and Epithelial Cell Retention. Cell monolayers based on Ca Ski or HEC-1-A cells were obtained as previously described.[31,32] Cells were seeded at a density of 3×10^5 per square centimeter over Millicell cell culture inserts (Merck-Millipore, Billerica, MA, USA). The inserts had a total area of 1.1 cm^2 and a 1 μm pore and were precoated with rat tail collagen type I. Cell monolayers were allowed to form under standard culture conditions over 7−8 days, and the medium was refreshed every 2−3 days. The formation of cell monolayers was monitored by measuring the transepithelial electrical resistance (TEER) with an EVOM epithelial voltohmmeter with "chopstick" electrodes (World Precision Instruments, Sarasota, FL, USA).

Film samples with a surface area of 0.16 cm^2 (corresponding to 300 μg of TFV) were gently immersed in 0.5 mL of HBSS placed on the apical side of the cell monolayers. The basolateral side was prefilled with 1.5 mL of HBSS. The inserts were maintained at 37 °C under orbital shaking (100 rpm) and the concentration of TFV on the basolateral side was monitored over time up to 4 h by periodically collecting 0.5 mL of samples and replacing the amount with fresh HBSS. At the end of the experiment, the inserts were collected and rinsed with HBSS and the TFV associated with the cell monolayers was extracted with 1 mL of dimethyl sulphoxide.[31,32] The permeability and retention in the cell monolayer of free TFV (300 μg) diluted in HBSS were also determined for comparison purposes. The amount of TFV in all samples was quantified by UV spectrophotometry at 260 nm. The apparent permeability coefficient (P_{app}) was calculated from permeability profiles according to

$$P_{app} = \frac{Q}{A \cdot C \cdot t} \quad (2)$$

where Q is the final amount of TFV permeated (μg), A is the area of the membrane (cm^2), C is the initial concentration of the drug on the apical side (μg/mL), and t is the experiment time (s).[32] All experiments were performed in triplicate.

2.5. Statistical Analysis. One-way ANOVA with Tukey's post-hoc test was used to compare mucoadhesion times. The mechanical properties calculated from texture analysis data were compared by two-way ANOVA with Tukey's post-hoc test. Permeability profiles were compared by one-way ANOVA with Tukey's post-hoc test. Two-way ANOVA with Dunnett's post-hoc test was used to compare values of P_{app} and drug associated to cell monolayers. The analysis was performed with Prism v. 5.03 (GraphPad Software, La Jolla, CA, USA). Values of $p < 0.05$ were considered as denoting significance. All results are presented as mean ± standard deviation (SD), unless otherwise specified.

3. RESULTS AND DISCUSSION

3.1. Technological Characterization. *3.1.1. Drug Release.* We have recently shown that HPMC and zein may be useful for producing vaginal films that allow the prolonged release of TFV for up to 120 h.[19] Contrary to the usual goal in the field of microbicides, which is to obtain films presenting almost complete drug release within a few minutes of being placed in aqueous media, HPMC and zein films may provide an interesting platform for developing coitus-independent microbicides. The successful blockage of transmission by this type of films may be further increased by enhancing drug release when the HIV is deposited in the vagina by semen. One specific way of achieving this goal is to use different plasticizers and their blends to confer pH-dependent drug release properties on HPMC and zein films. Indeed, our data highlighted the notable differences in TFV release depending on the plasticizer selected: films containing PEG sustained drug release in SVF for 144 h, while lactic acid reduced this time to only 12 h (Figure 1).[19] Glycerol and oleic acid had intermediate behaviors. These results can be explained by the nature of the plasticizers. Oleic acid is an amphiphilic molecule that can interact with zein by bonding the carboxylic acid group to the terminal glutamine residues of the protein,[33] thus providing a sandwich-like structure that makes films less permeable.[34] Lactic acid and glycerol are polar molecules which undergo solvation in aqueous media, irrespective of pH, and facilitate drug diffusion. PEG is also a polar molecule but has a roughly 4-times higher molecular weight, which may hinder penetration into the polymer/protein network and enable intermolecular interactions between HPMC and zein so the medium has less diffusivity within the film matrix.[35] PEG has also been reported as forming chemical bonds with zein via protein PEGylation.[36] It has further been observed that films containing acidic plasticizers (lactic or oleic acid) allow faster drug release in the SVF/SSF medium (Figure 1B). A particularly marked increase in TFV release at a higher pH was evident for films containing oleic acid, resulting in the only f_2 value below 50 for comparing drug release from films containing a single plasticizer (Table 2). This suggests that oleic acid may be a good initial candidate plasticizer for providing pH-sensitive behavior, while its combination with PEG may be relevant for slowing drug release at acidic pH. The comparison of f_2 values between films containing different single plasticizers also pointed to differences in TFV release, except in the case of films with glycerol or oleic acid in SVF (Supporting Information, Table S1).

