Research Article

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Therapeutic Delivery

Cross-linked hyaluronan films loaded with acetazolamide–cyclodextrin–triethanolamine complexes for glaucoma treatment

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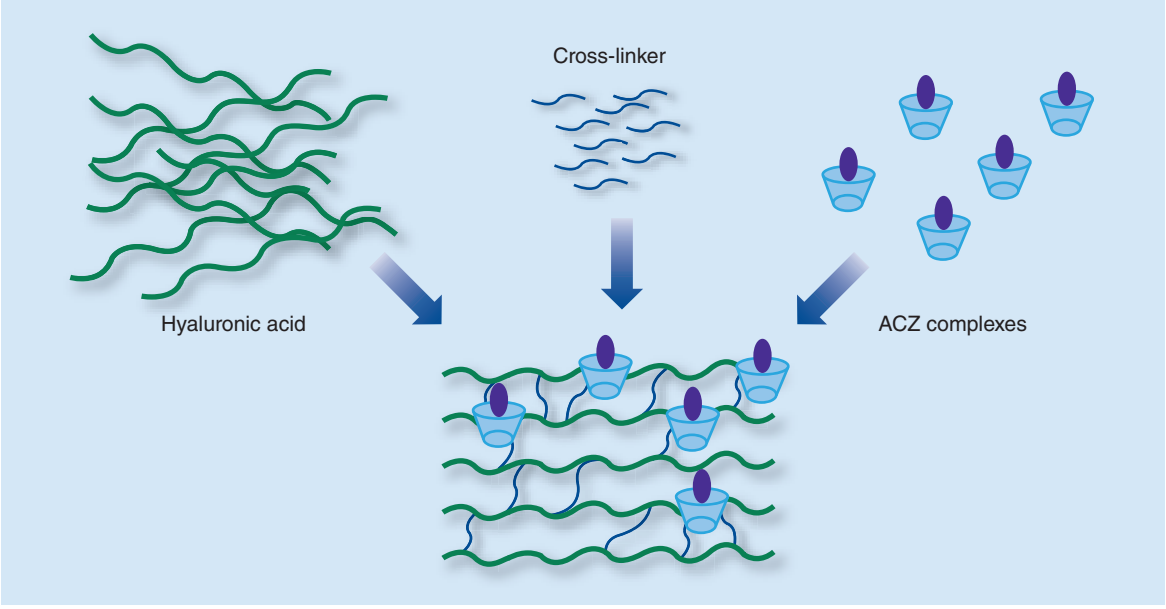
Aim: This work aimed to design and characterize cross-linked hyaluronic acid–itaconic acid films loaded with acetazolamide–hydroxypropyl β cyclodextrin–triethanolamine complexes. **Materials & methods:** Films were cross-linked with itaconic acid and poly(ethyleneglycol)-diglycidylether. Biopharmaceutical properties were assessed by evaluating *in vitro* drug release rate, biocompatibility in a human corneal epithelial cell line, bioadhesiveness with pig gastric mucin, *in vivo* bioadhesion and efficacy. **Results:** Showed good mechanical properties and oxygen permeability. Proliferation rate of corneal cells was affected by highest acetazolamide concentration. Bioadhesive interaction exhibited a water movement from pig mucin to the film; *in vivo* experiments showed strong bioadhesion for 8 h and hypotensive effect for almost 20 h. **Conclusion:** Experimental set showed promising performance and encouraged future studies to optimize formulation.



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Glaucoma includes a collection of neurodegenerative ophthalmic diseases with diverse clinical presentation whose common denominator, if left untreated, is progressive optic nerve atrophy eventually leading to irreversible blindness [1]. In 2013, near 64.3 million people (40–80 years) worldwide was estimated to be diagnosed with glaucoma, increasing to 76.0 million in 2020 and 111.8 million in 2040 [2]. It is the leading cause of global irreversible blindness and a major public health problem worldwide, demanding to health public services find better health policies and treatments.

The definition of glaucoma excludes any mention of intraocular pressure (IOP), although IOP remains an integral part of the diagnosis and management strategies in glaucoma [1]. The reduction of IOP by pharmaceutical treatments or surgical procedures has long been the standard treatment for glaucoma. A wide number of effective drugs like timolol maleate™ are available as eye drops. However, patient adherence to treatment can be poor due to occasional or frequent omitting and underestimation of defaulting level [3]. Thus, clinical efficacy of drugs could be compromised.

There are very efficient orally administrated drugs for IOP reduction, such as methazolamide or acetazolamide (ACZ) tablets. However, as result of the challenges involved with crossing the blood–retinal barrier, oral pharmaceutical forms have very low bioavailability in the eye structures. The topical administration of drugs to lower IOP remains the current standard for glaucoma treatment.

ACZ is a potent carbonic anhydrase reversible inhibitor, effective in controlling fluid secretion in the treatment of glaucoma [4]. To obtain the desired IOP reduction, large oral doses of ACZ must be administrated. Thus, a large number of side effects (due to its systemic distribution) such as diuresis, fatigue, electrolyte imbalance, systemic acidosis and dyscrasias in some severe cases, are generated.

The adverse systemic side effects of ACZ could be avoided if it was administered topically in the eye. However, its poor aqueous solubility (0.7 mg/ml) and low corneal permeability (4.1×10^{-6} cm/s) limit its ocular bioavailability [5]. In the past, attempts to improve residence time by using ACZ soaked contact lenses or mucoadhesive polymer gels were reported [6,7]. A few strategies to increase the solubility of this drug by using liposomes, cyclodextrins (CD) or liquid crystals have been also reported [8–10]. Recently, Granero *et al.* reported the significant increase in the aqueous solubility of ACZ by the simultaneous complexation and salt formation with triethanolamine (TEA) [11]. Nevertheless, a combined approach, combing both strategies seems to not be studied.

The need for maintaining sterility, and ensuring stability and security of the formulation throughout the treatment period still represent a big challenge to be overcome. Benzalkonium chloride is one of the most commonly used ophthalmic preservative drugs, well known for its permeability enhancer properties [12]. However, there is a growing interest to avoid its presence in ophthalmic formulations because of several side effects discovered in patients [13]. Preservative-free single-dose containers appear to be the best alternative; nevertheless, the market cost of these formulations amply exceeds that of conventional dose formulations.

Some of the challenges previously described are closely related to liquid dosage formulations and could be overcome by using new drug-delivery systems. Combining CD ability for solubilize many lipophilic water-insoluble drugs with structurally modified hyaluronic acid films could lead to an innovative solid, preservative-free, bioadhesive, oligosaccharide-based drug delivery platform for ACZ ocular topical administration (ACZ-loaded films). Polymer-based solid systems are capable of keeping the therapeutic agent attached to the mucosal surfaces of the eye like the conjunctiva, and previous sterilization could avoid preservative issues.

In the last 10 years, several attempts to produce polymer drug-delivery systems for ophthalmic route have been reported in the scientific literature, including synthetic [14–17], semisynthetic [18], natural [19–21] or combined polymers [22]. However, most of them are still formulated as liquid pharmaceutical dosage forms.

