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Eudragit® L100/chitosan composite thin bilayer films for intravaginal pH-responsive release of Tenofovir

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ABSTRACT

The high rate of HIV new infections and AIDS-related deaths each year make prevention tools still necessary today. Different dosage forms – including films – for vaginal administration of antiretroviral drugs have been developed for this purpose. Six batches of Tenofovir-loaded films were formulated based on Eudragit® L100 (EL100) and chitosan, containing triethyl citrate and glycerol. In all the cases films structured in two layers – the upper layer mainly attributed to EL100 and the lower layer to chitosan – were revealed by SEM. A higher content in EL100 and plasticizers improves the mechanical properties and control over drug release in the vaginal medium without affecting mucoadhesion. The EL100-based layer acts as a structuring agent that controls Tenofovir release for days in the vaginal medium while it occurs in a few hours in the presence of seminal fluid. Bilayer films with the highest tested content of EL100 and plasticizers would be the most suitable as vaginal microbicides as they are easier to administer due to their excellent mechanical properties and they offer more comfortable posology and enhanced protection against HIV during intercourse due to their pH-responsive release of Tenofovir.

1. Introduction

The latest data from the Joint United Nations Programme on human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) –UNAIDS – point to 1.5 million new HIV infections and 690,000 deaths from AIDS-related illnesses in 2020 (UNAIDS, 2021). The current COVID-19 pandemic will negatively influence people living with HIV, especially in sub-Saharan Africa, and a six-month interruption of the antiretroviral therapy supply could lead to up to 500,000 extra deaths and a doubling of mother-to-child transmission in over one year (Keene et al., 2020). In view of this, UNAIDS highlights the need to ensure that tools such as pre-exposure prophylaxis (PrEP) continue to be available at this current time of pandemic so that everyone can be safe and protect themselves against HIV (UNAIDS, 2020a, 2020b).

Bearing in mind that women are more vulnerable than men to HIV infections, and that most infections come from unsafe sex, vaginal dosage forms containing antiretroviral drugs can be seen as a good choice for reducing the risk of sexual transmission of the virus (Gong et al., 2017). Films for vaginal administration are an interesting option among these potential microbicides for the prevention of HIV (Guthrie et al., 2018). One of the main advantages of these polymeric thin dosage forms is their small footprint, which allows them to be easily concealed (Gong et al., 2017). This is particularly important for women who have little control over their sexual activity, as occurs in sub-Saharan Africa, the area most affected by HIV infection worldwide (Kharsany and Karim, 2016). Some vaginal films loaded with Tenofovir (TFV) as an antiretroviral drug have now reached Phase I clinical trials (U.S. National Library of Medicine, n.d.). This drug acts by inhibiting the reverse transcriptase of HIV-1 and has already been approved by the FDA as PrEP for the prevention of the sexual transmission of HIV, in an oral tablet for daily administration containing TFV disoproxil fumarate and Emtricitabine (Krakower and Mayer, 2015).

Vaginal films can dissolve fast when they come into contact with the vaginal fluid but can also be designed as mucoadhesive dosage forms that adhere to the vaginal mucosa, thereby increasing the residence time of the formulation at the site of administration (Rohan and Zhang, 2014)

Abbreviations: EL100, Eudragit® L100; TEC, Triethyl citrate; TFV, Tenofovir.

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and allowing the controlled release of the drugs they contain. This kind of vaginal film is very interesting for the development of microbicidal formulations. Certain characteristics of thin films such as their drug release profile, mucoadhesiveness and their mechanical properties are influenced by the film-forming polymer (Karki et al., 2016), which is the most important component of these systems (Rohan and Zhang, 2014). Chitosan is a natural polymer derived from chitin and obtained from the exoskeleton of crustaceans. It is the second most abundant polysaccharide in nature and has good film-forming and mechanical properties. It is a biodegradable, biocompatible and nontoxic polymer which also possesses antimicrobial and antioxidant activities (Singh et al., 2015; Xu et al., 2019). Chitosan can be used to obtain mucoadhesive and controlled release drug delivery systems (Valenta, 2005).

However, it is frequently a challenge for a single polymer to meet all the characteristics desired in a formulation, such as mucoadhesion and the drug release profile. For instance, previously developed TFV-loaded vaginal films based on chitosan showed good mucoadhesive properties but released the drug quickly in only 6 h (Cazorla-Luna et al., 2020a), while others containing Eudragit® L100 (EL100), which is associated with a lack of mucoadhesion, released the drug for up to 300 h (Cilurzo et al., 2003; Notario-Pérez et al., 2021). The combination of different polymers allows systems to be obtained with improved properties in comparison with the polymers alone (Abruzzo et al., 2017), and is the origin of bilayer films. These are systems consisting of two layers, each with different physicochemical properties, which gives the formulation greater functional versatility by merging the advantages and characteristics of both materials (Cazorla-Luna et al., 2020b; Slavutsky et al., 2018). Bilayer films composed of a mucoadhesive layer containing chitosan and another layer intended for controlled release based on ethylcellulose or Eudragit® S100 were formulated for the buccal administration of propranolol hydrochloride and the vaginal administration of TFV respectively (Abruzzo et al., 2017; Cazorla-Luna et al., 2020b). The use of pH-dependent polymers like some types of Eudragit® means the systems offer a controlled release depending on the pH (da Costa et al., 2015). EL100 is an anionic copolymer of methacrylic acid and methyl methacrylate that has previously been included in vaginal dosage forms. Since this polymer becomes soluble at a pH over 6, it represents an interesting excipient for achieving controlled release in the acidic vaginal environment (Cautela et al., 2019; Widjaja et al., 2018), and faster drug release at the time of intercourse due to the presence of semen, with a typical pH of 7.5 (Zhang et al., 2011). For this reason, we proposed EL100 as a potential polymer for the development of bilayer films with pH-responsive vaginal drug release. Although multilayer films are usually obtained by layer-by-layer assembly (Hu et al., 2017) as in the aforementioned cases, bilayer films have been also formulated in a single-step process (Cazorla-Luna et al., 2020b).

Other typical excipients in film formulations are plasticizers, which are located between the polymer chains and enhance the flexibility and hence the handling of the films (Akil et al., 2011; Vieira et al., 2011). Glycerol is one of the most widely studied plasticizers for chitosan films, and has been shown to improve their mechanical properties (Snejdrova and Dittrich, 2012). Triethyl citrate (TEC) is used as a plasticizer for Eudragit® in controlled release formulations (Wypych, 2004) and can be found specifically in the literature on EL100 films as a plasticizer. Like film-forming polymers, plasticizers also condition the films' dissolution, adhesive and mechanical properties (Bando and McGinity, 2006a).

Against this background, the aim of this work was to formulate, in a single-stage process, vaginal bilayer films based on chitosan and EL100 with mucoadhesive properties and pH-responsive release of TFV for the prevention of the sexual transmission of HIV; and to evaluate how the amount of EL100 and/or the proportion of plasticizers in the films can influence the suitability of these systems for the desired purpose.