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Table 2. Similarity Factor Values (f_2) Calculated from Drug Release Profiles in SVF and SVF/SSF[a]

film	f_2
HZ-PEG	64.5
HZ-G	81.1
HZ-LA	73.9
HZ-OA	**20.1**
HZ-G/LA	55.3
HZ-G/OA	**49.8**
HZ-PEG/LA	59.8
HZ-PEG/OA	**35.7**
HZ-PEG1/OA7	**30.8**
HZ-PEG1/OA3	**34.8**
HZ-PEG3/OA1	58.1
HZ-PEG1/OA7-20	61.3
HZ-PEG1/OA7-60	**22.8**
HZ-PEG1/OA7-80	**22.2**

[a]Values of f_2 denoting differences are shown in bold.

The study consisted of testing binary mixtures of plasticizers, one neutral and one acidic. Drug release profiles in SVF showed intermediate behavior between films with a single plasticizer (Figure 1C). Films including PEG still trended toward slower drug release, especially when combined with oleic acid. Films prepared with binary mixtures of plasticizers also showed an overall faster drug release in SVF/SSF than in SVF (Figure 1D). The comparison of f_2 values only revealed notable differences for films containing PEG and oleic acid, thus indicating the potential of this binary mixture to confer pH-dependent drug release behavior (Table 2). Finally, the comparison of f_2 values for the various films pointed to differences only for the mixtures of PEG/oleic acid in SVF (Supporting Information, Table S2).

Films with additional ratios (1:7, 1:3, and 3:1) of the PEG/oleic acid combination were prepared and tested and showed no significant differences in terms of TFV release in SVF (Figure 1E). We hypothesized that during the formation of the films, oleic acid first interacts with zein and only later with PEG, which explains the dominance of the structure formed by oleic acid and zein, as can be seen when comparing scanning electron microscopy micrographs of films prepared with oleic acid alone or combining PEG and oleic acid (Supporting Information, Figures S1 and S2). Values of comparison by f_2 (Supporting Information, Table S3) indicate negligible differences between films. When considering the data for SVF/SSF, increasing amounts of oleic acid led to a faster release of TFV (Figure 1F and Table 2). Only films containing PEG/oleic acid in a ratio of 3:1 proved to be significantly different from the others; higher amounts of PEG appeared to impair TFV release in this medium (Supporting Information, Table S3). Again, this supports the ability of oleic acid to yield pH-dependent drug release.

Overall, the greater differences between the media in drug release from films with PEG/oleic acid at a ratio of 1:7 indicates that this formulation may be suitable for biphasic drug release. Various matrix-forming material/plasticizer ratios were further evaluated in a final attempt to optimize the film formulation. Increasing amounts of plasticizers led to slower release of TFV in SVF (Figure 1G). The lowest tested concentration of plasticizers resulted in lower drug retention in SVF, suggesting poor plasticization of HPMC and/or zein and hence that the drug release is controlled mainly by these non-pH-sensitive matrix-forming materials. Curiously, changes in drug release were minimal in the case of SVF/SSF (Figure 1H). Film with 80% plasticizers (HZ-PEG1/OA7-80) had the largest sustained drug release in SVF and was selected as the most suitable for the pH-dependent release of TFV.