Reported studies of solid biocompatible ocular platforms were often carried on with water-soluble molecules such as, brimonidine tartrate, timolol maleate, pilocarpine nitrate [23–25]. Only, few studies with similar approaches were reported [26,27].

Present work aims to obtain a novel drug delivery platform able to gather desirable properties such as, lipophilic drug vehiculation, bioadhesion, absence of preservatives, biocompatibility and effectiveness in a single topical ocular drug formulation by combining a natural oligosaccharide such as HPβCD with a natural polymer such as hyaluronic acid (HA), and study its performance in a wide experimental set.

Materials & methods

Materials

HA sodium salt (Mw: 1,560,000 Da) was acquired from Parafarm (Buenos Aires, Argentina). HPβCD (Mw: 1325 Da; molar substitution: 7.0) was a gift from Ferroment S.A. (Roquette representative in Argentina). ACZ, TEA (98%), poly (ethylene glycol) diglycidylether (PEGD) (average Mn: 500) and 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) were acquired from Sigma-Aldrich Corp. (MO, USA). Penicillin, streptomycin, epidermal growth factor, insulin, foetal bovine serum, phenol red-free Roswell Park Memorial Institute medium and Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12) were supplied by Invitrogen-Gibco (Inchinnan, UK). AlamarBlue® reagent (resazurin) was purchased from AbD Serotec (Oxford, UK). Cell culture plates were from Nunc (Roskilde, Denmark). Pig gastric mucin (PGM) was purchased from Sigma (MO, USA) (Mw not reported by the supplier, product code: M1778) and was used without further purification. All other chemicals involved were of extra pure grade and used as received.

ACZ ternary inclusion complex preparation by freeze-drying

Preparation of the solid multicomponent complex ACZ–hydroxypropyl beta cyclodextrin–TEA (ACZ:HPβCD:TEA) at a 1:1:1 molar ratio was obtained by the freeze-drying method, previously described [28]. ACZ, HPβCD and TEA were weighed, dissolved in distilled water and stirred for 24 h. Then, solution was filtrated and frozen overnight before being lyophilized for 30 h by using a Labconco Freeze Dry System (KCK, USA). The ACZ inclusion complex with TEA was prepared by dissolving equimolar amounts of ACZ and TEA in ethanol. Then, ethanol was removed in a vacuum after ultrasound treatment for 1 h.

Film synthesis

Hyaluronic acid–itaconic acid films were synthesized from HA/IT/PEGD solutions using twice-distilled water as solvent. Reagents amount was adjusted to achieve (1:1:2) molar ratios and 2% (w/w) HA concentration. The reaction was done following a previously disclosed homogeneous cross-linking method [21]. Briefly, IT was dissolved in 80 % of total twice-distilled water, followed of HA addition. Solution was stirred until homogenous gel like solution was observed. Then, PEGDE (in 10% of total water) was added and let for 24-h reaction time under slight stirring at room temperature (RT). ACZ-CD-TEA complexes were used to incorporate lipophilic ACZ molecules into reacting solution. Thus, ACZ-CD-TEA complexes were incorporated (in 10% of total water) into the HA films during the cross-linking process to achieve a 0.3% w/w ACZ concentration (graphical abstract). Finally, cross-linked gels were cast at RT under an extraction hood in 7.0 cm diameter circular matrices. Different size and shape samples were cut for film characterization (for *in vivo* assays, the films were cut into 4 mm diameter disks). Resulting films were transparent but blurry, with a smooth surface and mean thickness of 155 ± 14 μm.

Physicochemical properties

Swelling

The swelling properties of ACZ-loaded films were analyzed at RT using distilled water as swelling medium. Disc samples of 7.45 mm diameter were dried until constant weight was achieved and then immersed in distilled water. Swelling ratio (SR) was calculated for three independent experiments in triplicate as weight or diameter increase of tested films by using the equation: SR = We/Wd, where We is the weight/diameter of the sample at equilibrium, and Wd is the weight/diameter of the dried sample.

Stress strain

Stress–strain behavior of the systems was studied in 1 × 6 cm rectangular strips of cross-linked ACZ-loaded films, cross-linked unloaded and unmodified unloaded films. Samples were carefully cut to avoid any imperfection on the sides, which could have originated a breakpoint, leading to premature break of the strips while stretching.

Ten samples in duplicate for each of the three films previously described were tested in an Instron 3369 tester (MA, USA) in traction mode at 2 mm/min at RT (23°C).

Fourier transform infrared spectroscopy in attenuated total reflectance

In order to confirm the presence of ACZ in the films, disk surfaces were analyzed by Fourier transform infrared spectroscopy in attenuated total reflectance mode (FTIR-ATR) with a Nicolet 5-SXC spectrometer (Thermo Fisher Scientific, MA, USA) using a ZnSe crystal with an incidence angle of 45 degrees. Different clean areas of five

samples were analyzed to confirm the homogeneity of each surface. All spectra represent the average of 42 scans recorded at 4 cm^{-1} resolution in a 4000–400 cm^{-1} range, using air as background.

x-ray diffraction, x-ray fluorescence & scanning electron microscopy

x-ray diffraction (XRD) and x-ray fluorescence spectrometry (XRF) studies were performed in cross-linked unloaded and ACZ-loaded film samples using a Philips PW1710 x-ray diffractometer and a Philips MagiX spectrometer (Amsterdam, The Netherlands), respectively. HA, IT, and ACZ powders were used as controls. XRD was performed from 2 to 70 degrees 2θ (diffraction angle) using a copper tube anode. After XRF, further semiquantitative analysis was performed by using IQ^+ standardless software from PANalytical (Almelo, The Netherlands).

Surface morphology of films was observed by scanning electron microscope (SEM) imaging using a Carl Zeiss AG Leo EVO-40XVP microscope (Oberkochen, Germany). Samples were swelled in distilled water for 1 h at RT and subsequently freeze dried, fractured and coated with Au in a PELCO 91000 sputter coater (CA, USA) before observation.

Oxygen permeability

The permeability to oxygen of films was determined by using a Mocon Inc. (MN, USA) Ox-Tran 2/21 oxygen transmission rate testing device at 50% relative humidity and RT. An open area of 0.5 cm^2 of masked films was exposed on one side to nitrogen (carrier gas) and the other side to oxygen (test gas). Both flowing gases were automatically set to the same working temperature. At least four tests were performed to each sample.

In vitro biocompatibility study

In vitro biocompatibility of developed platforms was assessed by viability and proliferation studies using the Araki-Sasaki SV40-immortalized human corneal epithelial (HCE) cell line derived from human corneal epithelial cells [29], at passages 30–35. Cells were cultured in DMEM/F-12 culture medium supplemented with 15% foetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 ng/ml epidermal growth factor, 0.5% DMSO and 5 μg/ml insulin, at 37°C in a 5% CO_2/95% air atmosphere. Media were changed every other day, and daily observation of cultures was made by phase contrast microscopy. Samples assessed in cell cultures were previously sterilized by immersion in 70% ethanol [21].