2. Materials and methods

2.1. Materials

EL100 (Lot: B110603006) was a kind gift from Evonik (Darmstadt, Germany). Chitosan (viscosity: 37 mPa*S; Lot: 0055790) was supplied by Guinama S.L.U. (Valencia, Spain). Its molecular weight was previously determined ($3.21 \cdot 10^1$kDa) and the degree of N-deacetylation of this polymer is 54.73 ± 4.26% (Cazorla-Luna et al., 2019b). TEC (Lot: BCBN8745V) and glycerol (Lot: 0000539368) were provided by Sigma-Aldrich® (St. Louis, MO, USA) and PanReac (Barcelona, Spain) respectively. TFV (Lot: FT104801501) was purchased from Carbosynth Limited (Compton, United Kingdom).

2-propanol (Lot: 0000788642) was acquired from PanReac (Barcelona, Spain). Demineralized water, obtained by a Milli-Q® system, was also used. All other products employed were of analytical grade.

2.2. Film manufacture

Six batches of films based on EL100 and chitosan were formulated. Their composition is shown in Table 1. TEC and glycerol were used respectively as plasticizers for these polymers, and TFV was included as drug in an amount of 30 mg per film in all cases. The batches differed in the amount of EL100 (200, 300 or 400 mg) they contained, and/or the proportion of plasticizers (60% or 100% of the dry weight of the corresponding polymer). The films were obtained by the solvent casting method. The different steps of the process are shown in Fig. S.1. First, the polymers and the drug were poured in a 4.5 cm-diameter silicone mould. The plasticizers were dissolved in 2-propanol for a final volume of 5 mL, which was added over the solid raw materials in the cast. The mixture of all the components was manually stirred until homogeneity was observed and then maintained at room temperature so the solvent evaporated. After 24 h, the resulting films were removed from the casts for their evaluation.

2.3. Scanning electron microscopy

In order to determine the distribution of both polymers in the films and the possible formation of bilayer structures, the microstructure of the systems obtained was observed by scanning electron microscopy (SEM). The samples were prepared following a previously described methodology (Cazorla-Luna et al., 2020b). Briefly, the films were immersed in simulated vaginal fluid (SVF; pH = 4.2 (Owen and Katz, 1999)) in a shaking water bath (P SELECTA® UNITRONIC OR, JP SELECTA S.A., Barcelona, Spain) at 37 °C and 15opm for 24 h, then freeze-dried. The freeze-dried films were cut into fragments of an adequate size to fix on the microscope sample holder, and then coated using a gold sputter module in a high-vacuum system to provide electronic conductivity. The cross-sectional microstructure was observed using a field emission scanning electron microscope (Hitachi S-4700, Tokyo, Japan) at 20.0 kV.

2.4. Texture analyses

The mechanical properties of the films that could influence their

Table 1
Composition in mg of the batches of films formulated.

Batch	EL100	Chitosan	TEC	Glycerol	TFV
E2Ca	200	100	120	60	30
E2Cb	200	100	200	100	30
E3Ca	300	100	180	60	30
E3Cb	300	100	300	100	30
E4Ca	400	100	240	60	30
E4Cb	400	100	400	100	30

handling and suitability for vaginal administration were studied through the tests described below.

Puncture test. This test was performed following a previously established methodology (Notario-Pérez et al., 2019), using a TA.XT*plus* Texture Analyser (Stable Micro Systems, Surrey, UK) with a 5 kg load cell and a spherical probe with a diameter of 5 mm. A piece of film was placed in a film support rig with the side based mainly on EL100 exposed to the probe, and hence to the externally applied force that would be expected at the time of administration of these films. The side based mainly on chitosan would be in contact with the mucosa. From an initial height of 85 mm, the probe descended in compression mode at 0.5 mm/s. After reaching a trigger force of 0.098 N, the sample was also pressed for a distance at 0.5 mm/s until the film burst. The probe was then returned to its initial height at 10 mm/s. The force *versus* time was measured. The maximum force applied for each sample to burst and the distance travelled by the probe at that point were recorded. This assay was carried out in quadruplicate for each batch. The data obtained were statistically analysed using ANOVA and Student's *t*-test (considering $p < 0.05$ as significant).

Tensile test. This test was also based on a previously described methodology (Notario-Pérez et al., 2021). The TA.XT*plus* Texture Analyser (Stable Micro Systems) with the 5 kg load cell and tensile grips was used. A 2 × 4 cm film fragment was placed between the grips, initially spaced 2 cm apart, to clamp the sample. The top grip moved up in a tension mode at 0.5 mm/s for a distance until the film ruptured. The stress *versus* deformation was measured. The maximum force applied for each sample to rupture and the elongation they underwent until this point were recorded. This assay was carried out in triplicate for each batch. The data obtained were statistically analysed using ANOVA and Student's *t*-test (considering $p < 0.05$ as significant).

2.5. Mucoadhesion test

The films were subjected to an *ex vivo* test according to a previously described methodology (Martín-Illana et al., 2019) to quantify their vaginal mucoadhesive properties, using the TA.XT*plus* Texture Analyser (Stable Micro Systems) with the 5 kg load cell and a cylinder probe with a diameter of 10 mm. A sample of bovine vaginal mucosa acquired from a local slaughterhouse was attached to the bottom of a 5 cm-diameter Petri dish using ethylcyanoacrylate (Loctite®) as adhesive material. The mucosa remained immersed in SVF until the start of the assay, when it was removed. The dish was placed on the table of the texture analyser. A 10mmx10mm fragment of film was fixed to the probe with double-sided tape, with the side containing mainly chitosan exposed to the mucosa, thus reproducing the conditions at the administration site. In compression mode, the probe descended at 1 mm/s from an initial weight of 30 mm and, after reaching a trigger force of 0.049 N, pressed the film against the mucosa with a force of 4.9 N at 0.1 mm/s for 30 s. The probe was then returned, also at 0.1 mm/s, to its initial height, thus ensuring the total detachment of the film sample from the mucosa. The force *versus* time was measured. The maximum force taken to detach each fragment of film from the mucosa was recorded. This assay was carried out in triplicate for each batch. The data obtained were statistically analysed using ANOVA and Student's *t*-test (considering $p < 0.05$ as significant).

2.6. Swelling test

This test was performed to characterize the structural changes undergone by the films due to the presence of vaginal fluid at the administration site, in order to explain how the release of the drug occurs in this medium. Three films from each batch were cut into quarters. According to (Mamani et al. (2012), the resulting samples were weighed and fixed to 3 cm-diameter stainless steel discs using ethylcyanoacrylate (Loctite®) as adhesive material. To replicate *in vivo* conditions, the side of the films attached to the disc was the one containing mainly chitosan, as this is the side that would be adhered to the mucosa at the site of administration. Each sample was then immersed in 100 mL of SVF in a beaker, which was placed in the oscillating water bath (P SELECTA® UNITRONIC OR) at 37 °C and 15opm to simulate *in vivo* conditions. At pre-set times the discs were extracted from the beakers and, after removing the excess SVF with a paper towel, they were weighed using a precision balance (METTLER® AT 200, Mettler-Toledo S.A.E., Barcelona, Spain). Eq. (1) was used to calculate the swelling ratio.

$$Swelling\ ratio\ (\%) = \{[(F_t - F_0)/F_0]/P_c\} \cdot 100 \quad (1)$$

where F_t refers to the weight of the film at a pre-set time, F_0 is the weight of the film before immersion in the medium (dry) and Pc is the proportion of chitosan in the films, corresponding to the swellable part.