3.1.2. Swelling Behavior. We studied the films' ability to hydrate and erode in order to gain a better understanding of the drug release results. The swelling behavior is mainly attributed to matrix-forming materials (typically poly-

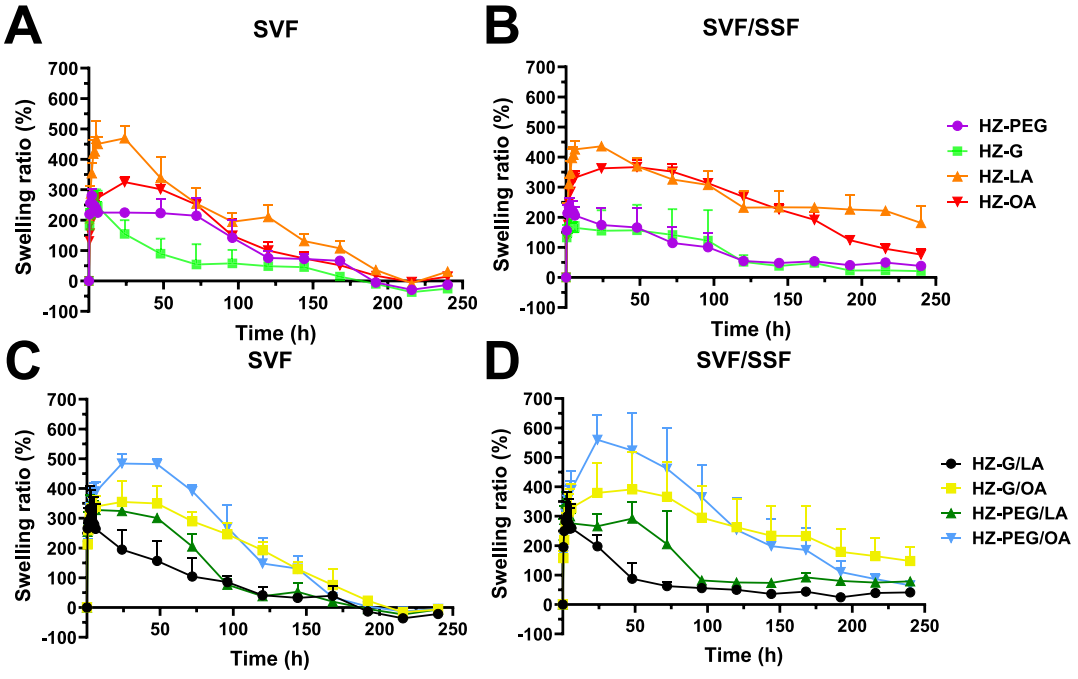

Figure 2. Swelling profiles of films prepared with a single plasticizer in SVF (A) and SVF/SSF (B) and films prepared with a combination of plasticizers in SVF (C) and SVF/SSF (D). The results are presented as mean ± SD (n = 3).

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mers).[37,38] In a previous study, we confirmed that films based on HPMC are able to capture large amounts of vaginal fluid, leading to the formation of a gel. Conversely, zein does not swell but is still able to capture water into its structure.[19] However, our data showed notable differences between films containing different plasticizers because they are able to modify the structure of the films, and as consequence, the fluid uptake of the formulation is modified. Films with lactic acid had the greatest ability to take up both SVF and SVF/SSF (Figure 2A,B), thus confirming their inferior plasticization efficiency and justifying their faster TFV release. Only films containing oleic acid revealed substantial differences in swelling when comparing different media. Specifically, these films were able to capture more fluid and maintain swelling for longer in SVF/SSF than in SVF. This suggests that changes to the sandwich structure of films plasticized with oleic acid are more pronounced with increasing pH, which may be implicated in the accelerated drug release observed in SVF/SSF.