Possible alterations in the cell proliferation rate induced by ACZ-loaded film exposure were measured by using the alamarBlue® assay. HCE cells were seeded in 24-well plates (40,000 cells/well) and grown for 24 h. Cells were then exposed to ACZ-loaded films with three different ACZ concentrations (0.1, 0.5 and 1 mg/ml), by introducing different ACZ-loaded films weights (16.7, 83, 3 and 166.7 mg respectively), for 24 h (ACZ concentration was considered total ACZ load of films). Afterward, films and medium were removed and cells incubated in 10% v/v alamarBlue in culture medium solution at 37°C for 4 h. alamarBluesolution was extracted and 100 ml aliquots were transferred into a 96-well plate for fluorescence measurement at 560 nm (excitation) and 590 nm (emission) wavelengths in a SpectraMax® M5 (Molecular Devices, CA, USA) fluorescence microplate reader. Cells were allowed to grow for additional 48 h, and the above-described procedure was repeated every 24 h. Unexposed cells were used as controls. Proliferation index was determined after 24, 48 and 72 h as a percentage by referring to control cells. Three independent experiments were performed in duplicate. Additionally, cells were observed and microphotographs taken at 24, 48 and 72 h after exposure using a Nikon Eclipse TS100 microscope (Tokyo, Japan) to evaluate morphological alterations.

Potential cytotoxic side effects of the formulations were assessed with the XTT test. HCE cells were seeded in 24-well plates (60,000 cells/well) and grown for 24 h. Cells were exposed to ACZ-loaded films using the same concentrations used for the proliferation test; 24 h later, XTT solution in phenol red-free Roswell Park Memorial Institute was added immediately after film removal and cells were incubated at 37°C for 15 h. Plates were read at 450 nm (reference wavelength: 620 nm). Unexposed cells and cells exposed to 0.001% benzalkonium chloride were used as controls. Cell viability was calculated as a percentage with regard to unexposed control cells. Each test was performed three-times in quintuplicate.

In vitro drug release

ACZ *in vitro* release rate was assayed in a specific device designed for studying release kinetics in small or low dosage pharmaceutical forms, currently under patenting process (Argentinean INPI patent pending, expedient: 20130100220) [30]. It is composed of six small containers. Each container can hold the sample and a small volume

of medium; the medium is mechanically stirred and the temperature is controlled by an external water bath. Tubes connected to each container collect the samples at desired times.

Three samples in triplicate of ACZ-loaded films (~20 mg) were immersed in 10 ml of degasified Ringer's solution as medium at 36°C (highest temperature measured on closed eyes) [31] under stirring. Samples were collected at 5, 10, 15, 30, 45, 60, 75, 90, 105, 120, 150, 180 and 240 min with fresh medium reposition and immediately diluted, filtered, and quantified by HPLC.

The HPLC system consisted of a Jasco chromatograph (OK, USA) equipped with a Jasco multiple wavelength ultraviolet-visible detector (Jasco UV-2077 Plus) and ChemStation software version ChromNAV. Chromatographic separations were performed on a Restek RP C18 column (250 mm x 4.6 mm i.d. filled with 5 µm particles; PA, USA). The UV-detector was set at 254 nm. HPLC measurements were performed using isocratic conditions. The mobile phase, at 1 ml/min flow rate, consisted of a mixture of water pH 4/acetonitrile/methanol (95:3:2 v/v/v), which was filtered through a 0.45 mm Millipore membrane and degassed prior to use. The column was thermostated at 40°C.

ACZ-loaded film-mucin bioadhesive interaction

Assessment of bioadhesive interaction between ACZ-loaded films and mucin was performed as follows. A piece of the ACZ-loaded film of approximately 0.5 cm x 0.5 cm was placed in 10 ml of a hydrated PGM solution in pH 7.4 PBS buffer at a concentration of 0.1 mg/ml, and allowed to equilibrate at RT for 24 h. Same solution was left at RT for 24 h and used as control. Under those conditions, PGM becomes into a material with elastic dominant gel like properties. Then, samples of the ACZ-loaded film and the ACZ-loaded film brought into contact with PGM were placed on a double-contact carbon tape, drying by vacuum and subsequently sputtered with gold before imaging in an SEM. Also, drops of the resultant mucin gels, in or without contact with ACZ-loaded films, were dried by vacuum and subsequently sputtered with gold before imaging in the SEM. Images were done using an accelerating voltage of 3 kV and the secondary electron detector on an electronic microscope.

Determination of IOP & bioadhesion in animals

Determination of IOP was performed on both eyes of nonsedated normotensive male New Zealand white rabbits (2–2.5 kg). White rabbits used in IOP measurements are usually chosen in this type of work because of their anatomical and physiological similarity to the human eye. In this way, the results could be carefully extrapolated.

Formulations were tested in ten animals (n = 20 eyes) with five animals (n = 10 eyes) as control. Animals were maintained with free access to water and food, in a controlled 12/12 h light/dark cycle in individual cages. Animal management procedures were conformed to the Association for Research in Vision and Ophthalmology resolution on the use of animals in research, the European Communities Council Directive (86/609/EEC), and the Institutional Care and Use Committee of the Chemistry Faculty of National University of Córdoba, reviewed and approved the protocols.

IOP was measured with an Icare, TonoVet rebound tonometer (Helsinki, Finland). This device allows IOP measurement without the need of topical anesthesia. IOP was set at 100% with two basal measurements taken to each eye 30 min before and immediately before 4 mm disc diameter ACZ-loaded film application. Then, a film was applied to both eyes. IOP measurements were performed once every hour for 8 h. Saline solution was used as control. The administration protocol included a washout period of at least 48 h between experiments.

In vivo bioadhesion experiments were performed by placing a 4-mm disc diameter ACZ-loaded film in the conjunctival fornix of each eye of six rabbits. Changes in adhesion intensity were observed by means of a Neitz IQ small pupils binocular indirect ophthalmoscope (Tokyo, Japan) and 20-diopter lens (Nikon, Tokyo, Japan). The magnitude of these changes was quantified according to the following detailed score: 0 = Spontaneously moved out of the eye; 1= Maintained in the fornix, but did not adhere to the palpebral or bulbar conjunctiva permanently; 2 = Remained in the fornix and attached to a particular conjunctive even when carrying out maneuvers on the eyelid; 3 = As previous but in this case the film remained attached even when rubbing manoeuvres took place on the palpebral; 4 = Remained attached even when trying to move it with a spatula.

Statistical analysis

Experimental results were expressed as the mean ± standard error of the mean. Differences were calculated by Student's *t*-test and considered to be significant when $p \leq 0.05$.