2.7. Drug release tests

Since the formulated films were intended for the controlled release of TFV in SVF, which was accelerated in the presence of seminal fluid, *in vitro* release tests were performed in both SVF and a mixture of SVF and simulated seminal fluid (SSF; pH = 7.7) in a 1:4v/v proportion (Zhang et al., 2011). SSF was also based on the medium proposed by Owen and Katz (Owen and Katz, 2005). Three films from each batch were placed in borosilicate glass flasks with 80 mL of the corresponding medium to ensure sink conditions. The films were arranged with their mainly chitosan side in contact with the bottom of the bottle and the mainly EL100 side totally exposed to the medium, thus simulating the *in vivo* situation. The flasks were immersed in the oscillating water bath (P SELECTA® UNITRONIC OR) at 37 °C and 15opm. Aliquots of 5 mL were extracted from each one at prefixed times, and this volume was replaced with clean medium. The amount of TFV released into the medium was quantified by UV–visible spectroscopy at a wavelength of 261 nm with an Evolution™ 60S spectrophotometer (Thermo Scientific™, Kyoto, Japan). The drug release profiles obtained were compared through similarity factor f_2.

The data resulting from these tests were processed to determine whether they fitted the Higuchi, Hopfenberg and Korsmeyer-Peppas kinetics (Costa and Sousa Lobo, 2001) to understand the mechanisms causing the release of the drug from the films.

Higuchi kinetics

This kinetic can be summarised by Eq. (2) known as the "simplified Higuchi model":

$$Q_t = K_H t^{1/2} \quad (2)$$

where Q_t is the amount of drug released at time t and K_H represents the Higuchi dissolution constant. Based on this model, the drug is released to the medium by a diffusion process according to Fick's first law, and proportionally to the square root of time.

Hopfenberg kinetics

The Hopfenberg release kinetic responds to the following equation Eq. (3):

$$M_t/M_\infty = 1 - [1 - (k_0 t/C_0 a_0)]^n \quad (3)$$

where M_t/M_∞ is the fraction of drug dissolved in the medium (M_t being the amount of drug dissolved at time t and M_∞ the dose), k_0 is the erosion constant, C_0 is the initial concentration of the drug in the dosage form, a_0 is the radius of the sphere or cylinder, the average thickness of the slab or the half-thickness of the film (depending on the formulation), and n is the exponent which varies according to the geometry (with a value of 1 for slabs and films (Shurshina et al., 2016), 2 for cylinders and 3 for spheres). Considering $k_1 = k_0/C_0 a_0$:

$$M_t/M_\infty = 1 - [1 - k_1 t]^n$$

According to this model, the drug release is explained by an erosive process of the dosage form, which can have different geometric shapes as already mentioned.

Korsmeyer-Peppas kinetics

In general, this model responds to Eq. (4):

$$M_t/M_\infty = at^n \quad (4)$$

where M_t/M_∞ is the fraction of drug released according to the dose, a is a constant that depends on the structural and geometric characteristics of the dosage form, t is time and n is the exponent indicating the mechanism responsible for the drug release. In this case, diffusion predominates when the value of n is less than or equal to 0.5; values of between 0.5 and 1.0 indicate an "anomalous transport" based on diffusion and the structural modification of the dosage form; n values equal to 1.0 ("transport case II") and over 1.0 ("transport super-case II") show drug releases that are due only to structural changes in the formulation.

3. Results and discussion

3.1. Film manufacture

During the process of obtaining the films, it was observed that EL100 dissolves in 2-propanol, so chitosan (which precipitates in it) can be seen in the bottom of the cast. This different behaviour of the polymers in the solvent is supported by the solubility of EL100 in alcohols (Sonje and Chandra, 2013) and the use of alcohol to precipitate chitosan (Uspenskii et al., 2010) found in the literature. This finding suggests that the two polymers will not be homogeneously distributed in the resulting films.

All the films obtained were flexible enough to be extracted from the casts and folded without breaking, although films containing a higher proportion of plasticizers showed enhanced properties in these terms (Fig. S.2). In all cases, they were white in colour, slightly brownish due to the presence of chitosan, and the upper surface was shiny while the lower one was matt.

3.2. SEM

The cross-sectional microstructure of the different batches is shown in Fig. 1. In all cases, the micrographs reveal porous structures that can be attributed to the sublimation of the aqueous medium during the freeze-drying process. However, some aspects are worth highlighting. First, two distinct areas with different thicknesses and pore size can be observed in all batches, especially in those containing 60% plasticizer. The right area of each microstructure shows small or even non-existent pores, while the pores on the left are larger, suggesting the formation of bilayer films. Chitosan, which is a swellable polymer (Shariatinia, 2018), is arranged in the lower layer, which explains its larger pore size, while EL100, which does not undergo any important changes in aqueous medium (Notario-Pérez et al., 2021), forms the upper layer with smaller pores. It should be noted that although it cannot be seen at the magnification of the micrographs in the figure, both layers have pores of different sizes. The presence of pores in the EL100 layer becomes more marked as the amount of the polymer increases, implying an increase in the amount of TEC, and/or the proportion of plasticizer. This could be attributed to the plasticizer content, as was also observed by Rabek et al.