Interestingly, the impact of acidic plasticizers was dissimilar in films containing binary mixtures of plasticizers. Films with lactic acid had the lowest ability to take up fluid (Figure 2C,D), which could be explained by the low interaction of lactic acid with matrix-forming materials and the consequent predominance of co-plasticizers (PEG or glycerol) in influencing swelling behavior. Conversely, an increase in swelling was observed for films containing oleic acid combined with either PEG or glycerol. As previously described by Xu et al.,[39] the interactions of oleic acid with zein during the manufacturing process appear to occur at an earlier stage than the interactions between glycerol and the protein. The same effect may presumably be valid for films containing oleic acid and PEG. Thus, the layer-by-layer structure established early between zein and oleic acid appears to be reinforced by the interaction of glycerol (or PEG) with the hydrophilic domains of the matrix-forming protein, thus enhancing the swelling ability. Overall, films with oleic acid and a neutral plasticizer also underwent more intense swelling in SVF/SSF than in SVF (Figure 2D).

3.1.3. Mucoadhesion. Drug dosage forms need to be retained in the vaginal cavity for a requisite amount of time after administration in order to effectively deliver active drug concentrations. The mucoadhesive properties of pharmaceuticals have long been regarded as beneficial in promoting vaginal residence and have mostly been related with their polymer content.[40] Polymers are mainly responsible for the mucoadhesion of the films. Mucoadhesion is likely dependent on the interpenetration of the polymeric chains with mucin.[41] Low-molecular-weight chains and hydrophilicity of the polymer facilitate its intimate interaction with vaginal fluids.[42] Interpenetration allows bonding between the polymer and mucin, which consolidates mucoadhesion.[43]

The renewal of the mucus and vaginal epithelium are parameters to be considered since they will clearly influence the retention of the formulation in the vagina. The fluid covering the vaginal epithelium is composed by a mixture of components, which include cervical mucus (mucin is the main component besides water), vestibular gland secretions, transudate from mucosal tissue, uterine secretions, cellular debris, and vestigial urine.[44] Turnover of vaginal fluids is low in the resting vagina: the typical production rate of the vaginal fluid has been estimated at approximately 6 g/day.[2,24]

The present work was set out to evaluate whether plasticizers could also affect the amount of time films remained adhered to the mucosa. We decided to evaluate the adhesion time rather than force of adhesion because these formulations are intended to be retained in the vagina for extended periods of time (up to several days), thus the considered parameter may be regarded as more useful. The results for films prepared with a single plasticizer indicated that lactic and oleic acid have the longest mucoadhesion time and remain attached to the mucosa throughout the experiment (Figure 3). Mucoadhesion

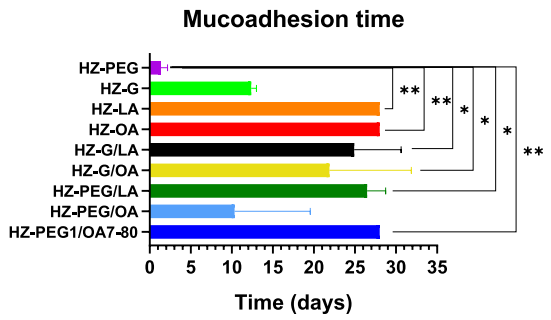

Figure 3. *Ex vivo* mucoadhesion time for films prepared with a single plasticizer, prepared with binary mixtures of plasticizers, and optimized for drug release (HZ-PEG1/OA7-80). Results are presented as mean ± SD (n = 2). (*) and (**) denote $p < 0.05$ and $p < 0.01$, respectively.

time was only 12.4 ± 0.6 days and 1.4 ± 0.8 days for films containing glycerol and PEG, respectively. The differences in swelling behavior between these films may account for these observations.[17,45] Acidic plasticizers may also contribute to the overall negative charge on the films' surface and thus favor the establishment of electrostatic bonding with the mucosa.[46] The co-incorporation of acidic and neutral plasticizers appeared to have an intermediate behavior. Again, excessive swelling of films prepared with binary mixtures of plasticizers may partially hinder interactions between HPMC/zein and mucosa.[47,48] Films optimized for drug release (80% of PEG/oleic acid at a ratio of 1:7) had a mucoadhesion time of 28 days, which further highlights the key role of oleic acid in enhancing adhesive interactions with mucosa.

