



## Layer-by-layer vaginal films for acyclovir controlled release to prevent genital herpes

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### ABSTRACT

Genital herpes is one of the most common sexually transmitted infections worldwide. It mainly affects women, as the rate of sexual transmission from male-to-female is higher than from female-to-male. The application of vaginal antiviral drugs could reduce the prevalence of genital herpes and prevent future infections. Layer-by-layer vaginal films were prepared by the solvent evaporation method using *iota*-carrageenan, hydroxypropyl methylcellulose and the polymethacrylates Eudragit® RS PO and Eudragit® S100, for the controlled release of acyclovir. The films were characterized by texture analysis and Raman spectroscopy. Swelling, mucoadhesion, and drug release studies were conducted in simulated vaginal fluid. The results show that Layer-by-Layer films exhibited adequate mechanical properties. The structuring of the layer-by-layer films allowed the controlled release of acyclovir and produced a prolonged mucoadhesion residence time of up to 192 h. The films formed in layer 2 by the combination of Eudragit® RS PO and S100 showed a controlled release of acyclovir for eight days, and adequate mechanical properties. These promising formulations for the prevention of genital herpes deserve further evaluation.

### 1. Introduction

Genital herpes is caused by the herpes simplex virus 2 (HSV-2) and is considered to be a global health issue (Garland and Steben, 2014). Approximately 20 million new cases occur each year and an estimated 400 million people worldwide are currently infected with HSV-2. The prevalence of HSV-2 infection is estimated to be the highest in Africa (31.5 %), followed by the Americas (14.4 %) (Looker et al., 2015; Nasrallah et al., 2019). HSV-2 infection is characterized by lifelong infection with intermittent periods of viral reactivation, and is incurable infection once acquired (Groves, 2016). It is also a leading cause of genital ulcer disease which mainly affects women, as sexual transmission of HSV-2 is more efficient from men to women than vice versa (World Health Organization (WHO), 2016). The infection has been implicated in increasing the risk of acquiring a new human immunodeficiency virus (HIV) infection by approximately threefold (Abu-Raddad et al., 2008; Freeman et al., 2006), particularly in sub-Saharan Africa (Abu-Raddad et al., 2008; Looker et al., 2017). Despite the

global interest in HSV-2 infection, women in these countries do not generally have access to effective methods to prevent its sexual transmission due to poorly/developed healthcare systems and to social and cultural factors. The development of effective methods such as vaginal microbicides will therefore prevent the spread of sexually transmitted infections (STIs), such as genital herpes, and will allow women to use them without their partner's consent (Notario-Pérez et al., 2017b). The prevention of HSV-2 infection by microbicides is recognized as an important tool for reducing the prevalence of HSV-2, following World Health Organization (WHO) guidelines (World Health Organization (WHO), 2016).

There is currently no effective treatment against HSV-2, and the vaginal microbicides developed so far do not provide an adequate response due to their multiple limitations, such as leakage, messiness, relatively low residence time, inadequate doses and low stability, resulting in ineffective formulations (Acarturk, 2009; World Health Organization (WHO), 2016). Acyclovir (ACV) is one of the most widely used antiviral drugs among the various treatment options available for

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herpetic infections. ACV is a guanosine analogue, active against the herpes simplex virus 1 (HSV-1), herpes simplex virus 2 (HSV-2), and varicella zoster virus (VZV) by interfering with DNA synthesis and inhibiting viral replication (Baker, 1999; O'Brien and Campoli-Richards, 1989). ACV is a safe and effective drug for vaginal administration and is used in the treatment of primary or recurrent genital herpes lesions, shortening virus elimination and accelerating the healing of some herpetic infections (Corey et al., 1982a, 1982b; Sánchez-Sánchez et al., 2015).

However, the drug's poor physicochemical properties, such as its limited solubility, results in low bioavailability, so current formulations of ACV cannot maintain the required drug levels at the site of action (Cortesi and Esposito, 2008). A variety of pharmaceutical formulations have been developed that seek to improve the delivery properties of ACV (Klysik et al., 2018), but these formulations have in many cases failed to produce the expected results. There is therefore an urgent need to develop new ACV delivery systems that will improve the treatment and prevention of genital herpes. The development of new vaginal formulations such as films would allow the controlled release of ACV and thus constitute safe and effective methods for preventing the acquisition of HSV-2.

Vaginal films are solid dosage forms composed primarily of various kinds of polymers mixed with plasticizers (Palmeira-de-Oliveira et al., 2015). They offer several potential advantages over traditional formulations such as gels and creams, including precise dose administration, efficient release of the drug on contact with vaginal fluid, good stability and viable large-scale production. Patients would enjoy such benefits as easy application (without an applicator), good portability, easy storage, no product leakage, a low cost and discreet use, which is useful in situations where women have little control over sexual activity, leading to better adherence to treatment or prevention of STIs (Elias and Coggins, 2001; Machado et al., 2013; Nel et al., 2011). However, to ensure correct adherence to the use of these systems, it is essential to develop vaginal films that control the release of drugs. The use of the layer-by-layer (LbL) technique allows the controlled release of various drugs (Choi and Hong, 2014; Wohl and Engbersen, 2012). The LbL technique is a versatile method for manufacturing multilayer films based on electrostatic interactions, hydrogen bond interactions, covalent bonds and hydrophobic interactions (Boudou et al., 2010; Decher, 1997; Gates and Shukla, 2017). It is a powerful method that allows the manufacturer to modulate the characteristics of the films, making it possible to develop bilayer films that contain a highly mucoadhesive layer – generally by using one or more natural or semi-synthetic polymers to prolong the residence time of the formulation in the area of action – and another layer based on a polymer capable of controlling the release of the drug (Cazorla-Luna et al., 2020b, 2020a).

The polymers used in the development of bilayer vaginal films must satisfy certain qualities to ensure the production of effective films: they must have good mucoadhesive capacity and be flexible, biocompatible, biodegradable and non-toxic (Duan and Zhang, 2017). Among these polymers, it is worth highlighting carrageenans, which are a group of linear sulphated polysaccharides present in the cell structure of *Rhodophyceae* algae and used in the pharmaceutical industry for the development of hydrogels due to their high viscosity, gelling ability and stabilizing properties (Li et al., 2014; Yegappan et al., 2018; Zia et al., 2017).

Carrageenans have also been extensively studied in vaginal administration (Pacheco-Quito et al., 2020a; Perino et al., 2019; Sánchez-Sánchez et al., 2015; Ugaonkar et al., 2015), due to their antiviral properties that inhibit the human papilloma virus (Novetsky et al., 2016; Perino et al., 2019) and offer protection against HSV-2 transmission by binding to herpes virus receptors (Boulou et al., 2017; Calenda et al., 2017; Fernández-Romero et al., 2012; Kizima et al., 2014; Pacheco-Quito et al., 2020b). There is little evidence of vaginal films based on this polymer (Gu et al., 2015; Traore et al., 2018).

Another polymer commonly used in a variety of formulations is

hydroxypropyl methylcellulose (HPMC), a non-ionic water-soluble polymer derived from cellulose. HPMC has been shown to control the release of antimicrobial drugs thanks to its swelling and dissolving capacity in aqueous medium; several studies have also confirmed its mucoadhesive capacity, making it an excellent excipient that is present in a wide variety of vaginal formulations (Dangi Amish et al., 2011; Gafitanu et al., 2017; Notario-Pérez et al., 2017a; Pacheco-Quito et al., 2020a; Sánchez-Sánchez et al., 2015; Tuğcu-Demiröz, 2017), including vaginal films (Ghosal et al., 2016; Grammen et al., 2014; Notario-Pérez et al., 2019).