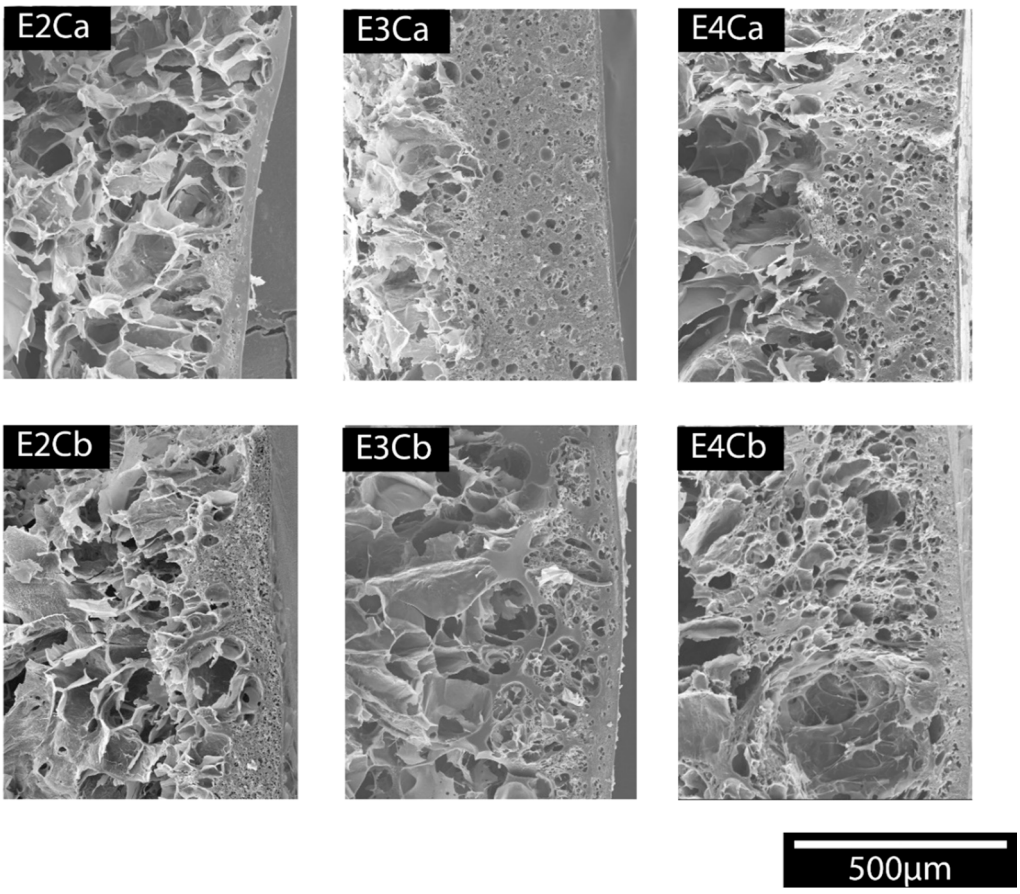

Fig. 1. Cross-sectional structure of the batches of films developed after hydration and freeze-drying by SEM at 100 times magnification. The films are arranged so their upper face, based mainly on EL100, is shown on the right and the bottom face, composed mainly of chitosan, is on the left in each image.

(2014) for films containing TEC. Moreover, although both layers are anchored in all cases, the interface between them is less differentiated and the cross-sectional structure seems more homogeneous in the systems containing a higher proportion of plasticizer, thus pointing to the important role of these excipients in blending the two polymers. Specifically, increased interpenetration could occur between EL100 and chitosan while the solvent evaporates during the manufacturing process due to the greater presence of plasticizers and hence the enhanced flexibility of the polymer chains.

Based on the above, the different arrangement of chitosan and EL100 in the films, as predicted during the manufacturing process, was confirmed through SEM micrographs. These films can therefore be classified as "bilayer structures", since they are composed of two layers: one based mainly on EL100 and the other on chitosan. This behaviour was previously observed for films based on ethylcellulose or EL100 and various biopolymers (Cazorla-Luna et al., 2020b; Martín-Illana et al., 2021), and can be related to the different solubility of the two types of polymer in the films in the solvent.

3.3. Texture analysis

Puncture test. The data on the maximum force applied and the distance travelled by the probe to puncture the films were processed to obtain deformability values. The results are shown in Fig. 2.

The deformability of the films increases with the amount of EL100. These results can be explained considering the fact that the higher the content of EL100, the higher the content of the TEC plasticizer. Given that the plasticizers are located between the polymer chains, this would produce a weakening of the intermolecular interactions and a rise in flexibility (Dobaria et al., 2009; Vieira et al., 2011). However, not only does the amount of TEC increase in this case, but also that of the polymer. On this basis, and considering that the side of the films exposed to the probe in this test was the one based mainly on EL100, the data suggest that EL100 polymer chains have good mobility, thus improving the mechanical properties of the films by increasing their polymer content. These differences in deformability according to the amount of EL100 can be seen to be larger among batches containing plasticizers in a proportion of 100% over the dry weight of the corresponding polymer than among those with 60%. Moreover, one-way ANOVA proved that the differences were statistically significant both for batches containing 60% (p-value $= 5.82 \cdot 10^{-3}$) and 100% plasticizers (p-value $= 1.23 \cdot 10^{-5}$).

The deformability of the films also becomes higher as the proportion of plasticizers increases (b > a), regardless of the amount of EL100, with statistically significant differences for batches E3C (p-value $= 4.00 \cdot 10^{-3}$) and E4C (p-value $= 5.21 \cdot 10^{-4}$), although not in the case of E2C by Student's t-test. This means that when the plasticizer content is higher, the probe travels a greater distance when the same force is applied to break the films. These results can be explained by the effect of the plasticizers described above, which is enhanced by increasing the plasticizer content, thus improving the flexibility and mechanical properties of the films (Zullo and Iannace, 2009). It should be noted that these differences in deformability between films containing 60% and 100% plasticizers become more marked as the amount of EL100 in the systems increases. For this reason, and based on the differences depending on the amount of EL100 mentioned above, there could be said to be a synergistic effect between plasticizers and EL100 in the mechanical properties of these films, as can be observed in Fig. 2. In fact, the batches containing the lowest amount of EL100, although handleable, can be considered to be systems that are not well-defined and with no clear influence of the proportion of plasticizer.

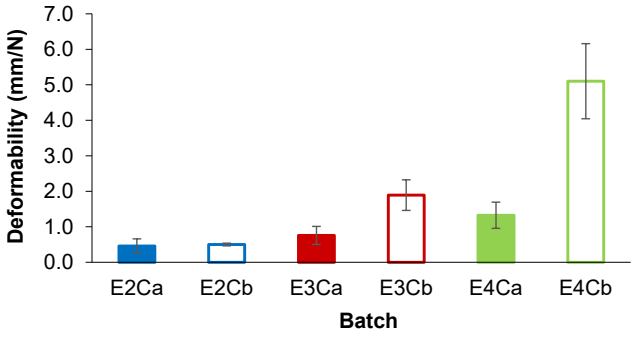

Fig. 2. Results of deformability obtained from the puncture of the films. Each data is presented as mean ± SD ($n = 4$).

Tensile test. The data on tensile strength (maximum force applied per area, i.e. the maximum force provided for the elongation) and breaking strain (percentage of elongation until the film breaks) are shown in Fig. 3.

As can be seen in Fig. 3A, the higher the amount of EL100 in the film, the lower the tensile strength, with statistically significant differences by one-way ANOVA among batches with 60% (p-value $= 9.17 \cdot 10^{-4}$) and batches with 100% (p-value $= 4.77 \cdot 10^{-3}$) plasticizer. As described for deformability, the increase in the amount of TEC explains the weaker interactions among the polymer chains and the subsequent lower force applied per area for the elongation of the films. The proportion of plasticizers in the system also determines its tensile strength, which decreases when it rises from 60% to 100%, whatever the amount of EL100. However, statistically significant differences can be established only for E2C batches according to Student's t-test (p-value $= 4.19 \cdot 10^{-2}$). These results agree with the results for deformability and are caused by the same behaviour. The higher the amount of plasticizer, the greater the flexibility of the polymer chains, which enhances the deformability and diminishes the tensile strength (Dobaria et al., 2009). Contrary to what can be seen in Fig. 2 (deformability), but as expected, differences in tensile strength between batches containing 60% and 100% plasticizers and among batches with the same proportion tend to be lower when the amount of EL100 and the proportion of plasticizer is increased respectively, thus confirming the synergistic effect between plasticizers and EL100 as suggested previously.

As seen in Fig. 3B, the amount of EL100 does not influence the percentage of elongation in films containing 60% but it does in films with 100% plasticizers (p-value $= 1.84 \cdot 10^{-2}$ by one-way ANOVA). The higher amount of this polymer implies an increase in the elongation of these films. The percentage of elongation also increases with the proportion of plasticizer, as could be expected, and as indicated by Dobaria et al. (2009). Student's t-test showed statistically significant differences in the case of batches E2C (p-value $= 9.30 \cdot 10^{-3}$) and E4C (p-value $= 6.01 \cdot 10^{-4}$). Although the elongation is mostly conditioned by the proportion of plasticizer, higher values are observed when both the amount of EL100 and the proportion of plasticizer is increased, as in the case of deformability.