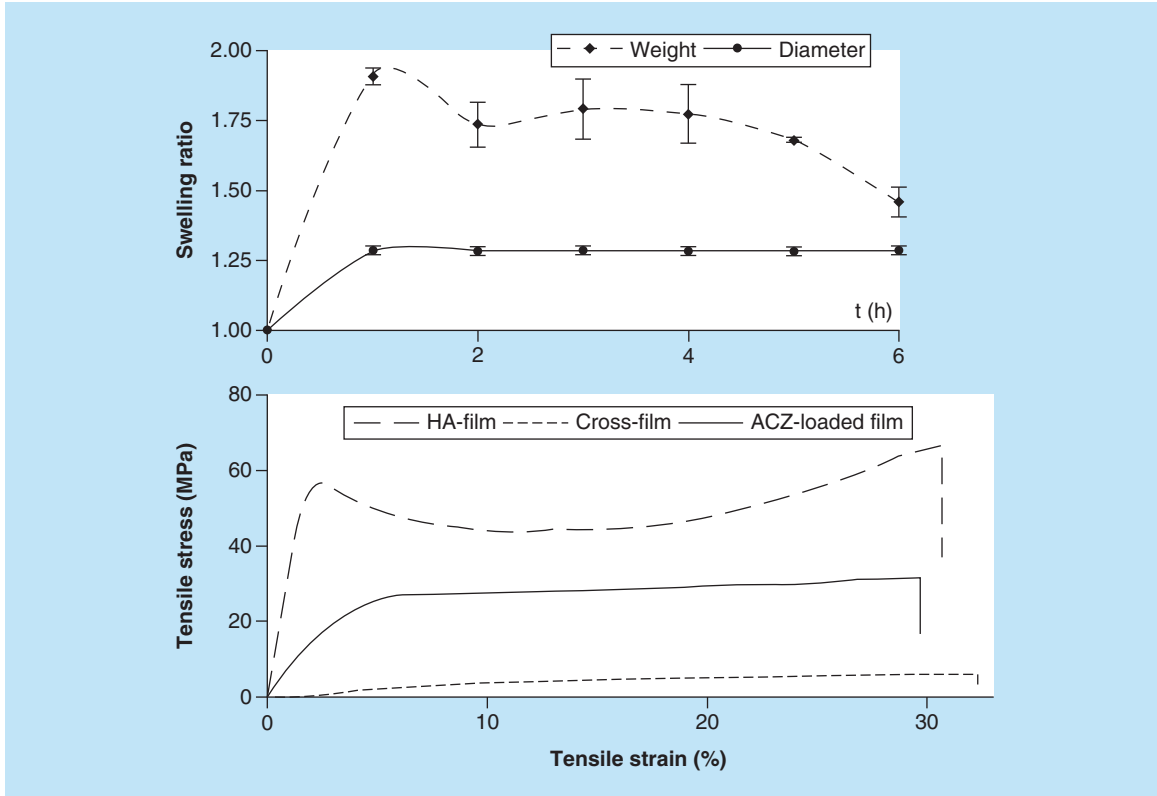

Figure 1. Swelling – tensile stress. (Top) Swelling assay of ACZ-loaded films measured in terms of weight and diameter (n = 3). ACZ-loaded films showed small changes in size and weight; suggesting successful cross-linking reaction. **(Bottom)** Stress–strain test at RT of ACZ-loaded films, Cross-film (unloaded cross-linked films), and HA-film (unloaded and noncross-linked films) (n =10). Modified HA films maintained plastic deformation after cross-linking due to the flexible PEG chains present in PEGD. ACZ-CD- did not affect ductility, but tensile stress and remarked the elastic to plastic transition.
ACZ: Acetazolamide; CD: Cyclodextrin; HA: Hyaluronic acid; PEGD: Poly (ethylene glycol) diglycidylether; RT: Room temperature.

Results & discussion

Swelling

The swelling behavior of a polymer provides information related to the cross-linking degree of the material; thus, poorly cross-linked polymers will have a little less restriction of movement and therefore greater swelling.

ACZ-loaded films showed high stability in an aqueous environment with small changes in size and weight. SR in terms of weight showed a lower than two-fold increase over the whole experiment, showing oscillation and a maximum peak at 1 h. SR in terms of size was constant after 6 h, showing a slight diameter increase (Figure 1.1). These findings suggest that the cross-linking chemical reaction was successful in avoiding immediate dissolution of unmodified HA films in water. PEGD and IT were efficient HA cross-linkers providing a highly stable material, but not strong enough to totally prevent swelling.

Generally, the process by which fluids enter a system is diffusion, resulting from random molecular motion and interaction of existing forces between the fluid and the polymer. This mechanism is considered in wetting theory, one of the theories postulated to explain the phenomenon of bioadhesion.

Bioadhesive interactions between polymers and the mucin lining living tissues require intimate contact between the two surfaces, and the presence of fluids. The latter is essential for swelling of the polymer to occur. Thus, chain mobility is increased, facilitating interpenetration with the mucus layer [32].

Similar findings were found for other polymers formulations. Longer swelling studies were conducted with casted chitosan films [23], showing initial swelling index of 75%, followed by stable period of 2 days until reduction, probably due to polymer dissolution.