Other functional excipients that have been studied as film-forming polymers are the various types of Eudragit®, a group of synthetic polymethacrylate copolymers (Morales and McConville, 2011), among which we will particularly describe Eudragit® RS PO (ERS) and Eudragit® S100 (ES). ERS is a vitreous copolymer synthesized from acrylic acid and methacrylic acid esters with 5 % functional groups of quaternary ammonium that has been widely used in pharmaceutical forms for its biocompatibility and safety (Albarahmeh et al., 2016; Jafariazar et al., 2015; Notario-Pérez et al., 2017a). ERS is a water insoluble polymer, and the drug delivery systems prepared from it show sustained drug release due to its low permeability in aqueous media (Albarahmeh et al., 2016; Haznedar and Dortunç, 2004; Jafariazar et al., 2015). Eudragit® S100 (ES) is a copolymer based on methacrylic acid and methyl methacrylate. It is non-soluble in acids and water and pH responsive, being only soluble in dissolutions of pH 7 or higher (Kumari et al., 2018). Its characteristics have allowed the development of pH-sensitive drug delivery systems (Qindeel et al., 2019), and its application in vaginal films is currently under study (Cazorla-Luna et al., 2020a).

Against this background, and since women are more affected by HSV-2, the aim of this study was to develop vaginal films loaded with ACV using the LbL technique, in order to obtain vaginal films with a high mucoadhesion capacity owing to the presence of the *iota*-carrageenan/HPMC layer, and controlled drug release thanks to the polymethacrylate layer, in order to protect women from the high incidence of HSV, in compliance with WHO guidelines (World Health Organization (WHO), 2016).

## 2. Materials and methods

### 2.1. Materials

Acyclovir (ACV, lot: 701376) was obtained from Lab. Reig Jofré (Toledo, Spain). *Iota*-carrageenan (*iota*-CG, lot: SLBP8536V) was provided by Sigma-Aldrich (St. Louis, MO, USA). Hydroxypropyl methylcellulose—Methocel® K 100 M CR (HPMC, lot: DT430329), was kindly supplied by Colorcon Ltd. (Kent, England). Eudragit® RS PO (ERS, lot: G120238035) and Eudragit® S100 (ES, lot: B071005090) were supplied by Evonik (Darmstadt, Germany). Glycerol (lot: 0000539368) was acquired from Panreac (Barcelona, Spain). All other reagents used in this study were of analytical grade and used without further purification. Demineralized water was used in all cases.

### 2.2. Methods

#### 2.2.1. *Iota*-carrageenan/HPMC based-films

**2.2.1.1. Films preparation.** Films were prepared using *iota*-CG, HPMC or different combinations of both. Glycerol was incorporated as a plasticizing agent. The films were manufactured by the solvent casting method (Machado et al., 2013) in a mixture of methanol/water, using 45 mm-diameter silicon moulds. All the films contained 20 mg of ACV. Blank films (without drug) were also prepared in order to evaluate the possible influence of ACV on film formation and subsequent tests. The composition of each batch is shown in Table 1.

**Table 1**

Composition (mg) of the *iota*-CG and HPMC based-films manufactured with the solvent casting method.

Batch	<i>Iota</i> -carrageenan	Hydroxypropyl methylcellulose	Glycerol	Acyclovir
I	250		200	
H		250	200	
IH1	150	100	200	
IH2	125	125	200	
IH3	100	150	200	
IA	250		200	20
HA		250	200	20
IH1A	150	100	200	20
IH2A	125	125	200	20
IH3A	100	150	200	20

The prepared films were visually evaluated to verify their appearance, colour, flexibility and handling. Films that were not fully formed, had surface cracks or bubbles or were not smooth were discarded.

**2.2.1.2. Drug release.** The release of ACV from the batches was evaluated with the method described by Sánchez-Sánchez *et al.* (Sánchez-Sánchez *et al.*, 2015). Each sample was inserted in a borosilicate glass bottle containing 80 mL of the simulated vaginal fluid (SVF, pH = 4.2) (Owen and Katz, 1999) and placed in a thermostatic shaking water bath (Selecta® UNITRONIC320 OR, Barcelona, Spain), with an experimental temperature of  $37 \pm 0.1$  °C and at 15 opm. The test was performed in triplicate. Samples of 5 mL were removed from each bottle at pre-established times and filtered, and the medium was replaced with the same volume of SVF at the same temperature. ACV concentrations in the SVF were quantified by UV-vis spectroscopy at a wavelength of 251 nm in a Shimadzu® UV-1700 spectrophotometer (Kyoto, Japan).

## 2.2.2. Layer-by-layer films

**2.2.2.1. Films preparation.** Although the *iota*-CG/HPMC films had acceptable characteristics, they were unable to sufficiently control the release of ACV. The same proportions of each polymer-based film were therefore selected to manufacture the LbL films, adding different proportions of ERS or ERS/ES to prepare the second layer (Table 2).

These LbL films were developed using the solvent casting method. First, *iota*-CG/HPMC films were prepared according to the method described above, then ACV was added after the complete evaporation of the solution, followed by a solution of ERS or ERS/ES in acetone. When this solvent was completely dry at room temperature, the resulting films were stored until further assessment. Layer 2 was prepared by only dissolving ERS or ERS/ES in acetone for its evaluation with Raman spectroscopy.

**2.2.2.2. Raman spectroscopy.** Raman spectroscopic measurements were taken on the surface of each film using a 50x light microscope, with an inVia Raman microscope (Renishaw®, Wotton-under-Edge, UK). The individual layers of the LbL films without drug, the whole LbL films without drug and the whole LbL films loaded with ACV and pure drug

**Table 2**

Composition (mg) of the LbL films manufactured with the solvent casting method.

Batch name	Layer 1			Drug	Layer 2	
	<i>Iota</i> -carrageenan	Hydroxypropyl methylcellulose	Glycerol		Acyclovir	Eudragit® RS PO
IH1A-ERS	150	100	200	20	100	
IH2A-ERS	125	125	200	20	100	
IH3A-ERS	100	150	200	20	100	
IH1A-ERS/ES	150	100	200	20	50	50
IH2A-ERS/ES	125	125	200	20	50	50
IH3A-ERS/ES	100	150	200	20	50	50

were analysed. The excitation source was a laser operating at a wavelength of 532 nm with a power of 25 mW. The interval analysed was  $3500\text{--}100\text{ cm}^{-1}$ . The areas of the bands of the Raman microscope were analysed using the Raman spectrometer analysis program.

**2.2.2.3. Texture analysis: resistance and elasticity.** To quantify the resistance and elasticity of the LbL films, the force required to break the film and the distance it can be deformed until rupturing were measured with the method described by Notario-Pérez *et al.* (Notario-Pérez *et al.*, 2019) using a TA.XTplus Texture Analyser (Stable Micro Systems®, Surrey, UK) with a 30 kg load cell. This test was performed in quadruplicate. Each film was fixed to a support rig, and a 5 mm-diameter spherical stainless-steel probe with an activation force of 5 g applied increasing force to each film to maintain a moving rate of 0.5 mm/s. 500 points per second were monitored during the data collection, and the force applied (N) and the distance travelled by the probe (mm) were registered at each point. The measurement ended when the film burst at the maximum registered force. The distance travelled by the probe when the film burst was also recorded.

**2.2.2.4. Swelling behaviour.** Each LbL film was evaluated in SVF using the method described by Ruiz-Caro *et al.* (Ruiz-Caro and Veiga-Ochoa, 2009). Three-centimetre diameter fragments of each batch were fixed to a stainless-steel disc of the same size with cyanoacrylate adhesive (Loctite®, Henkel, Austria), with the hydrophilic layer (*iota*-CG/HPMC) facing the disc and the hydrophobic layer facing outward, thus reproducing the expected conditions after administration. This preparation was placed in a beaker containing 80 mL of SVF, then in a thermostatic shaking water bath (Selecta® UNITRONIC320 OR, Barcelona, Spain) at  $37 \pm 0.1$  °C and 15 opm to simulate physiological characteristics. At specific time intervals, the discs were removed from the medium, placed on filter paper to eliminate the excess liquid and weighed on a precision balance. Each LbL film was tested in triplicate. The swelling ratio (SR%) for each sample was calculated according to Equation (1) below (Haupt *et al.*, 2006):

$$SR(\%) = \left( \frac{F_s - F_d}{F_d} \right) \times 100 \quad (1)$$

where  $F_s$  corresponds to the weight of the swollen film and  $F_d$  to the weight of the dry film.