From the data obtained from the texture analysis, it can be derived that both the amount of EL100 and the proportion of plasticizers are critical parameters in the mechanical properties of these films. A minimum amount of both excipients is required if suitable deformability and elongation results are to be attained. The batch with the best mechanical properties in terms of deformability, tensile strength and breaking strain, and hence the most suitable for vaginal administration, is the batch containing the highest amount of EL100 and the greatest proportion of plasticizer, that is, E4Cb. The preferred mechanical properties that can be found in the literature on vaginal films are sufficient tensile strength to withstand mechanical stress during handling and administration, and high elongation at break (Kawarkhe and Poddar, 2010; Mishra et al., 2016; Yoo et al., 2006). Nevertheless, it could be also assumed that the lower force required to elongate or deform the film could be translated into easier insertion of the dosage form in the vagina.

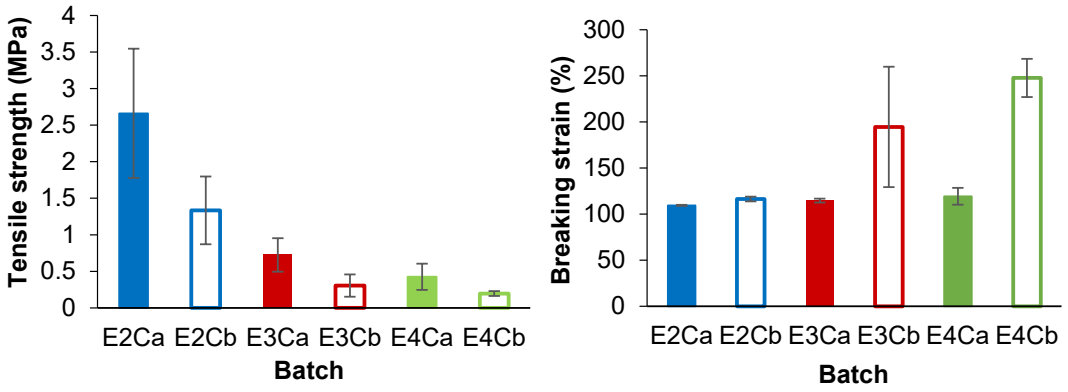

Fig. 3. Tensile strength (A) and breaking strain (B) data from the texture analysis. Each data is presented as mean ± SD (n = 3).

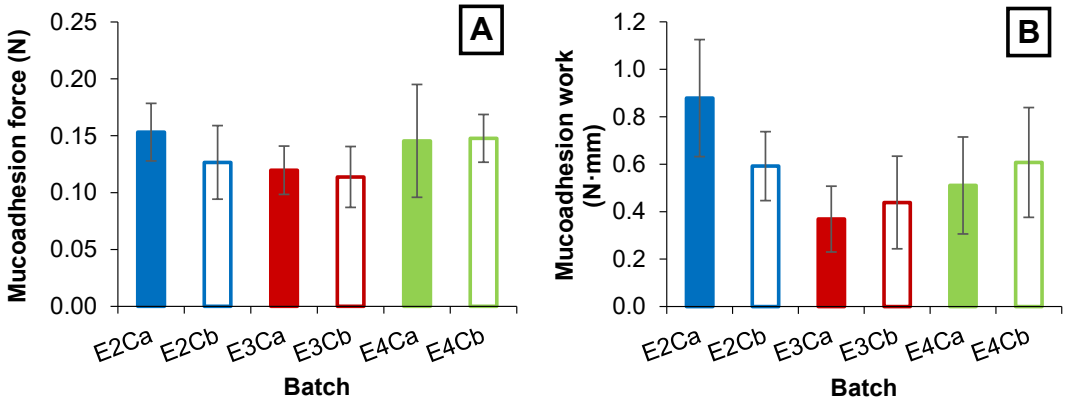

Fig. 4. Results of the mucoadhesion test. Fig. 4A collects the mucoadhesion force values while the work of mucoadhesion is represented in Fig. 4B. Each data is presented as mean ± SD (n = 3).

3.4. Mucoadhesion test

The mucoadhesive properties of the films are attributed to chitosan as indicated in the introduction section. The mucoadhesion mechanism of this polymer responds mainly to ionic interactions and hydrogen bonds between its positively charged amino groups and the negatively charged groups of the mucus gel layer (Sandri et al., 2012). The results of the mucoadhesion test are shown in Fig. 4. All the films obtained were mucoadhesive, showing similar values to some previously developed layer-by-layer films containing Eudragit® S100 and a chitosan derivative (Cazorla-Luna et al., 2020a) and chitosan-based tablets for vaginal administration of TFV (Cazorla-Luna et al., 2019b). Abilova et al. (2020) found that the mucoadhesive properties of vaginal films containing chitosan and poly(2-oxazoline) increased with the proportion of chitosan. The batch with the lowest content of chitosan showed similar mucoadhesion force values to those obtained for the films developed in the present work. According to these data, we could expect all the batches formulated to have a good potential as mucoadhesive drug delivery systems.

As seen in Fig. 4A, all the batches show similar mucoadhesive force values, so it can be affirmed that neither the amount of EL100 nor the proportion of plasticizers are defining parameters of this force. No statistically significant differences were confirmed by two-way ANOVA. Considering that the side of the films based mainly on chitosan, which is the mucoadhesive polymer included in a fixed amount in all the batches, is the side exposed to the mucosa in this test, it was to be expected that the mucoadhesion would not be affected by the amount of EL100 in the film.

Some differences can be established in terms of mucoadhesion work (Fig. 4B). This parameter tends to decrease in the case of films E2C and increase in E3C and E4C as the proportion of the plasticizers raises. An increase in the proportion of the plasticizers could result in greater mucoadhesion due to a decrease in the viscosity of the polymer that would lead to a higher ability to spread onto the vaginal mucosa (Snejdrova and Dittrich, 2012). However, an over-hydrating of the films may occur if the amount of plasticizer is too high, thus causing a lower mucoadhesion (Karki et al., 2016). Based on the above, the slightly higher mucoadhesion work -although no significant- of the batches E3Cb and E4Cb over E3Ca and E4Ca, respectively, could be explained by the diminishing of the polymer viscosity due to plasticizer gain. In the case of batches E2C, the amount of EL100 is lower so that the water entry could be easier, and an over-hydrating of the film could respond to the decrease in the average mucoadhesion work by increasing the proportion of plasticizer. However, two-way ANOVA showed statistically significant differences in the mucoadhesion work only based on the amount of EL100 in the films (p-value = 4.08 · 10-2) while one-way ANOVA confirmed no differences related to this parameter both for batches containing 60% of plasticizers and for those with 100% of plasticizers. It could therefore be stated that neither the proportion of the plasticizers nor the amount of EL100 condition the mucoadhesive properties of the films.

Based on the results obtained, it could be said that all the developed batches show similar mucoadhesive force and, therefore, similar adhesiveness. Regarding the mucoadhesion work, the batches could be considered statistically no different and, thus, equally sticky and suitable for the desired purpose.