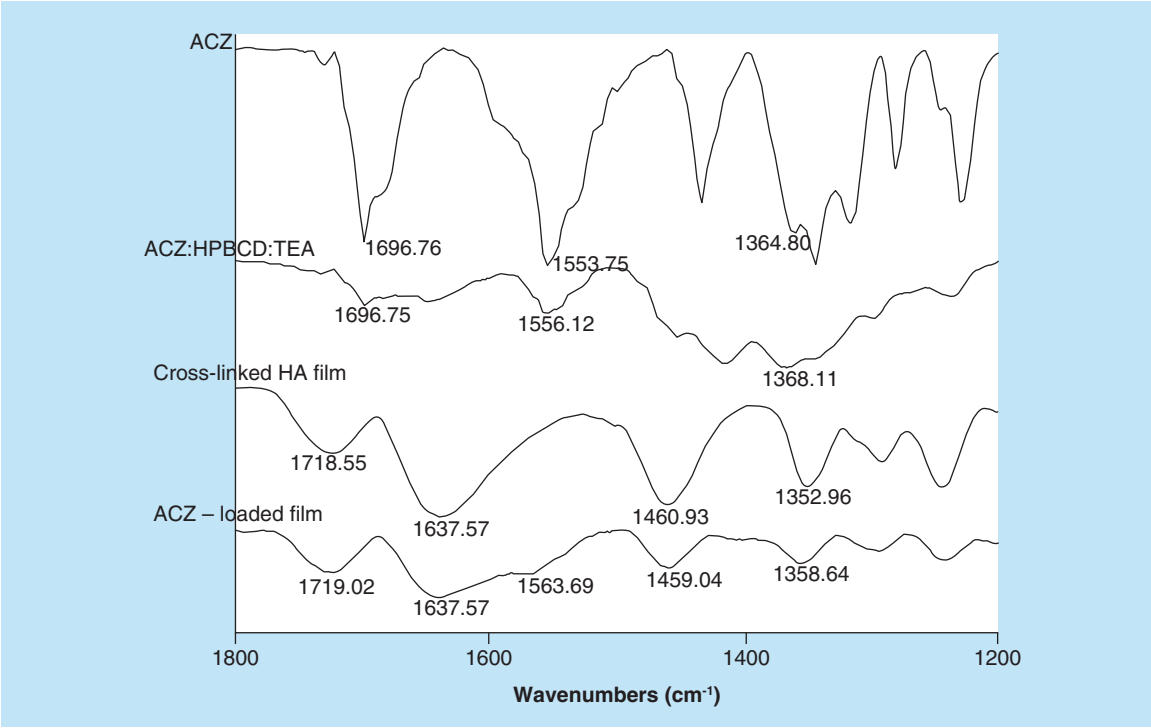

Figure 2. Fourier transform infrared spectroscopy in attenuated total reflectance mode spectra of acetazolamide formulations and unloaded/cross-linked HA film. New peak at 1564 cm^{-1} in ACZ-loaded film spectrum and the peak at 1358 cm^{-1}, plus band located at 1352 cm^{-1} (carbonyl group of the amide III of cross-linked HA film), suggest the presence of ACZ on the surface of the ACZ-loaded film. The absence of band located at 1696 cm^{-1}, (C = O group of the ester of ACZ), might suggest interaction with the HA film and carbonamide group of ACZ.
ACZ: Acetazolamide; HA: Hyaluronic acid; TEA: Triethanolamine.

Stress strain

Stress–strain experiments (Figure 1 [bottom]) showed differences in the tensile behavior of the three samples. All samples showed plastic deformation. However, the yield point of each sample was reached at a different tensile stress value. Unmodified unloaded films (HA-film) showed the sharpest change from elastic to plastic deformation and the highest tensile stress. In contrast, both cross-linked samples exhibited smooth yield points with lower tensile stress values. The cross-linked unloaded film (cross-film) showed the lowest values.

Despite cross-linking processes usually leading to an increase in material stiffness as a consequence of the restriction of polymer chain movements, the structure of PEGD with long and flexible PEG chains can reduce these effects. Thus, PEGD can be used as cross-linker agent for natural polymers like HA without missing intrinsic ductility as previously disclosed [21].

The presence of the ACZ–CD–TEA inclusion complexes in loaded films did not affect HA film ductility, which was expected considering the low drug presence in the film. However, the presence of the ACZ complexes affected the tensile stress and remarked the elastic to plastic transition, bringing the ACZ film tensile behavior closer to that exhibited by unmodified unloaded films.

Fourier transform infrared spectroscopy in attenuated total reflectance

Figure 2 shows the FTIR-ATR spectra of the unloaded cross-linked HA film and ACZ formulations. Here, it is possible to observe the presence of the peak at 1719 cm^{-1}, assigned to the carbonyl group of the ACZ amide group. Also, absorption peaks were found at 1637 and 1459 cm^{1}, attributable to the symmetric and asymmetric stretching vibration bands of carbonyl groups of the carbonamide and sulphonamide groups, respectively [33].

The appearance of a new peak at 1564 cm^{-1} in the FTIR-ATR spectrum of the ACZ-loaded film and the peak at 1358 cm^{-1}, which might be mixing of the band at 1364 cm^{-1} corresponding to the $\nu(SO_2)$ stretching mode of ACZ [28], plus the band located at 1352 cm^{-1} attributed to the carbonyl group of the amide III of the cross-linked HA film [34], suggest the presence of ACZ on the surface of the ACZ-loaded HA film. In addition, the absence of

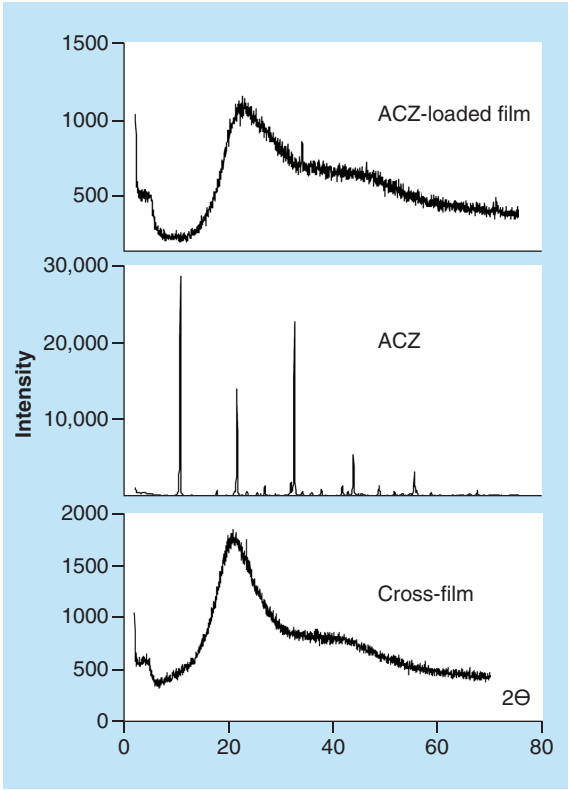

Figure 3. x-ray diffraction of acetazolamide-loaded films, cross-film (unloaded cross-linked films) and acetazolamide (powder). No crystallographic differences between cross-linked ACZ-loaded and unloaded films, except for the presence of a weak peak at 30° 2θ were observed.
ACZ: Acetazolamide.

the band located at 1696 cm^{-1}, assigned to the C=O group of the ester of ACZ, might suggest that the carbonamide group of ACZ is involved in the interaction with the HA film.

X-ray diffraction, x-ray fluorescence & scanning electron microscopy

XRD did not show crystallographic differences between cross-linked ACZ-loaded and unloaded films, except for the presence of a weak peak at 30° 2θ. Thus, the large presence of amorphous polymers like HA could be masking the presence of all other substances and suggest that crystalline drug was partially converted to an amorphous state when incorporated into HA polymeric matrix (Figure 3). Literature reports similar effects for other drug/polymer combinations, such as brimonidine/chitosan [23], timolol/chitosan [35], hydrocortisone/PLGA, prednisone/PLGA [36].

Nevertheless, the much more sensitive XRF technique showed increased intensity for sulphur atoms present in the ACZ structure for ACZ-loaded films, confirming the ACZ presence in the films. The intensity of S atoms in ACZ-loaded films was 0.33% higher than that measured in the cross-linked unloaded formulations.

General aspect of ACZ-loaded films was transparent but blurry with a smooth surface, suggesting homogeneous ACZ distribution into the film. On the contrary, attempts to load ACZ into cross-linked films without complexing leaded to nonhomogeneous and irregular films (data not shown).

SEM images (Figure 4) of ACZ-loaded films showed a compact appearance with a low presence of pores on exposed surfaces after cryofracture. Nonfractured surfaces showed a smooth and homogeneous morphology. These findings correlated with a cross-linked material with limited swelling.