**2.2.2.5. Drug release.** The release of ACV from the LbL films was evaluated according to a previously established methodology (Sánchez-Sánchez *et al.*, 2015). Each LbL film was inserted in a borosilicate glass bottle containing 80 mL of SVF, with the hydrophilic layer of the film (layer 1) in contact with the glass and the Eudragit® based layer (layer 2) in contact with the medium, as it would be positioned in the vagina. This preparation was then placed in a thermostatic shaking water bath with an experimental temperature of  $37 \pm 0.1$  °C and at 15 opm. The test was performed in triplicate. Samples of 5 mL were removed from each bottle at pre-established times and filtered to eliminate the particles in the suspension medium; the medium was replaced with the same volume of SVF at the same temperature. ACV concentrations in the SVF

were quantified by UV–vis spectroscopy at a wavelength of 251 nm in the spectrophotometer.

The release profiles obtained from our LbL films were compared using a model independent index described by Moore and Flanner to determine whether there are significant differences between the hydrophobic layers (Moore and Flanner, 1996). This similarity factor ( $f_2$ ) was calculated according to Equation (2).

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

Where  $n$  is the number of samples for each dissolution test,  $R_j$  and  $T_j$  correspond to the drug release percentage at each time for the reference and test product respectively, and  $W_j$  is a weight factor ( $W_j = 1$  in this work). A value for  $f_2 > 65$  indicates similarity between profiles higher than 95 %, while  $f_2 < 65$  denotes non-similar profiles (Mamani et al., 2012).

**2.2.2.6. Ex vivo mucoadhesion.** To establish the LbL films' capacity to adhere to the vaginal mucosa throughout the administration time, the work and force necessary for detachment were assessed using an *ex vivo* method previously described by Cazorla-Luna et al. (Cazorla-Luna et al., 2020a). The mucoadhesion test was assessed with the TA.XTplus Texture Analyser. The LbL films were fixed to a 10 mm stainless-steel probe with the *iota*-CG/HPMC layer (layer 1) facing the vaginal mucosa, as expected at the time of administration. Square fragments of 2 × 2 cm of bovine vaginal mucosa (obtained from a local slaughterhouse) were fixed to a Petri dish with cyanoacrylate adhesive and hydrated with 5 mL of SVF. The probe with the LbL film was moved at a speed of 1 mm/s until it came into contact with the vaginal mucosa, applying a contact force of 500 g for 30 s. The probe was then separated from the mucosa at a speed of 0.10 mm/s until the complete detachment of the LbL film. The force applied during the detachment of the formulation was measured at a rate of 500 pps. The force applied vs the distance covered by the probe was measured, and the maximum force required to separate the LbL film from the mucosa was recorded as the mucoadhesion force. Each batch was evaluated in triplicate.

An *ex vivo* mucoadhesion test was applied to determine how long the LbL films remained adhered to the vaginal mucosa, according to the method described by Notario-Pérez et al. (Notario-Pérez et al., 2017a). A sample of bovine vaginal mucosa was fixed to an 8.5 cm × 5 cm stainless-steel plate with cyanoacrylate adhesive. Each LbL film was then adhered to the mucosa with the hydrophilic layer (layer 1) facing the mucosal surface, applying a pressure of 500 g for 30 s. The preparation was placed at an angle of 60° inside a beaker containing SVF, and then in the thermostatic shaking water bath at 37 ± 0.1 °C and 15 rpm. All batches were evaluated in duplicate, and the residence time of each batch was assessed by visual observation of the samples.

**2.2.2.7. Cytotoxicity.** Three human cell lines were used to evaluate the cytotoxicity: a lymphoblastic cell line, MT-2 (Harada et al., 1985); a macrophage–monocyte derived cell line, THP-1 (ATCC® TIB-202); and a uterine/endometrial epithelial cell line, HEC-1A (ATCC® HTB-112™), kindly provided by María Ángeles Muñoz. All the cells were grown and propagated in RPMI 1640 medium supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, and 50 mg/mL streptomycin (all Whittaker M.A. Bio-Products, Walkersville, MD, USA) at 37 °C with a humidified atmosphere of 5 % CO<sub>2</sub>. HEC-1A cells were detached by removing the medium and rinsing the flask during 10 min with 1 to 2 mL of trypsin 0.25 % – EDTA 0.03 % solution (Merck, St Quentin Fallavier, Lyon, France). The medium was replaced every-three days after cell centrifugation at 1500 rpm for 5 min.

Cell toxicity was measured using the CellTiter Glo (Promega®, Madison, WI, USA). Briefly, cells were incubated in 96-well plates at a density of 10 × 10<sup>5</sup> cells per well (MT-2 and THP-1) and 2 × 10<sup>4</sup> (HEC-

1A) in complete medium. To assess the cytotoxic effects of the materials used to prepare the films, cells were exposed to a fresh medium containing different concentrations of ERS, ES, ERS/ES (1:1), and *iota*-CG, or the same concentration of PBS 1 × as control. The materials were suspended in PBS 1 × following a standard method, in which the materials are pre-incubated for 48 h in 5 % CO<sub>2</sub> atmosphere and 37 °C to mimic the conditions of release in the human uterus (Krug, 2011). This pre-incubated mix was added to the cell culture and maintained at 37 °C and 5 % CO<sub>2</sub> humidified atmosphere. After incubating for 48 h, the medium was removed from cell cultures and 50 µL of CellTiter Glo reagent was added to each well of the plate. Supernatants were then carefully removed and transferred to a white microplate. Relative luminescence units (RLUs) were measured in a luminometer (Sirius®, Berthold Detection Systems). Cytotoxic concentration 50 (CC<sub>50</sub>) values were calculated using GraphPad Prism Software (non-linear regression, log inhibitor versus response). The results of the cytotoxic assay are shown as the average of at least three individual experiments.

### 3. Results and discussion

#### 3.1. *iota*-CG and HPMC based film

##### 3.1.1. Films manufacture

In the present study, ten batches of films with varying concentrations of *iota*-CG, HPMC and *iota*-CG/HPMC with ACV and without drug were prepared and their characteristics were evaluated (Table 3). The films developed with *iota*-CG or HPMC presented some defects in their formation. *iota*-CG films were not fully formed, and the structures were very thin and difficult to handle, while HPMC films had numerous bubbles and cracks on the surface, mainly in the central area of the structure, making it difficult to use them for future evaluations. In contrast, the *iota*-CG/HPMC films were found to have an adequate formation; they were homogeneous, smooth, easy to handle and bubble-free, as on contact with the solvent (methanol/water) the polymers relax their chains and form a homogeneous solution which, when the solvent evaporates completely, leaves a well-structured film at the bottom of the mould. When ACV was added to the formulation, the drug did not improve the formation of the single polymer films, as bubbles and cracks could still be seen in the structure. The films formed by the

**Table 3**  
Characteristics of *iota*-CG, HPMC and *iota*-CG/HPMC based films.

Batch	Ratio (%)	Characteristics	Comments
I	100	Semi-transparent. Beige. Very thin, difficult to handle.	Incomplete film formation.
H	100	Semi-transparent. Off-white color. Smooth and easy to handle.	Incomplete film formation. Bubbles and surface cracks.
IH1	60:40	Semi-transparent. Light beige. Smooth and easy to handle.	No bubbles and cracks.
IH2	50:50	Semi-transparent. Light beige. Smooth and easy to handle.	No bubbles and cracks.
IH3	40:60	Semi-transparent. Light beige. Smooth and easy to handle.	No bubbles and cracks.
IA	100	Semi-transparent. Light beige. Very thin, difficult to handle.	Incomplete film formation.
HA	100	Semi-transparent. Off-white color. Smooth and easy to handle.	Incomplete film formation. Bubbles and surface cracks.
IH1A	60:40	Semi-transparent. Off-white color. Smooth and easy to handle.	No bubbles and cracks.
IH2A	50:50	Semi-transparent. Off-white color. Smooth and easy to handle.	No bubbles and cracks.
IH3A	40:60	Semi-transparent. Off-white color. Smooth and easy to handle.	No bubbles and cracks.

combination of polymers (*iota*-CG/HPMC) and ACV were observed to be adequate, since when the ACV was dissolved with the polymers, they captured the drug in their polymeric networks, allowing the formation of a semi-transparent, soft, easy-to-handle film with an off-white colour due to the presence of the drug. In all cases the presence of the plasticizer (glycerol at 80 % w/w) produced completely flexible structures. Finally, the films selected for further testing were IH1, IH2, IH3, IH1A, IH2A and IH3A.