3.5. Swelling test

Fig. 5 shows the profiles obtained from the swelling test. In all cases

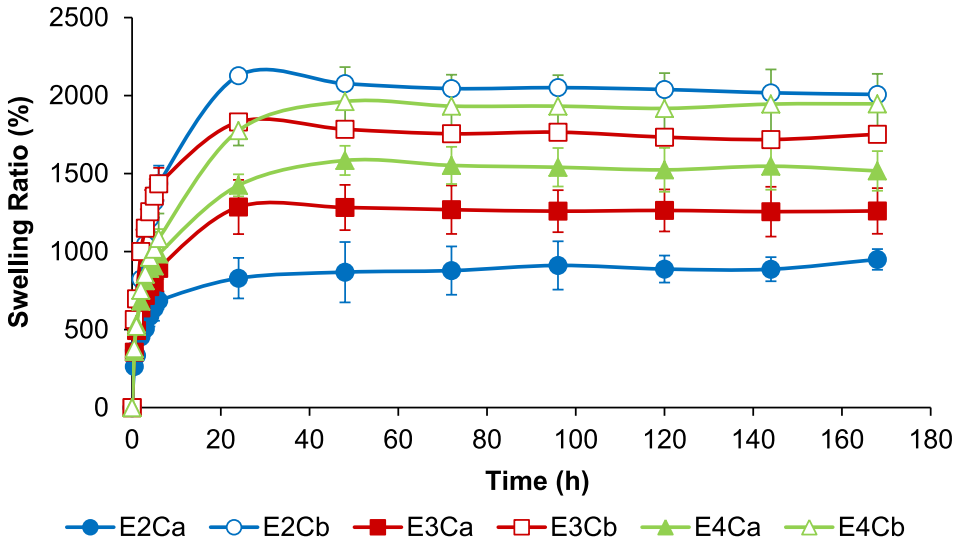

Fig. 5. Swelling profiles of the batches in SVF. Each data is presented as mean ± SD ($n = 3$).

there is an initial increase in swelling ratio followed by a plateau stage that continues until the end of the test. The first phase responds to the capture of water from the medium by chitosan, which forms a gel in the presence of diluted acids. However, this gel is not homogeneous in SVF since its acidity is insufficient to cause the perfect gelation of the polymer. In this case, chitosan takes up water from the medium, giving rise to a flocculated system (Cazorla-Luna et al., 2019a). EL100-based films barely swell in the presence of SVF as was previously demonstrated (Notario-Pérez et al., 2021). The plateau stage can be explained by the insolubility of EL100 at pH values below 6 (thus, at the pH of SVF). This hinders the weight loss and destructuring of the chitosan-based layer of the films which is usually observed in the final stage of the swelling profiles due to the erosion and/or dissolution of the dosage form in the presence of the medium. This behaviour was visually confirmed during the test.

As shown in Fig. 5, some differences can be established based on the proportion of plasticizers and the content of EL100 in the films. The amount of polymer influences the swelling of the films in a different way depending on the proportion of plasticizer. Batches containing 60% plasticizers have a higher swelling ratio when the amount of EL100 increases. Batches E3Ca and E4Ca exhibit significantly greater values than E2Ca during almost the entire test. Films E4Ca swell more than E3Ca, although there are no significant differences between them at several points of the test. This behaviour is because the layer composed mainly of EL100 acts as a structuring agent that hinders the erosion in the medium of the layer containing mainly chitosan, allowing this polymer to capture a greater volume of water. Nevertheless, these differences seem to disappear in the case of films with a higher proportion of plasticizer. Although there are some significant differences between batches E2Cb, E3Cb and E4Cb, they have similar swelling profiles. It can thus be affirmed that the amount of EL100 barely influences the swelling ratio in the case of films with a higher proportion of plasticizer.

The swelling ratio is higher when the proportion of plasticizers is increased from 60% to 100%, regardless of the amount of EL100. In the case of films containing 200 or 300 mg of EL100, batches with a greater proportion of plasticizers undergo significantly higher swelling than those with a lower proportion throughout almost the entire test (E2Cb > E2Ca, E3Cb > E3Ca). However, no significant differences are observed between batches E4Ca and E4Cb until 24 h of the test. These differences in swelling according to the proportion of plasticizers can be explained by the fact that plasticizers modify the three-dimensional structure of the polymers, filling the intermolecular spaces and diminishing the hydrogen bonds between polymer chains, thus increasing the free volume and mobility of the polymers. A high concentration of hydrophilic plasticizers such as glycerol can increase water diffusion into the polymer, as indicated by Vieira *et al.* (Vieira et al., 2011). In this case, the plasticizer expands the spaces between the polymer chains, allowing greater water uptake and consequently higher swelling (Hafezi et al., 2019). This is to be expected, as glycerol would mostly plasticize chitosan, that is, the swellable polymer in the systems. Moreover, the higher interpenetration of EL100 and chitosan observed by SEM when the proportion of plasticizers increases could also explain these differences, as the films are more structured, thus reducing the erosion of the chitosan-based layer. Nevertheless, it should be noted that the differences in the swelling ratio according to the proportion of plasticizer decrease when the amount of EL100 in the films is increased, so the influence of the proportion of plasticizers in the swelling is lower when the amount of EL100 is higher. This could be because it becomes more difficult for water to enter the films from the medium when the amount of polymer increases (Radhika et al., 2009), and hence the amount of TEC, which can also act to reduce water capture by the films as it is hydrophobic (Snejdrova and Dittrich, 2012).

3.6. Drug release tests

Fig. 6 shows the data obtained from the TFV release tests in both SVF (Fig. 6A) and the SVF/SSF mixture (Fig. 6B). The controlled release of the drug in SVF, which is faster in the SVF/SSF mixture, is observed for all the batches. The f_2 factor indicated no equivalence between the release profiles in one medium and the other in any of the cases. The percentages of TFV released are significantly higher in the SVF/SSF mixture than in SVF throughout the entire assay generally for all the batches. These clear differences in drug release from one medium to another can be attributed to the pH-dependent solubility of EL100. As mentioned previously, this polymer is insoluble at the pH of SVF (below 6) and soluble at the pH of the SVF/SSF mixture (above 6), which causes the layer based mainly on EL100 to dissolve and accelerate the release of TFV in the presence of SSF. This pH-dependent release of TFV was previously observed for layer-by-layer films composed of one layer of Eudragit® S100 and another layer of chitosan derivatives (Cazorla-Luna et al., 2020a).