3.1.4. Mechanical Properties. All the films evaluated showed suitable mechanical properties for vaginal administration and use. The results obtained in the mechanical characterization are discussed in the Supporting Information (S2. Supplementary Results).

3.2. Cell Studies. *3.2.1. Cytotoxicity.* Safety is a key aspect of microbicide development, and early *in vitro* assessment of formulation prototypes provides valuable hints on possible toxicity issues.[49] Complete cell viability profiles are presented in the Supporting Information (Figure S4). TFV and the excipients included in the optimized films were initially screened (Table 3 and Supporting Information, Figure S4), and the results generally confirm the relatively low toxicity potential of all the ingredients, including TFV.[50−52] A marked decrease in cell viability was mainly observed only at the highest concentrations tested (10 mg/mL except for HPMC) and was correlated to changes in the properties of the cell culture media. For example, we previously observed that PEG affects cell viability by significantly increasing osmolarity.[51] Oleic acid showed the highest decrease in cell viability, which may be due to the marked decrease in the pH of the media. However, this should not be a problem for human use due to the naturally acidic pH of the vaginal fluid.

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Table 3. CC_{50} Values of Raw Materials Included in Optimized Films as Determined Using HeLa, Ca Ski, and HEC-1-A Cell Lines

	CC_{50} (mg/mL)		
raw material	HeLa	Ca Ski	HEC-1-A
TFV	6.7	2.7	2.5
HPMC	>1	>1	>1
zein	6.7	7.8	>10
oleic acid	1.2	0.2	1.6
PEG	>10	4.4	3.5

More importantly, we assessed the cytotoxicity potential of optimized films by testing their extracts.[53] The data are shown in Figure 4. The extracts presented considerable cytotoxicity except for the 10% dilution (cell viability of approximately 70% or higher); these results may be explained by the higher content of oleic acid in the optimized films. Again, changes in the pH of the media may have influenced cell viability while in culture and additional—including *in vivo*—toxicological assessment are recommended for optimized films.[50] We also noted that optimized films were safer than the commercially available VCF Vaginal Contraceptive Film (Apothecus, Oyster Bay, NY, USA).

3.2.2. Permeability and Epithelial Cell Retention. Various films, including the formulation optimized for pH-dependent drug release, were tested for their ability to influence the permeation of TFV across two relevant genital cell monolayer models. These experiments serve as a proxy for systemic exposure (amount of permeated drug) and accumulation in the mucosa (amount of drug associated with the cell monolayer).[31,32] It should be noted that the conditions used in these experiments mimic those observed on ejaculation (the pH of HBSS is 7.0−7.4). In general, the transport of TFV across both cell monolayers was similar or lower for films compared to the free compound (Figure 5A,B). TFV incorporated into films is not immediately available to cross the cell monolayer, and this alone could account for the permeability profiles observed. However, no correlation was apparent for drug release data and permeability when comparing different films, implying that transport across cell monolayers is governed by complex phenomena and may even be influenced by interactions between films or their ingredients and cells. The transport of TFV was generally faster across the Ca Ski model, agreeing with our previous observations.[31,32] The P_{app} values calculated (Figure 5C) were in the range of 6.1×10^{-6} to 9.5×10^{-6} and 3.1×10^{-6} to 6.1×10^{-6} cm/s for Ca Ski and HEC-1-A cell monolayer models, respectively, indicating that TFV is only mildly permeable.[54] We are unaware of any permeability data reported for TFV using these two models. However, our results were consistent with the findings on the permeability of TFV across Caco-2 colorectal cell monolayers in solutions (P_{app} = 0.1 to 0.4 $\times 10^{-6}$ cm/s)[55−57] or ectocervical tissue incorporated in vaginal gels (P_{app} = 2 to 3 $\times 10^{-6}$ cm/s).[58]