### 3.1.2. Drug release

The ACV release profiles of the selected formulations are shown in Fig. 1. Sustained release of more than 12 h occurs in all cases but does not exceed 24 h. This reveals the inability of these films to control the release of the drug for several days. However, the samples have a very similar profile, releasing more than 70 % of ACV up to 12 h. This behaviour can be attributed to the fact that when the formulation is in contact with the medium, the polymers (*iota*-CG/HPMC), due to their hydrophilic nature and the presence of the divalent ion of the SVF ( $\text{Ca}^{2+}$ ), cause the polymer chains to swell rapidly and form a homogeneous gel with a fluid consistency which allows the release of the ACV trapped in the polymer structures.

No significant differences were found in the release profiles of IH1A, IH2A and IH3A, regardless of the amount of *iota*-CG or HPMC; these data were corroborated in a previous study (Pacheco-Quito et al., 2020a). For this reason, and in order to modulate the gelation rate of the film components and hence the release of ACV, it was decided to develop LbL films using two types of polymethacrylate: ERS and ES.

## 3.2. Layer-by-layer films

### 3.2.1. Film manufacture

In order to improve the characteristics of *iota*-CG/HPMC films, LbL films were developed using the films initially evaluated (IH1A, IH2A and IH3A) as layer 1, with the addition of ERS or ERS/ES as layer 2. Six different batches were obtained, adding 100 mg of ERS dissolved in acetone directly on layer 1 or a mixture of ERS/ES (1:1). The layers were uniformly joined in all cases. The union between the layers forming the LbL films is ensured by hydrogen bridges, sulphate groups and electrostatic interactions (Choi and Hong, 2014; Wohl and Engbersen, 2012).

A visual analysis of the batches was carried out on their removal from the mould. It was observed that the LbL films have an off-white colour, are flexible and easy to handle. One of the differences found between the ERS layer and the ERS/ES layer was that the mixture of ERS/ES produced films with greater flexibility compared to films covered only by

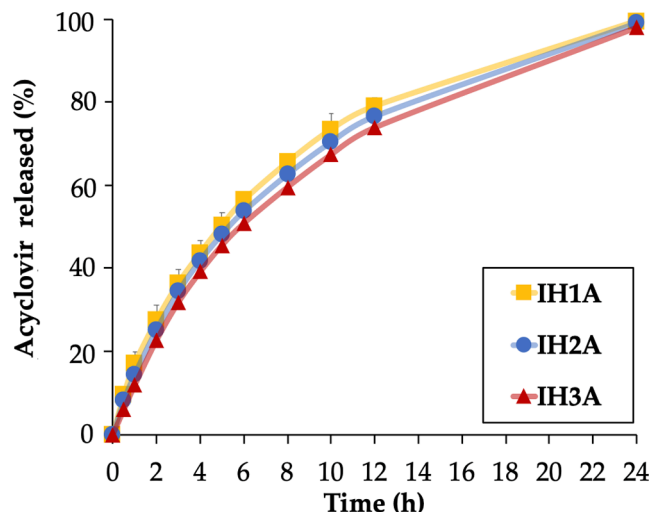


Fig. 1. Acyclovir release profile in SVF from *iota*-CG/HPMC based films.

ERS, pointing to the feasibility of using ES to improve not only the ACV release conditions but also the flexibility of the film, without the need for plasticizers in layer 2. Another characteristic that differentiated LbL films is that layer 1 had an opaque appearance while layer 2 was shiny.

### 3.2.2. Raman spectroscopy

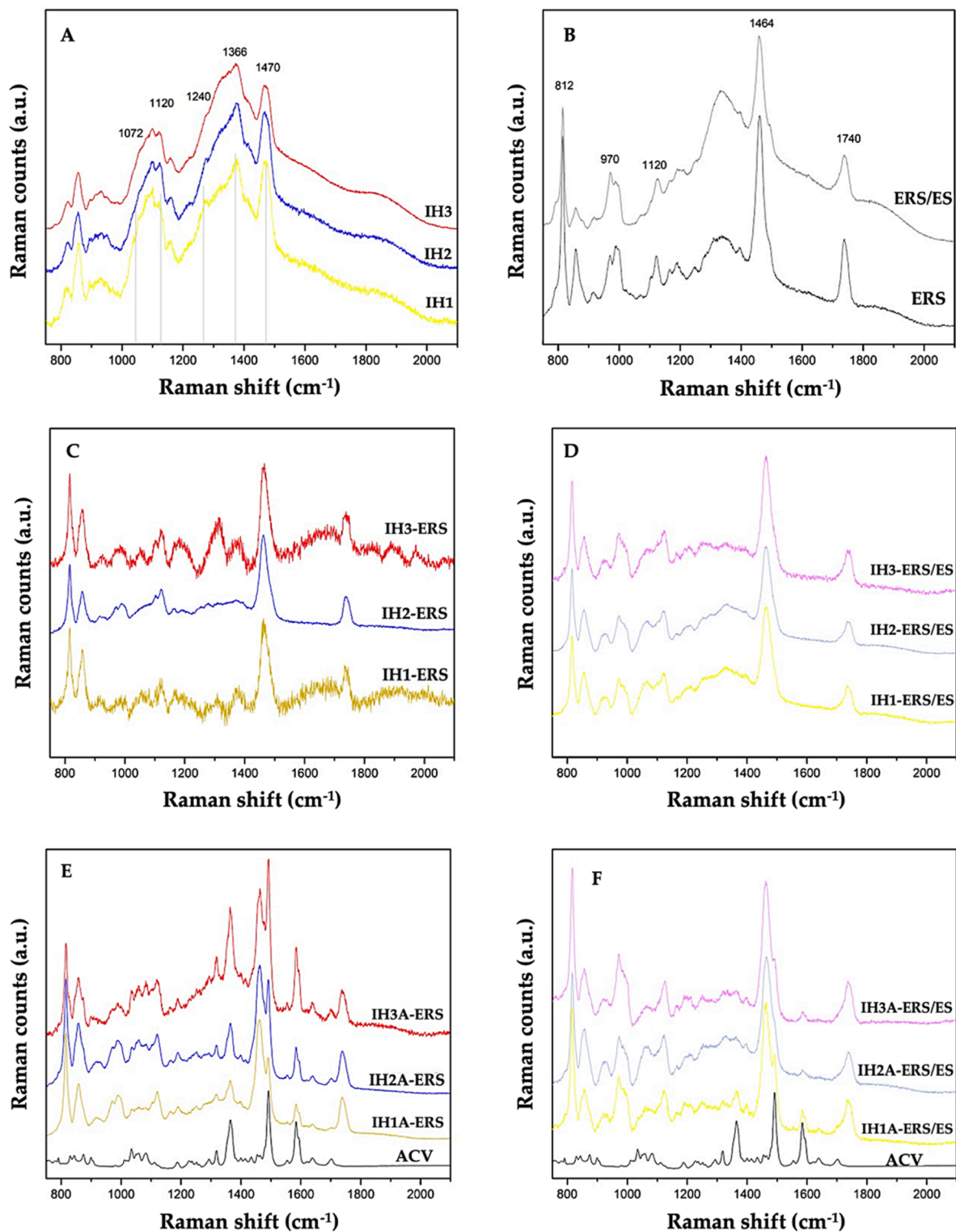
Raman spectra were recorded for the individual film layers (Fig. 2) and the characteristic peaks identifying their components were determined. Fig. 2A shows the characteristic spectra of the components of the films formed only by *iota*-CG/HPMC. The spectra appear quite similar due to their partial overlapping as the amount of *iota*-CG in the film increases. The most prominent bands in the HPMC are located at  $1120\text{ cm}^{-1}$  and assigned to the stretches  $\nu(\text{C}-\text{O})$ ,  $\nu(\text{C}-\text{C})$  and bending vibration  $\nu(\text{C}-\text{O}-\text{H})$ ; and  $1366$  and  $1452\text{ cm}^{-1}$  assigned to the asymmetric and symmetric bending deformation  $\nu(\text{C}-\text{O}-\text{H})$  of methylene and methyl groups in the glucose ring (De Veij et al., 2009; Song et al., 2016). *Iota*-CG presents characteristic peaks at  $1072$  and  $1240\text{ cm}^{-1}$  corresponding to the C—O stretching of 3,6-anhydrogalactose and the S=O vibration of the sulphate esters (Pereira et al., 2003). Glycerol peaks are also observed around  $820$ – $930\text{ cm}^{-1}$ . The most significant variation in the spectra is the decrease in the band centred at about  $1470\text{ cm}^{-1}$ , while the  $1328\text{ cm}^{-1}$  band increases as the HPMC content in the films rises, suggesting conformational changes in the HPMC molecules. The bands located at about  $1080$  and  $1240\text{ cm}^{-1}$  decrease in parallel with the amount of *iota*-CG. No evidence of interaction between the two molecules is observed. Fig. 2B also shows the spectra of the two types of Eudragit®, where slight changes can be seen in the position of the peaks attributed to the conformational changes in the polymer. In any case, the most prominent bands in the spectra are located at  $1464\text{ cm}^{-1}$  and assigned to the  $\text{CH}_2$  symmetric bending, and  $812\text{ cm}^{-1}$  for the C—C stretching vibration, and the bands assigned to the ester groups and located at  $1120$  and  $1740\text{ cm}^{-1}$  for the C—O and C=O stretching of the ester groups respectively (Agrawal and Samal, 2018; Martín-Illana et al., 2021; Sipos et al., 2008). In the ERS spectra, an increase can also be seen in the intensity of the band centred at about  $970\text{ cm}^{-1}$  and assigned to the symmetric stretching vibration of the  $(\text{CH}_3)_3\text{N}^+$  groups.