Oxygen permeability

The permeability to oxygen is an important parameter to know in topically applied ocular devices. Materials used in these devices must exhibit a good permeability to oxygen to prevent hypoxia-induced problems. The oxygen transmissibility (Dk/t) is a parameter commonly studied in contact lenses. Holden and Mertz [37] predicted that contact lenses under daily wear conditions should provide a minimum Dk/t of 34 Barrer/cm, whereas this value would increase upto 87 Barrer/cm to prevent corneal hypoxia and thus limit corneal oedema to physiological levels. Studied samples exhibited good permeability to oxygen, with a measured mean of 56.00 ± 8.30 $cm^3.mm/m^2.day.atm$ (equivalent to 82.80 ± 12.27 Barrer/cm).

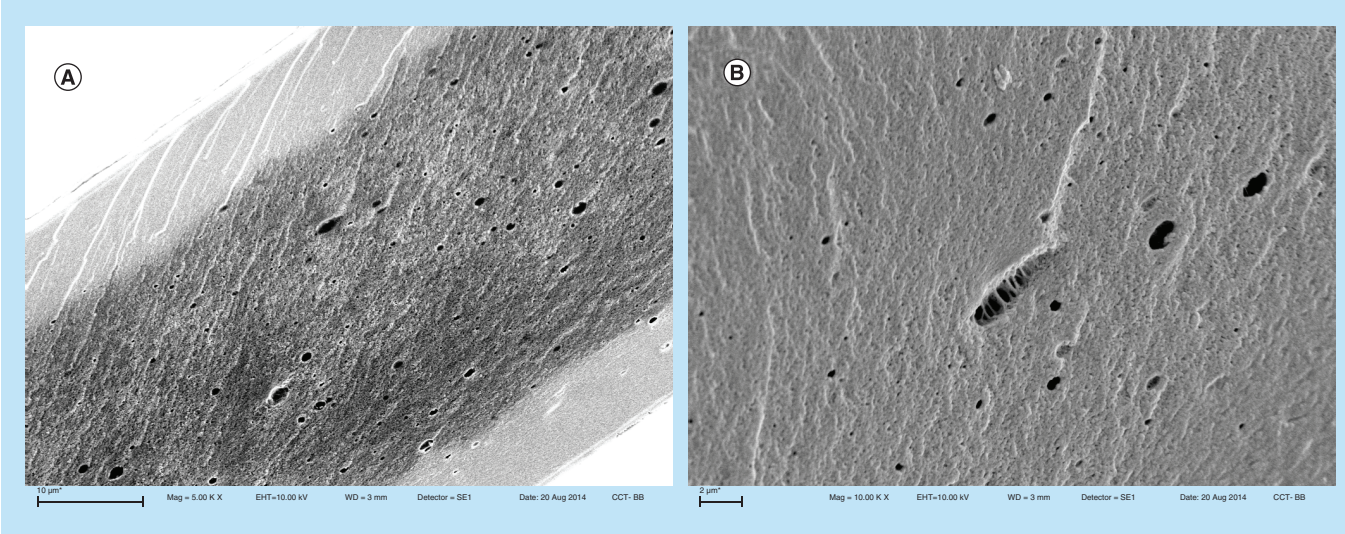

Figure 4. Scanning electron microscope images of the exposed surface of acetazolamide-loaded films after cryofracture. (A) 5000×; **(B)** 10,000×. Acetazolamide-loaded films exhibited compact appearance with low presence of pores on surfaces revealed by cryofracture. Nonfractured surfaces presented smooth and homogeneous morphology.

In vitro biocompatibility

In vitro studies focused on materials and living tissue or cell culture interactions appear as a crucial tool to design novel materials with biomedical applications, including drug delivery systems. Some reports in the scientific literature refer to similar techniques for HA cross-linking [34,38]. As far as we know, there is just one report showing no ocular toxicity of HA films cross-linked with PEGD and IT [21]. Nevertheless, we considered it important to study the biocompatibility of the novel platform as novel compounds such as HPβCD and TEA are included in the proposed film.

Our *in vitro* biocompatibility studies (Figure 5 [top]) revealed no differences in HCE cell viability after 24 h of exposure to sterilized ACZ-loaded films. All conditions showed the same cell viability percentages as those observed in unexposed control cells. Indeed, morphological characteristics of exposed cells remained without alterations (Figure 5 [middle]). On the other hand, cell proliferation rate (Figure 5 [bottom]) experienced a significant reduction after exposure to the higher concentration of ACZ (1 mg/ml). Both 0.5 and 0.1 mg/ml concentrations shown to be safe in any of the biocompatibility tests.

In vitro drug release

Figure 6 shows ACZ release from the loaded HA films. ACZ release was found to be biphasic, with an initial burst release in the first 15 min (~80%), followed by sustained release. The initial burst release may be due to the complex adsorbed on the film surface. This observation is in agreement with the previous results obtained by FTIR-ATR and XRF [28]. After 15 min, release of the remaining drug in the film was constant during all the experiments due to the decreased drug concentration in the film, thereby reducing the concentration gradient and hence the drug release. The initial rapid release is commonly observed in polymer formulations [39–41], and may have a functional use by providing an attack dose during drug delivery, reducing any lag period. Same phenomenon was described for HA films functionalized with β-CD [42]. Moreover, in a topical eye drop formulation, the drug is quickly released and completes clearance from the eye within few minutes, in contrast, the biofilm allows enhancing the precorneal drug residence time and subsequent diffusion of drug molecules into the cornea, because of a greater initial driving force for ACZ diffusion due to the larger concentration gradient, but this becomes less significant over the course of the release. This mechanism is in agreement with the remarkable decrease of IOP in normotensive rabbits over the time, which reached the lowest point 6 h after the biofilm administration.

ACZ-loaded film-mucin bioadhesive interaction

A strategy to increase the drug transport into ocular tissues has been to utilize mucoadhesive formulations that enhance the bioavailability of the drug in the immobilized mucin layer by increasing the drug residence time on