Microbicide drugs are assumed to exert their activity at the mucosal level. It is therefore important to assess films' ability to provide suitable high levels of TFV locally. Overall, the films tested provided at least similar drug levels associated to cell monolayers in both Ca Ski and HEC-1-A models, as compared to the free drug (Figure 5D). No differences were observed in the case of the optimized film. These data appear to suggest that drug release under conditions mimicking the pH observed on intravaginal ejaculation is fast enough to achieve suitable TFV levels for protection. It is worth noting that films prepared with glycerol as a single plasticizer led to a significantly higher amount of TFV associated with both types of cell monolayers, as compared with free TFV. Although further investigation is required, we hypothesize that glycerol may interact directly with cells and facilitate the intracellular accumulation of TFV. For instance, glycerol is known to inhibit multidrug resistance-associated proteins (MRPs) that may be involved in TFV efflux.[59] This film could represent an option for a vaginal microbicide. Although it does not offer pH-dependent release of TFV, it could increase protection against viral infection through the higher drug levels associated to cells. Glycerol has also proved to be nontoxic to female genital cells such as HEC-1-A.[19,60] This formulation may therefore also be considered for future *in vivo* trials.

4. CONCLUSIONS

Films with pH-dependent drug release can potentially provide interesting microbicide products. The addition of different plasticizers to films prepared with the same matrix-forming ingredients (HPMC and zein) can modify the release profile of TFV. Films optimized with an 80% plasticizing mixture of oleic acid and PEG in a ratio of 1:7 showed promising characteristics for developing semen-triggered microbicides. The presence of PEG in these films helps sustain drug release in SVF, while the content in oleic acid doubles the TFV release rate when the medium is neutralized by seminal fluid. Oleic acid ensures that the formulation adheres properly to the

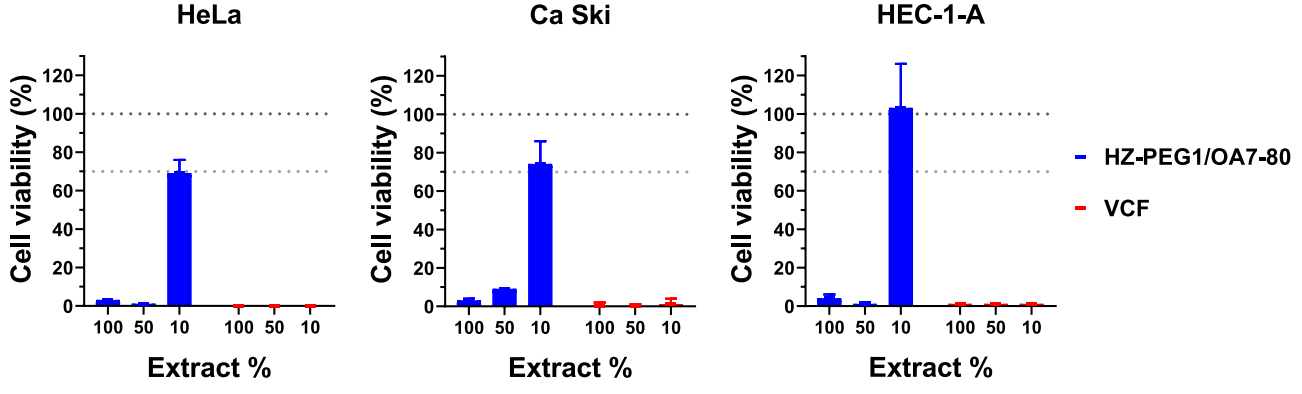

Figure 4. Viability of HeLa, Ca Ski, and HEC-1-A cells upon exposure to extracts of optimized films and VCF. Results are presented as mean ± SD (n = 3).