Fig. 2C and D show the spectra of the LbL films with the characteristic peaks of each component of the formulation clearly distinguishable, since no significant interactions are identified when the spectra overlap. The most significant peaks of ACV are observed in both cases after the incorporation of ACV to the LbL films (Fig. 2E and 2F), and the ring stretching mode located at about  $1485$ – $1490\text{ cm}^{-1}$  is the most intense in all cases. At this point it is clear that the greatest incorporation of ACV occurs in the LbL films produced with ERS and, in the films produced with ERS/ES, the intensity of the bands corresponding to ACV increases from the IH1A-ERS/ES film to the IH3A-ERS/ES film, suggesting a greater incorporation of ACV in the films with the lowest amount of *iota*-CG.

### 3.2.3. Texture analysis: resistance and elasticity

Fig. 3A shows the resistance to fracture of the LbL films. The films formed by *iota*-CG/HPMC and ERS broke easily with the application of 1 to 5 N of force, and deformed less than 1 mm before rupturing. Of the three batches covered with ERS, IH1A-ERS is the batch with the lowest resistance to fracture and elasticity of all three batches, possibly because this batch has the highest amount of *iota*-CG in layer 1. There are no significant differences between IH2A-ERS and IH3A-ERS, confirming that flexibility can be conditioned by the amount of layer 1 polymers, since the film is more flexible as it contains more HPMC, supporting the results of other studies (Jafariaraz et al., 2015; Notario-Pérez et al., 2019). Layer 2 (ERS) requires the addition of a plasticizer to improve its mechanical properties, and although the visual evaluation of the LbL films composed of ERS showed they had flexibility, it did not meet the conditions for its application.

LbL films made up of a mixture of ERS/ES (1:1) had better mechanical properties than films containing only ERS, due to the



**Fig. 2.** Raman spectra of the individual films: (A) Layer 1: IH1, IH2, IH3; (B) Layer 2: ERS and ERS/ES. LbL films without drug: (C) LbL films of *iota*-CG/HPMC and ERS; (D) LbL films of *iota*-CG/HPMC and ERS/ES. LbL films loaded with drug: (E) LbL films of *iota*-CG/HPMC and ERS; (F) LbL films of *iota*-CG/HPMC and ERS/ES. Raman spectra of the drug ACV has been also added for comparison purposes.

incorporation of ES in layer 2. These batches are an improvement on those with only ERS, both in resistance to fracture and elasticity. The batches with the highest amount of *iota*-CG presented a lower resistance to fracture and less elasticity, which would confirm that the LbL films are influenced not only by layer 2 but by both layers in terms of improvements or decreases in their mechanical properties. In general terms,

IH2A-ERS/ES and IH3A-ERS/ES can be regarded as having the best mechanical properties, as they can withstand more than 20 N of force and more than 2 mm of deformation, which will guarantee their easy application and comfort for patients, as mentioned in a previous study (Notario-Pérez et al., 2019).

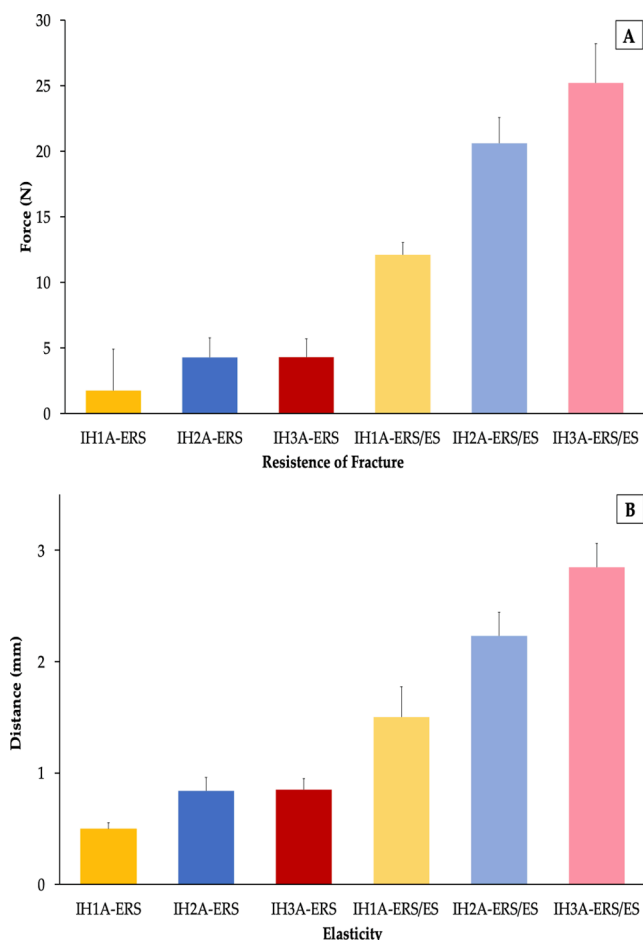


Fig. 3. Texture analysis: resistance of fracture (A) and elasticity (B).