Differences in the release according to the proportion of plasticizers and the amount of EL100 in the films can be found both in SVF and the SVF/SSF mixture. The release in SVF (Fig. 6A) tends to be slower with higher amounts of EL100, regardless of the proportion of plasticizer. Batch E2Ca releases the drug for 24 h with statistically significant higher percentages of TFV released than E3Ca and E4Ca, which offer a release of TFV for 192 h (8 days) and 216 h (9 days) respectively. The f_2 showed

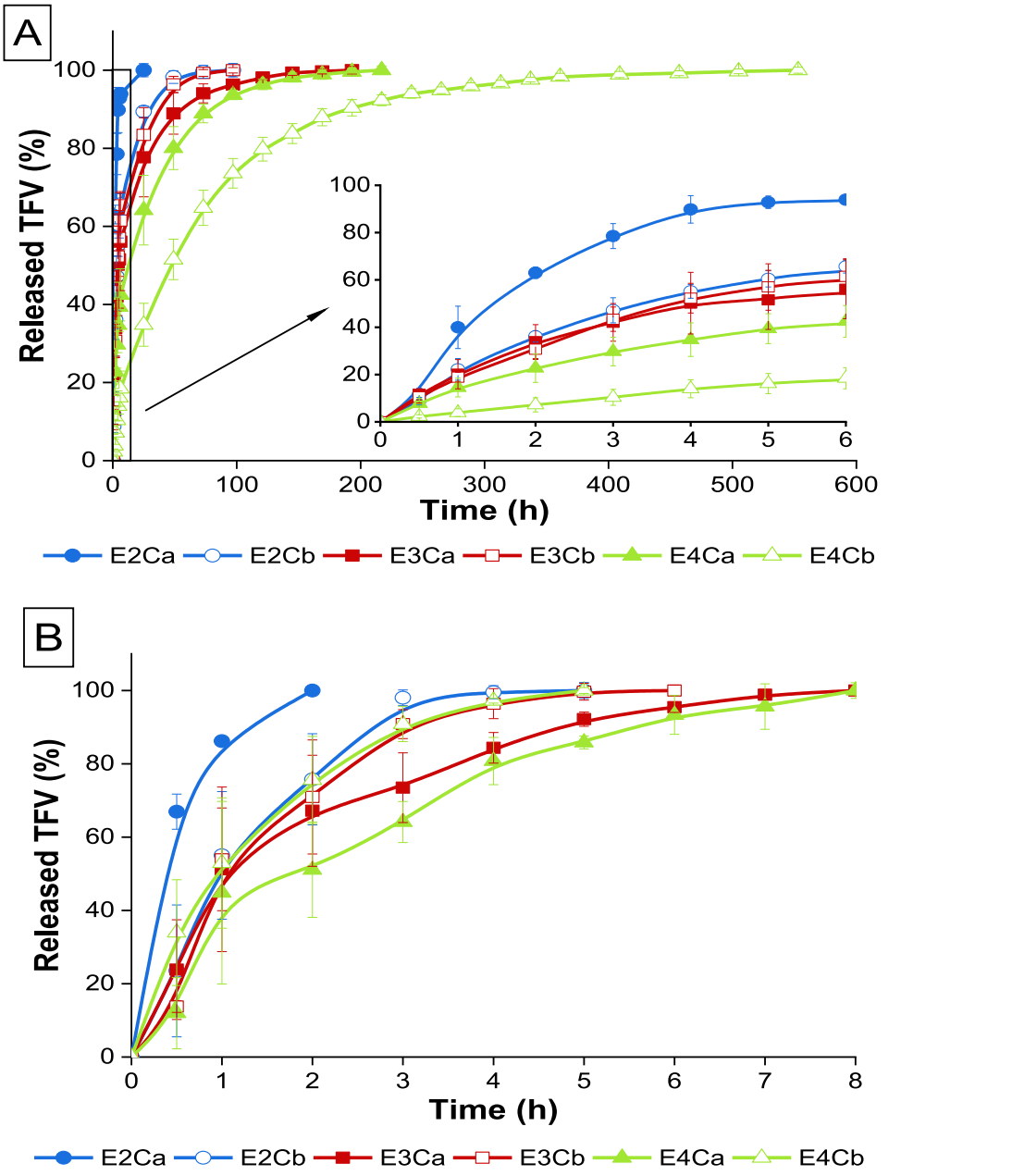

Fig. 6. Drug release profiles obtained from the *in vitro* release tests in SVF (A) and the SVF/SSF mixture (B). The first 6 h of the test in SVF are amplified at the right of the figure. Each data is presented as mean ± SD (n = 3).

no equivalence between the E2Ca profile and the E3Ca or E4Ca profiles. Nor was any similarity detected between E3Ca and E4Ca, although only a few significant differences can be observed between them. For films containing the highest proportion of plasticizers (100%), both E2Cb and E3Cb allow a controlled release of 96 h, with no significant differences between them at any time during the test. These batches release the drug faster than E4Cb, which has significantly lower values than the previous ones throughout the test due to a sustained release of 552 h (23 days), although 90% of the dose is released in 192 h. The f_2 confirmed these differences. Since EL100 is insoluble at the pH of SVF, it could be expected that the greater the content of this polymer in the films, the more controlled the release of TFV in this medium. This agrees with the results of the swelling test, where EL100 was assumed to be a structuring agent that prevented the erosion of the films. Since the amount of TEC rises with the EL100 content, the hydrophobic character of this plasticizer could also support the more controlled release of the drug in this case. Based on these results, it can be affirmed that a minimum amount of EL100 is required to obtain good control over the release of the drug.

In terms of the proportion of plasticizer, E2Ca releases the drug faster than E2Cb, with significant differences throughout almost the entire test. The f_2 factor confirmed that these profiles are not similar. The same occurs for films containing 400 mg of EL100, where the release from E4Ca is faster than from E4Cb. Significant differences between their profiles can be found at any point in the assay, and no equivalence was statistically proven by f_2. However, this does not occur for E3C batches. Although a few significant differences were found between E3Ca and E3Cb, f_2 indicated that their profiles are similar. According to these results, the release of TFV can be said to be generally more controlled in SVF when the proportion of plasticizers in the films is increased from 60% to 100%. These data suggest that the release process in this medium is influenced by TEC when the proportion of plasticizers increases, as its hydrophobicity would make it more difficult for the drug to pass

through the layer based mainly on EL100, thus slowing the release into the medium from the films containing a higher proportion of TEC. Moreover, the higher swelling rate observed in the corresponding test for the batches containing 100% plasticizers and attributed to the role of glycerol could also be translated into a thicker chitosan-based layer, which would require longer for TFV to be released from the film. In addition, the greater structuring of the systems due to the higher interpenetration of both polymers could also enable more control over the release of the drug.

In the SVF/SSF mixture (Fig. 6B), the amount of EL100 in the films conditions the release of TFV in the case of batches with 60% plasticizer. As also occurred in SVF, the higher the amount of this polymer in the films, the slower the release of the drug, since this layer takes longer to dissolve. Although E3Ca and E4Ca show similar profiles according to f_2, with a release of the dose in 8 h and significant differences between them only at 5 h of the assay, both batches allow a significantly slower release of TFV than E2Ca, which releases the drug in 2 h in this medium, as was statistically confirmed by f_2. However, when the plasticizer content is 100% over the dry weight of the polymers, the release of the drug in the SVF/SSF mixture is not influenced by the amount of EL100 in the films. The E2Cb, E3Cb and E4Cb profiles are similar, and very few significant differences can be observed between them. As can be seen in the figure, the release occurs in 5 h from E2Cb and E4Cb and in 6 h from E3Cb. The rapid release of the drug due to the dissolution of EL100 and the leaching of TEC at the pH of the SVF/SSF mixture could explain the fact that the release profiles are more similar and less influenced by the amount of EL100 in this medium, especially when the proportion of plasticizer is higher.