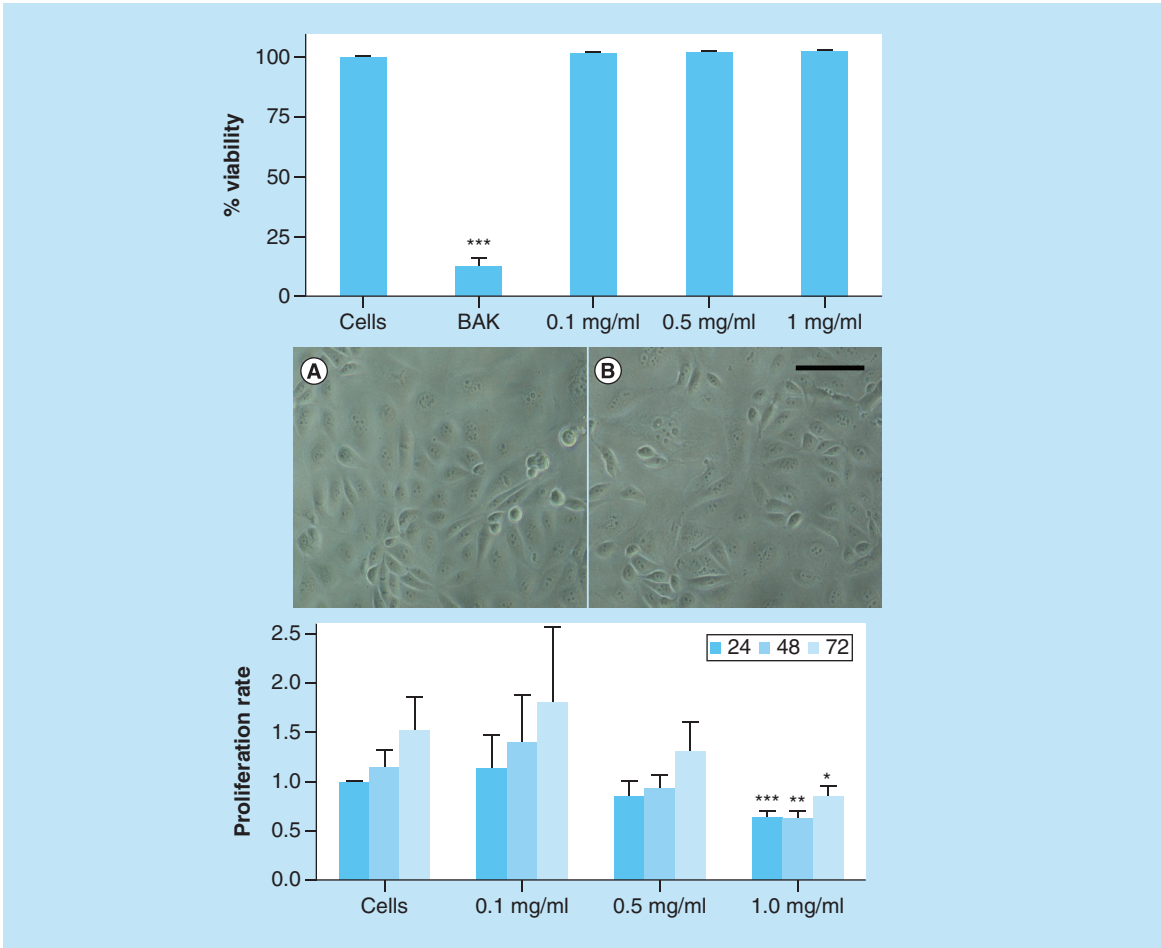

Figure 5. ***In vitro* biocompatibility experiments. (Top)** Cell viability of human corneal epithelial (HCE) cells after exposure to acetazolamide-loaded films for 24 h. ***$p \leq 0.001$ ($n = 3$). No differences in HCE cell viability after 24 h of exposure to sterilized acetazolamide (ACZ)-loaded films were observed. **(Middle)** Phase contrast microscopy image of HCE cells (20×). **(A)** Unexposed cells; **(B)** ACZ-loaded film-exposed cells. Scale bar = 50 μm. Morphological details of exposed cells remained intact with no appreciable alterations. **(Bottom)** Proliferation rate of HCE cells after 24 h exposure to films in relation to basal level ($n = 3$). Significant differences were determined between exposed and unexposed cells at each time point. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$. Significant reduction in cells proliferation was observed after exposure to the higher concentration of ACZ (1 mg/ml).

the ocular surface. Mucins are glycosylated polymers, secreted by surface epithelial cells that form a matrix of fibers entangled and joined together by multiple weak noncovalent bonds such as hydrogen bonding and electrostatic interactions, forming a fully hydrated viscoelastic gel layer.

In order to explore the mucoadhesive behavior of ACZ-loaded films, SEM was used to study the changes that occurred with PGM particles brought into contact with ACZ-loaded film.

Herein, assays were performed using PGM as mucins of different origins have similar structural and functional properties [43]. Also, samples for SEM images were air-drying without any drying steps to observe PGM in a hydrated state, to mimic the physiological state of ocular mucins.

Figure 7A shows hydrated PGM images formed voluminous and highly viscoelastic fibers spread on the surface. Fibers of mucin also appear expanded and occupy the entire surface of the gel layer. Figure 7B displays SEM images of the surface of the ACZ-loaded film without brought into contact with PGM. As shown in the 7B image, the film showed homogeneous with a relatively smooth surface. However, SEM images of the ACZ-loaded film brought into contact with PGM (Figure 7C & D) displayed that the surface of the film is heterogeneous. Therefore, we speculate that this effect might be due to adsorption of fibers of PGM to the film surface coating it. On the other hand, crystal, probably of ACZ molecules, could be distinguished on the film surface brought into contact with

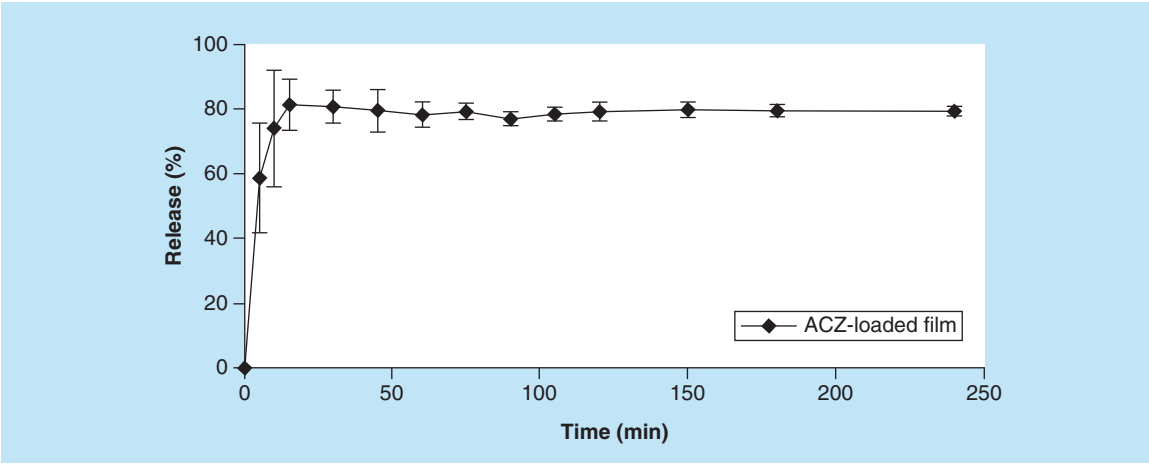

Figure 6. *In vitro* release assay of acetazolamide from acetazolamide-loaded films in Ringer's solution at 36°C (n = 3). Initial burst release in the first 15 min, followed by sustained release was observed.
ACZ: Acetazolamide.