### 3.2.4. Swelling tests

Fig. 4 shows the swelling profiles assayed in simulated vaginal fluid (SVF). Fig. 4A displays the swelling behaviour of the films developed with *iota*-CG/HPMC and ERS, which have a faster and higher swelling profile compared to batches with ERS/ES. This profile is conditioned by two factors: the quantity and type of polymers in layer 1; and the behaviour of ERS. The films present three types of swelling: IH3A-ERS has a high swelling rate due to the greater amount of HPMC, whose hydrophilic nature allows the gradual uptake of medium, leading to greater swelling (Kulinowski et al., 2011; Notario-Pérez et al., 2017a); IH2A-ERS has an intermediate swelling rate, which is also conditioned by the hydrophilic nature of the polymers (*iota*-CG/HPMC); and finally IH1A-ERS has the lowest swelling rate of the three batches. This is possibly because it has more *iota*-CG in the formulation, which forms a fluid gel thanks to the presence of the divalent ion of SVF ( $\text{Ca}^{2+}$ ) (Tako et al., 1987), allowing the formation of intermolecular bridges between the sulphate groups of carrageenan (Thrimawithana et al., 2010). It should be noted that ERS did not greatly influence the swelling behaviour, since it is insoluble in aqueous medium and has low permeability and swelling regardless of the pH (Haznedar and Dortunç, 2004); this indicates that the swelling behaviour is influenced by the composition of layer 1.

Fig. 4B shows the swelling profiles of the batches developed with *iota*-CG/HPMC and ERS/ES (1:1), revealing a similar swelling profile in all three cases, but lower than the batches developed by ERS. In this case the swelling is influenced by the combination of the two types of Eudragit®, because although ERS has lower permeability, ES is insoluble in acidic media (Kumari et al., 2018). This means that the uptake of medium is gradual and they swell less. The rapid swelling of layer 1

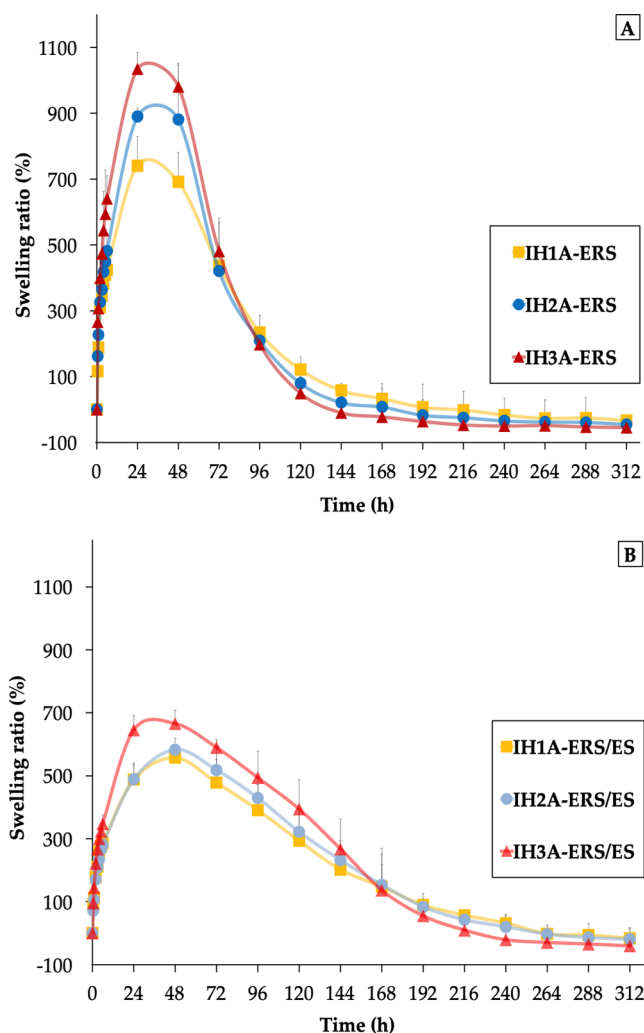


Fig. 4. Swelling profiles obtained in SVF from LbL films with ERS (A) and LbL films with ERS/ES (B).

polymers was therefore reduced in layer 2, unlike the batches developed with ERS.

### 3.2.5. Drug release

The ACV release profiles of LbL films are shown in Fig. 5. Sustained release of more than 72 h is observed in all cases.

The LbL films made up of *iota*-CG/HPMC and ERS have a very similar release profile in all cases and control the release of ACV for up to 96 h, although there are slight differences between one batch and another. This implies that the batches were not influenced by the different composition of layer 1 and layer 2 when it comes to releasing the drug. These findings are closely related to the swelling behaviour of LbL films, which is higher than in ERS/ES films. Layer 1 polymer chains have greater penetration of the medium, allowing them to form a mixed gel that supports the drug so the drug is released by diffusion, as described in other studies when HPMC is mixed with carrageenans (Pacheco-Quito et al., 2020a; Sánchez-Sánchez et al., 2015). Because their layer 2 is developed with ERS, this allows the controlled release of ACV as the drug penetrates through the pores of this low-permeability layer (Haznedar and Dortunç, 2004).

In contrast, the films formed by *iota*-CG/HPMC with ERS/ES have much more sustained drug release profiles for up to 192 h in all cases. When the formulation contained Eudragit® RS PO/ Eudragit® S100, the diffusion of the drug was more modulated because Eudragit® S100 has a pH-dependent solubility (soluble at pH greater than 7). Thus, in this

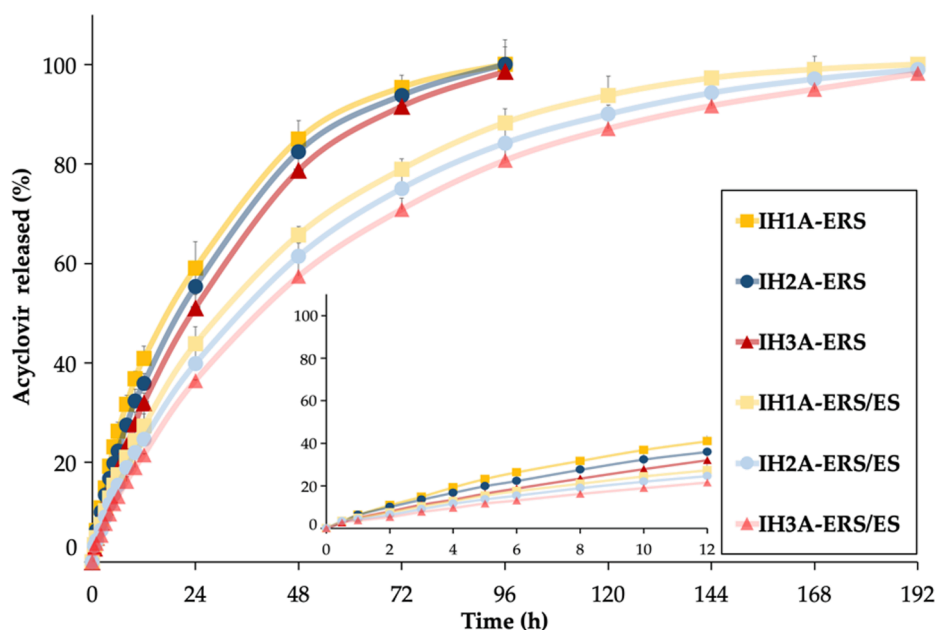


Fig. 5. Acyclovir release profile in SVF from LbL films based on *iota*-CG/HPMC, and ERS or ERS/ES.

layer, only the part corresponding to Eudragit® RS PO (which has low permeability) is able to permeate the vaginal environment and dissolve the ACV, but Eudragit® S100 does not dissolve or swell at the pH of the vaginal environment and acts by hindering the characteristic behaviour of Eudragit® RS PO. Therefore, the combination of the two types of Eudragit® produces a synergy of their characteristics, preventing the ACV from rapidly passing through the layer two of the vaginal layer. These findings are also closely linked to the swelling behaviour of LbL films, since they showed only slight swelling and their erosion took longer, which is a factor in sustaining the release of ACV and confirms that layer 2 controls the swelling and therefore the release of the drug (Cazorla-Luna et al., 2020a; Qindeel et al., 2019).