Differences based on the proportion of plasticizers can be also established in the SVF/SSF mixture. In the case of films with 200 mg of EL100, the release is faster for the batch containing the lower proportion of plasticizers (E2Ca), which shows significantly higher values than E2Cb throughout the test. No equivalence between their profiles was statistically confirmed by f_2. The opposite is observed for films with 300 mg and 400 mg of EL100; the batch containing the higher proportion of plasticizers offers a faster release. According to f_2, the E4Cb profile is not similar to the E4Ca profile, although no significant differences can be established between them at the first points of the test. While equivalent release profiles were obtained from both proportions in the films with 300 mg of EL100, E3Cb shows significantly higher data than E3Ca from 3 h to 6 h. Based on these results, it could be said that once the film contains a minimum amount of EL100, the increase in the proportion of plasticizers leads to a faster release of the drug in the SVF/SSF mixture, which could be attributed to the amount of TEC. Bando *et al.* (Bando and McGinity, 2006b) found that film coatings composed of EL100 and Eudragit® S100 plasticized with TEC underwent a rapid leaching of the plasticizer at pH 6, leading to a porous polymer structure. They also observed that organic cast films with this composition simultaneously suffered disintegration of the acrylic polymers and leaching of TEC from the films at pH 7, and that these processes were more marked when the proportion of TEC increased (Bando and McGinity, 2006a). These findings account for the faster release of TFV in the SVF/SSF mixture, where EL100 dissolves, due to the increase in the amount of TEC in the films, which can be seen more clearly by increasing the EL100 and thus the amount of TEC.

The results obtained by processing the data from the drug release test in SVF to determine whether they fitted the Higuchi, Hopfenberg or Korsmeyer-Peppas kinetics are shown in Table 2. Since the release was very rapid in the SVF/SSF mixture, the release profiles in this medium were not adjusted to these mathematical models.

In view of the data collected in Table 2, it can be stated that these release profiles generally show the best fit to the Korsmeyer-Peppas kinetics ($r^2 > 0.93$ in all cases). The values of n between 0.5 and 1 indicate that diffusion through the EL100-based layer and the structural modifications due to swelling of the chitosan-based layer can explain the release of TFV from these films. However, some differences can be noted. When the amount of EL100 in the systems increases, the value of the Higuchi dissolution constant becomes higher, while k_1 from Hopfenberg and n from Korsmeyer-Peppas generally decrease. These data indicate that the erosion or structural modification of the films progressively gives way to diffusion as the main mechanism responsible for the release of the drug, by raising the amount of EL100 in the films. These results are consistent with the TFV release profiles in SVF since the batches with a higher amount of EL100 – where diffusion becomes more important – were those that allowed a more controlled release.

Depending on the proportion of plasticizer, batches E3Ca and E3Cb show similar values for all the kinetic parameters. These results agree with the equivalence obtained by f_2 between the E3Ca and E3Cb release profiles. The slightly higher value of n for E3Cb could indicate that structural modification is more present in this batch than in E3Ca, thus responding to the greater values of released TFV exhibited by E3Cb at certain points of the test. However, n tends to decrease when the plasticizer content rises (E2Ca > E2Cb, E4Ca > E4Cb). This agrees with the decrease in k_1 of Hopfenberg and the increase in the Higuchi dissolution constant, and justifies the best adjustment of E2Cb and E4Cb to Higuchi kinetics. The value of n over 1.0 in the case of E2Ca means that the release occurs by a "super-case II transport" and responds to structural changes in the film, which supports the best adjustment of this batch to Hopfenberg kinetics. These results are confirmed by the swelling and release profiles in SVF, since the greater the proportion of plasticizer, the more structured the films and the more controlled the release of the drug, thus highlighting the greater importance of diffusion.

Based on the above, it is confirmed that both the amount of EL100 and the proportion of plasticizers influence the release of TFV from the bilayer films. Bearing in mind the release profiles in both SVF and the SVF/SSF mixture, E4Cb is the most suitable batch for the proposed objective as it allows a controlled release for 23 days in SVF (reaching 90% of the dose at 8 days) and achieves the total release of TFV in 5 h in the presence of SSF. Comparing to the TFV-based vaginal microbicides that are at the clinical phase (from pericoital or daily administration gels to 3-monthly intravaginal rings), these bilayer films would be an interesting alternative for HIV prevention by offering a different but comfortable posology as well as a pH-dependent protection.

Table 2
Main parameters resulting from fitting the drug release profiles in SVF to Higuchi, Hopfenberg and Korsmeyer-Peppas kinetics. The correlation coefficients of the kinetic offering the best adjustment for each batch are marked in bold.

Batch	Higuchi		Hopfenberg		Korsmeyer-Peppas		
	K_H	r^2	k_1	r^2	a	n	r^2
E2Ca	1.9485	0.9348	0.2694	**0.9556**	0.3092	1.2125	0.9347
E2Cb	3.3704	**0.9802**	0.1084	0.9392	0.1844	0.8641	0.9676
E3Ca	5.5004	0.8873	0.0249	0.6231	0.1970	0.6363	**0.9820**
EC3b	4.9545	0.8840	0.0277	0.6279	0.1830	0.7445	**0.9961**
E4Ca	8.3011	0.9454	0.0139	0.7750	0.1350	0.6775	**0.9903**
E4Cb	13.441	**0.9962**	0.0060	0.9339	0.0451	0.6758	0.9692

4. Conclusions

Combining EL100 and chitosan in the presence of 2-propanol makes it possible to obtain bilayer films with one layer based mainly on EL100 and the other on chitosan in a single stage process as confirmed by SEM. The interpenetration of the two layers due to the presence of the plasticizers (TEC and glycerol) allows both layers to remain joined during the periods tested. Both the amount of EL100 and the proportion of plasticizers condition the mechanical properties, swelling and release of TFV from the films, but not their mucoadhesion, due to their layer based on a fixed amount of chitosan.

The best mechanical properties were achieved by increasing both the amount of EL100 and the percentage of plasticizer, as they exert a synergistic effect on each other. The swelling and release profiles are mainly defined by the layer based on EL100, which acts as a structuring agent that hinders the erosion of the chitosan layer. The presence of EL100 allows the sustained release of TFV in the vaginal medium – and this control becomes greater as the amount of polymer increases –, and the release in a few hours in the vaginal/seminal fluid mixture due to its solubility at a pH over 6. The higher proportion of TEC also helps control the release of the drug in the vaginal medium due to its hydrophobic nature and hastens the process in the presence of seminal fluid by promoting the destructuring of the EL100-based layer at this pH.

Based on the above, bilayer films of EL100 and chitosan containing 400 mg of EL100 and 100% plasticizers (called E4Cb) are the most interesting option for vaginal microbicides for the prevention of the sexual transmission of HIV. Future studies such as *in vivo* tests should be performed on these films since they could offer patients easier administration, a more comfortable posology – potentially resulting in greater therapeutic compliance – and increased protection against HIV during intercourse.

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CRediT authorship contribution statement

A. Martín-Illana: Formal analysis, Investigation, Methodology, Writing – original draft. **R. Cazorla-Luna:** Investigation, Methodology. **F. Notario-Pérez:** Investigation, Methodology. **J. Rubio:** Conceptualization, Methodology. **R. Ruiz-Caro:** Methodology, Supervision, Validation, Writing – review & editing. **A. Tamayo:** Conceptualization, Investigation, Methodology, Supervision, Writing – review & editing. **M. D. Veiga:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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