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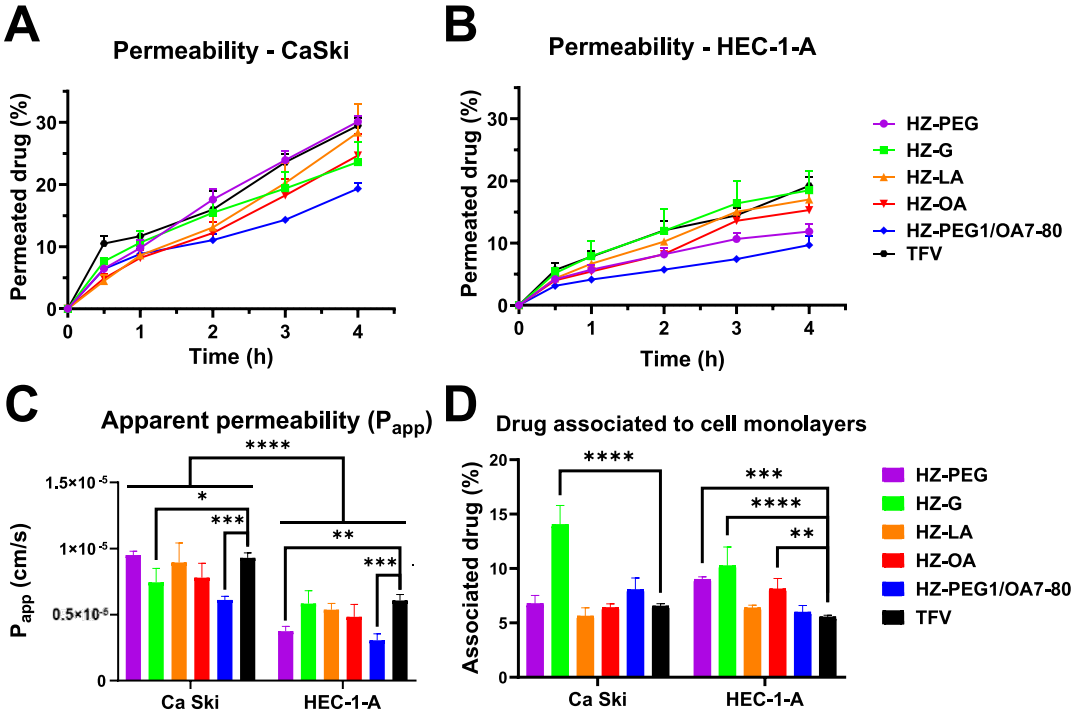

Figure 5. Permeability profiles of TFV across Ca Ski (A) and HEC-1-A (B) cell monolayers and apparent permeability (C) and drug associated to cell monolayers (D), as mediated by different films or the free drug. Results are presented as mean ± SD (n = 3). (*), (**), (***), and (****) denote $p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively.

vaginal mucosa, and the presence of PEG confers mechanical properties that make it suitable for comfortable intravaginal administration. The formulation containing these plasticizers also allows similar association levels of TFV to genital epithelial cell monolayers to those observed for the drug in solution but possibly with lower systemic exposure.

Films plasticized only with glycerol may be appropriate as a microbicide for the immediate release of TFV. This film was able to double the amount of TFV associated to both Ca Ski and HEC-1-A cell monolayers compared to free TFV and could therefore increase women's protection against the sexual transmission of HIV. The nature of this plasticizer also gives the films acceptable mucoadhesive and mechanical properties, although more frequent administration would be required due to the non-pH-dependent release of TFV.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.0c01609.

Methodology and results for mechanical test and scanning electron microscopy; f_2 comparison of drug release data; and viability of HeLa, Ca Ski, and HEC-1-A cell lines upon exposure to raw materials (PDF)

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Author Contributions

F.N.-P.: Conceptualization, investigation, and writing—original draft. R.C.-L.: Investigation. A.M.-I.: Investigation. J.G.: Investigation. R.R.-C.: Conceptualization, writing—review and editing, and supervision. B.S.: Conceptualization, writing—review and editing, supervision, and funding acquisition. J.d.N.: Conceptualization, writing—review and editing, and supervision. M.-D.V.: Conceptualization, writing—review and editing, supervision, project administration, and funding acquisition. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CC_{50}, half-maximal cytotoxic concentration; DMEM, Dulbecco's modified Eagle medium; HBSS, Hank's balanced salt solution; HIV, human immunodeficiency syndrome; HPMC, hydroxypropyl methylcellulose; MRPs, multidrug resistance-associated proteins; PEG, polyethylene glycol 400; SD, standard deviation; SR, swelling ratio; SSF, simulated seminal fluid; SVF, simulated vaginal fluid; TEER, transepithelial electrical resistance; TFV, tenofovir

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