LbL films with ERS/ES fulfil several of the characteristics evaluated, so they would be ideal candidates for future tests and for the prevention of sexually transmitted infections such as genital herpes. As far as we know they are the first LbL films that control the release of ACV for vaginal application.

Similarity factor ( $f_2$ ) were used to compare the experimental results (Table 4).

According to the  $f_2$  statistic, there are differences between IH1A-ERS and IH3A-ERS. As mentioned above, this is due to the composition of

Table 4

Similarity factors ( $f_2$ ) values for the release profiles. Comparisons with significant difference ( $f_2 < 65$ ) are in bold.

Batch	IH1A-ERS	IH2A-ERS	IH3A-ERS	IH1A-ERS/ES	IH2A-ERS/ES	IH3A-ERS/ES
IH1A-ERS		74.115	<b>59.911</b>	<b>49.303</b>	<b>44.974</b>	<b>41.168</b>
IH2A-ERS			72.933	<b>56.125</b>	<b>50.431</b>	<b>45.721</b>
IH3A-ERS				<b>61.816</b>	<b>58.015</b>	<b>51.876</b>
IH1A-ERS/ES					77.729	<b>63.492</b>
IH2A-ERS/ES						77.619
IH3A-ERS/ES						—

each formulation, and in this case the differences are influenced by the amount of polymers. HPMC originates a more viscous gel than *iota*-CG, so the formulation with higher amount of HPMC (IH1A-ERS) controls better the ACV release than the formulation with lower amount of HPMC (IH3A-ERS). The same behaviour was observed when comparing IH1A-ERS/ES with IH3A-ERS/ES, since the HPMC/*iota*-CG 1.5/1 or 1/1.5 w/w ratio controls the drug release, obtaining  $f_2$  values lower than 65.

On the other hand, the comparison of the batches with ERS and the ones with ERS/ES does not reveal any similarity, as expected due to the nature of layer 2 (ERS/ES), which controls the release of ACV from these batches.

### 3.2.6. Mucoadhesion assessment

Fig. 6 shows the results for the mucoadhesion forces and mucoadhesion works for all the LbL films. It can be seen that all the formulations can bind to the vaginal mucosa, and in general there are no great differences between one batch and another, as confirmed by a previous study evaluating this combination of polymers (*iota*-CG/HPMC) which reported good mucoadhesiveness (Pacheco-Quito et al., 2020a). Due to its adhesion mechanisms mediated by hydrogen bonds, this mucoadhesiveness groups sulphate and other potential interactions (Cook and Brown, 2018). Layer 2 has an influence as a substrate in maintaining layer 1 adhered. One interesting finding is that the batches with proportions (1:1) of polymers have a slightly higher work of mucoadhesion than the other batches, possibly because they share the same mucoadhesion mechanisms (chemical bonds) in similar polymer concentrations.

Finally, these LbL films were assessed to determine whether they have the capacity to remain at the site of action to fulfil their therapeutic objective. The results for the *ex vivo* mucoadhesion residence time indicate that all films remain adhered to the mucosa for 6–10 days (Fig. 7).

These results were predictable since a previous study evaluating the mucoadhesive capacity of vaginal tablets formed by the *iota*-CG/HPMC combination revealed that the formulations remained adhered to the mucosa for long periods of time, and were not influenced by the polymer concentration (Pacheco-Quito et al., 2020a). The mucoadhesion periods were therefore expected to be very similar in this study. However, the results showed that the ability of the LbL films to remain adhered to the mucosa was influenced not only by layer 1, but also by the layer 2. The LbL films that remained adhered to the mucosa for the longest time were

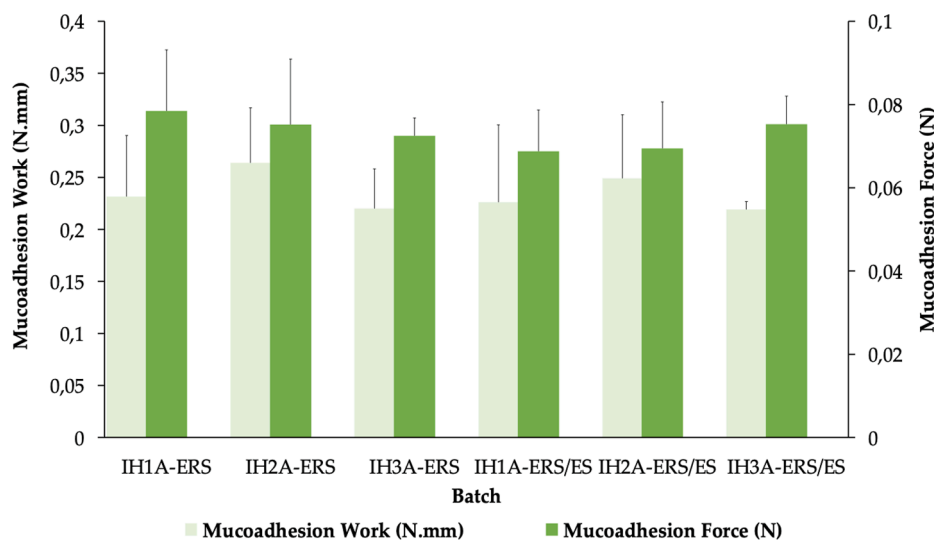


Fig. 6. Mucoadhesion work and force observed for the batches tested on bovine vaginal mucosa.

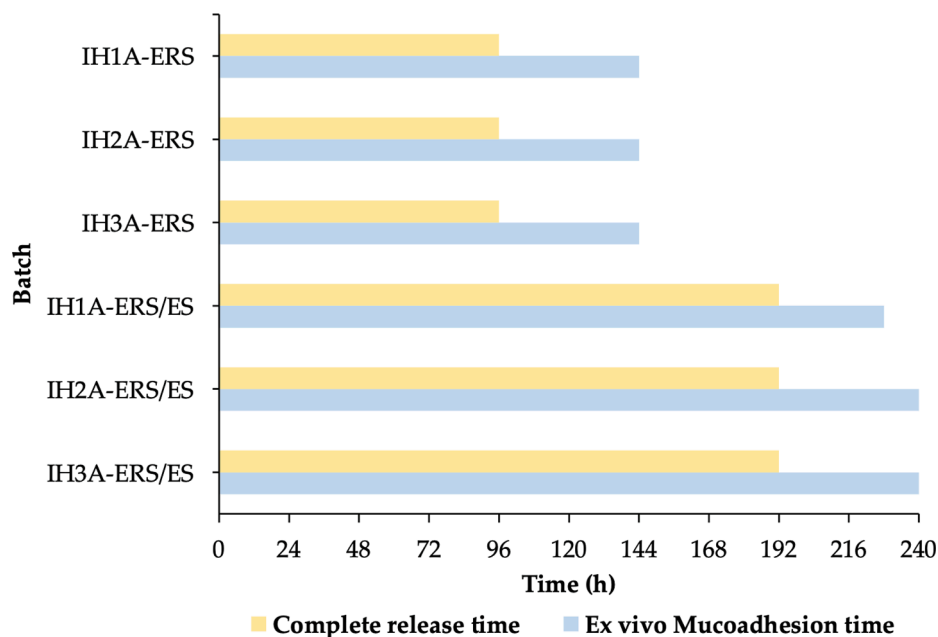


Fig. 7. Summary of the data obtained from ex vivo mucoadhesion residence time and drug release for each batch.

those whose layer 2 is formed by the combination of ERS/ES, possibly because these films have a certain hydrophobic character (Kumari et al., 2018) because ES is not soluble at vaginal pH, meaning that they take longer to erode. This is corroborated by the swelling tests, which show that the three batches had a lower swelling rate compared to the films with ERS only in the second layer. It is important to mention that the LbL films composed only of ERS in layer 2 remained adhered for up to six days, which is also corroborated by the swelling test, where it is seen that these films tend to swell more quickly and therefore their erosion is faster.

In all cases it was observed that the LbL films completely release the drug before the formulation is fully detached from the mucosa, which is a suitable characteristic for vaginal administration.

### 3.2.7. Cytotoxicity

To study cell toxicity, the raw materials were incubated in PBS 1x at 37 °C and 5 % CO<sub>2</sub> for 48 h before the assay to ensure that any potential toxic component would be present in the samples to be tested. The cell

culture was then treated with the suspension at different concentrations (maximum concentration of 1000 µg/mL). Experiments were performed in lymphoblastic (MT-2) and macrophage-monocyte (THP-1) derived cell lines to evaluate toxicity on the immune cells present in vaginal or uterine mucosae, and in a uterine epithelial cell line (HEC-1A) to assess the potential damage to the integrity of the mucosae.

As shown in Fig. 8 and Table 5, all the materials tested were non-toxic in the three cell types, even at the maximum concentration tested, with CC<sub>50</sub> values greater than 1000 µg/mL.

## 4. Conclusions

The combination of two types of layers in the development of vaginal films makes it possible to obtain suitable and effective formulations capable of controlling the release of ACV, thereby reducing the dosage frequency and avoiding possible adverse effects. Layer 1, made up of the combination of *iota*-CG/HPMC, produces a layer with a high mucoadhesive capacity which remains adhered to the mucosa for prolonged

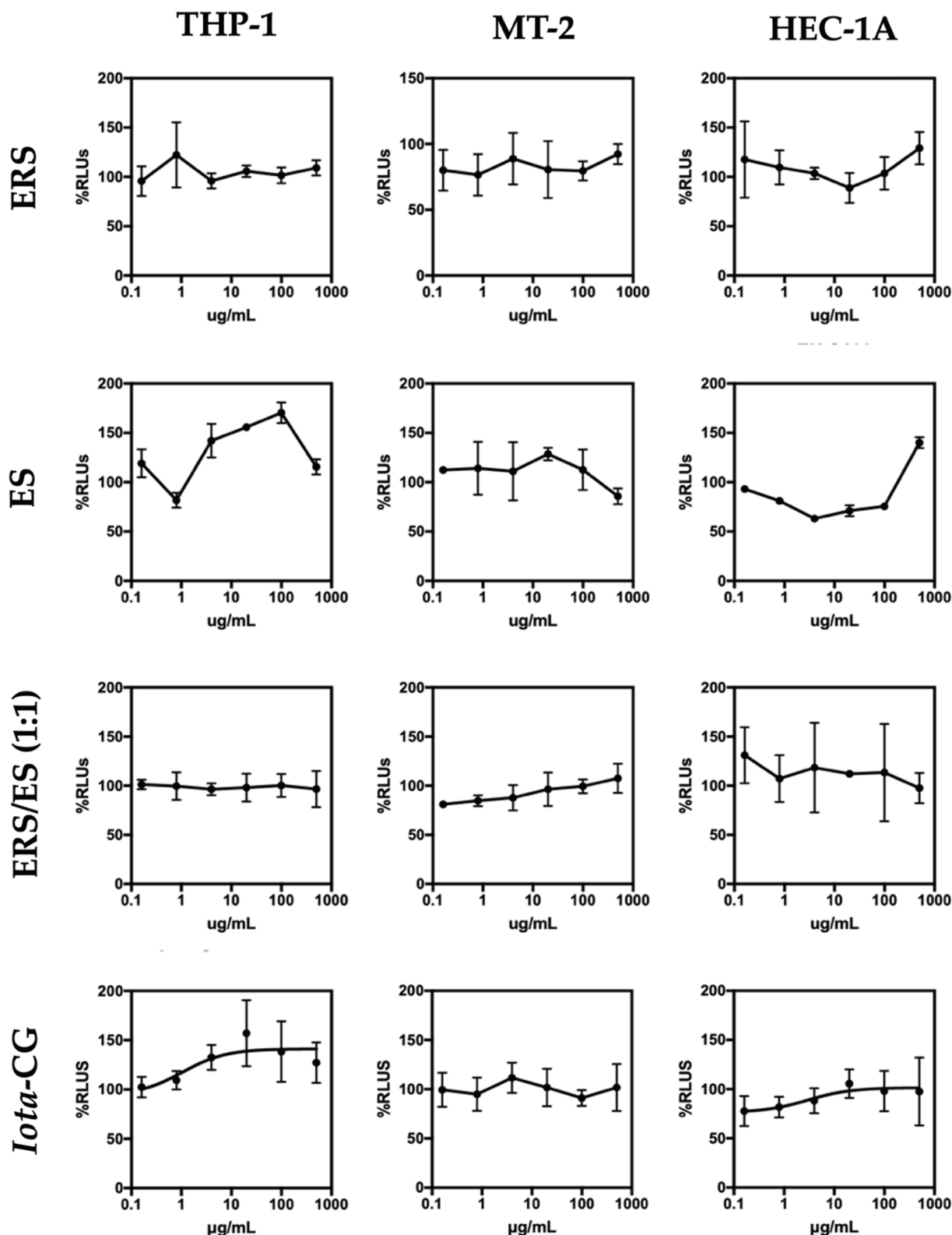


Fig. 8. Graphic representation of the cytotoxic evaluation of the film materials ERS, ES, ERS/ES (1:1) and *Iota-CG* in THP-1, MT-2 and HEC-1A cells. 100% shows the cell viability of the untreated (solvent vehicle treated) culture. Experiments were performed in triplicate.

periods of time, allowing the complete release of the drug. The second layer, developed from a combination of Eudragit®, forms a mixed layer that modulates the diffusion of the drug through it, producing the controlled release of ACV. This is due to the different behaviour of the two Eudragit® in simulated vaginal fluid, ERS shows low permeability

and ES is not soluble.

Of all the formulations tested, we can report that the LbL films with ERS/ES had the best performance, as they enable the controlled release of ACV and have good strength and elasticity, a moderate swelling profile and an adequate residence time on the mucosa. They would

**Table 5**

Results of the cytotoxicity analysis of ERS, ES, ERS/S and iota-CG in THP-1, MT-2 and HEC-1A cells. Cytotoxic concentrations 50 (CC<sub>50</sub>) were calculated using GraphPad Prism software.

Batch	CC <sub>50</sub> µg/mL (CI95%; R <sup>2</sup> )		
	TPH-1	MT-2	HEC-1A
Eudragit® RS PO	>1000	>1000	>1000
Eudragit® S100	>1000	>1000	>1000
Eudragit® RS PO/ Eudragit® S100 (1:1)	>1000	>1000	>1000
Iota-carrageenan	>1000	>1000	>1000

therefore be suitable candidates for future tests to allow new forms of total protection to be offered against genital herpes, and thus reduce the high prevalence of this infection, especially in women from sub-Saharan Africa.

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### CRediT authorship contribution statement

**Edisson-Mauricio Pacheco-Quito:** Formal analysis, Methodology, Investigation, Writing – original draft. **Luis-Miguel Bedoya:** Investigation, Methodology. **Juan Rubio:** Conceptualization, Methodology. **Aitana Tamayo:** Investigation, Methodology, Supervision, Writing – review & editing. **Roberto Ruiz-Caro:** Methodology, Supervision, Validation, Writing – review & editing. **María-Dolores Veiga:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

### Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: 'Maria Dolores Veiga reports financial support was provided by Spanish Ministry of Economy, Industry and Competitiveness. Maria Dolores Veiga reports financial support was provided by Spanish Research Agency and the European Regional Development Fund (AEI FEDER, UE).'

### Data availability

Data will be made available on request.

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