PGM (Figure 7D), which could indicate that mucoadhesive interactions may play a significant role in the release mechanism of ACZ loaded in the film. Figures 7E & F show images of the remaining soluble PGM after contact with the ACZ-loaded film. In this case, PGM looks dehydrated, displaying a filamentous structure aggregated together and numerous pores with very small interfiber spacing. These noticeable changes in the mucin network after incubation with the hydrophilic ACZ-loaded film may be attributed to the water movement from the PGM gel layer to the contacting partially hydrated film, leading to the dehydration of the mucin gel to form strong adhesive joints at low hydration [44–47]. Interactions between PGM and the ACZ-loaded film are believed to be due to hydrogen bonding between carboxylic groups of polymers forming the film and hydroxyl groups in the oligosaccharide residues present in mucin [48].

Results are in line with those obtained from the swelling studies demonstrating the low swelling characteristics of the ACZ-loaded film and support the hypothesis that polymers with low swelling degrees show higher mucoadhesion characteristics, which could be attributed to the dehydration of the mucus gel to form adhesive joints at low hydration. Thus, as the swelling degree of the polymer is reduced, the mucoadhesion increased [49].

Determination of intraocular pressure & bioadhesion in animals

In previous works, we have explored the use of these types of complexes but in aqueous formulations [9]. We were able to achieve a decrease of approximately 4 mmHg at 2 h, but the peak was transient, returning the IOP to normal values immediately.

On the other hand, we also explore the use of films using HA as carrier material [21].

For this reason, progress was made in the design of a strategy to keep the complex anchored on the ocular surface.

With this in mind, for this work combining both strategies, an experiment was designed to control the decrease of IOP and the adhesion of the films, simultaneously

The films placed in the fornix exhibited adherence to the bulbar conjunctiva throughout the experimental procedures. The results of the effects of the films on IOP are showed in Figure 8, where it can be observed, that the ACZ loaded film leaded to a remarkable IOP decrease in normotensive male rabbits, reaching the lowest point 6 h after application, with similar values to those obtained with the liquid formulation described above.

Furthermore, as can be seen in Figure 8, the maximal hypotensive effect is maintained for a further 4 h and then returns to normal in a slow mode with low IOP values for almost 20 h. The maximum and sustained effect can be attributed to the adhesion of the film to the surface of the eye. As can also be seen in Figure 8 when bioadhesion begins to decrease the hypotensive effect becomes less intense. These results are in agreement with the tests carried out with mucin using SEM. It is important to note that throughout *in vivo* IOP determinations no irritation was verified.

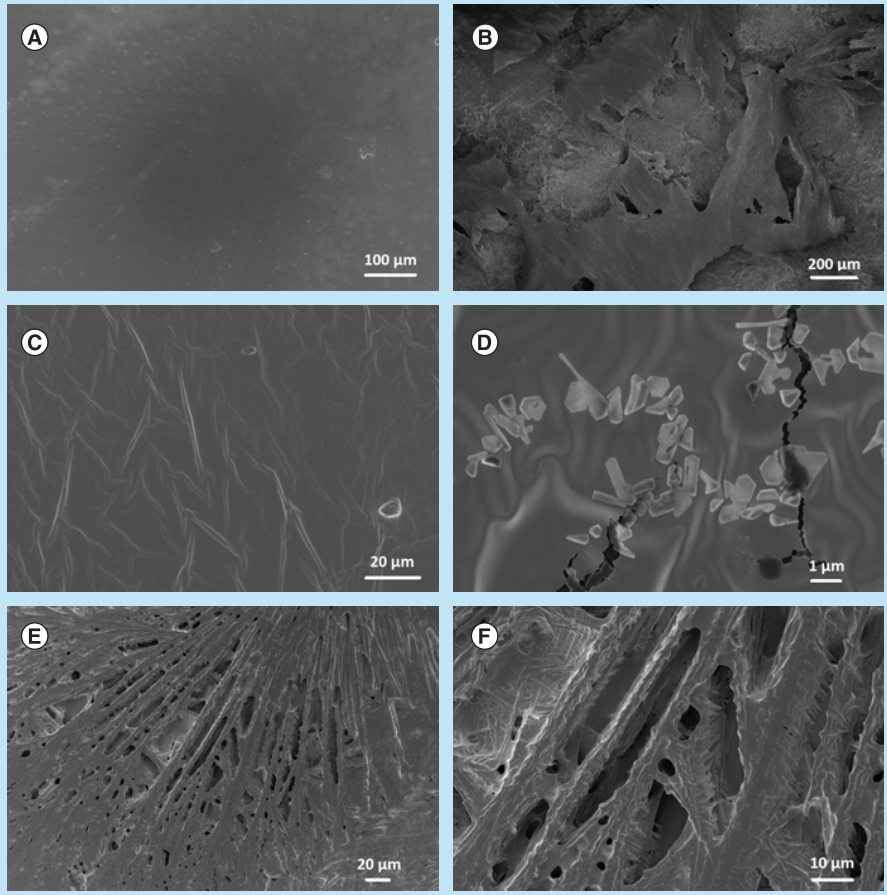

Figure 7. Mucoadhesive behavior of the acetazolamide-loaded films brought into contact with pig gastric mucin (n = 3). Changes in the mucin network after incubation with the hydrophilic acetazolamide (ACZ)-loaded film may be attributed to the water movement from pig gastric mucin (PGM) gel layer to the contacting partially hydrated film, leading to the dehydration of the mucin gel to form strong adhesive joints at low hydration. **(A)** Hydrated PGM; a homogeneous surface is displayed in this figure (452x). **(B)** ACZ-loaded film without PGM (200x). **(C)** ACZ-loaded film in contact with PGM; this figure shows the presence of PGM fibers (2440x). **(D)** ACZ-loaded film in contact with PGM; this figure shows the presence of ACZ particles (25540x). **(E)** PGM after being in contact with ACZ-loaded film; this figure shows a PGM as a dehydrated structure with filamentous shape and numerous pores (1070x). **(F)** PGM after being in contact with ACZ-loaded film; this figure shows a PGM as a dehydrated structure with filamentous shape and numerous pores (1070x).

Conclusion

Previous studies support the idea of HA as a solid material for topical ocular drug delivery. It was successfully modified to an insoluble material and loaded with an ACZ ternary inclusion complex. The final formulation exhibited good mechanical properties and biocompatibility with human corneal cells. However, higher concentrations of ACZ in cell cultures led to a reduction in cell proliferation.

ACZ-loaded films did not show long-term *in vitro* drug delivery, probably due to decomplexation of ACZ during cross-linking reactions. Nevertheless, *in vivo* experiments exhibited good bioadhesive and antihypertensive performance.

HA showed the capacity to be easily modified by a mild chemical reaction and drug-loaded without losing its intrinsic properties. Despite the decomplexation of ACZ, the use of CD enabled it to be loaded in a hydrophilic reaction medium with HA.

Presented formulation showed to be an innovative drug delivery platform able of combining desirable properties such as, lipophilic drug loading, bioadhesion, absence of preservatives, biocompatibility and effectiveness in a new single topical ocular drug formulation. Those properties were addressed by combining a natural polymer

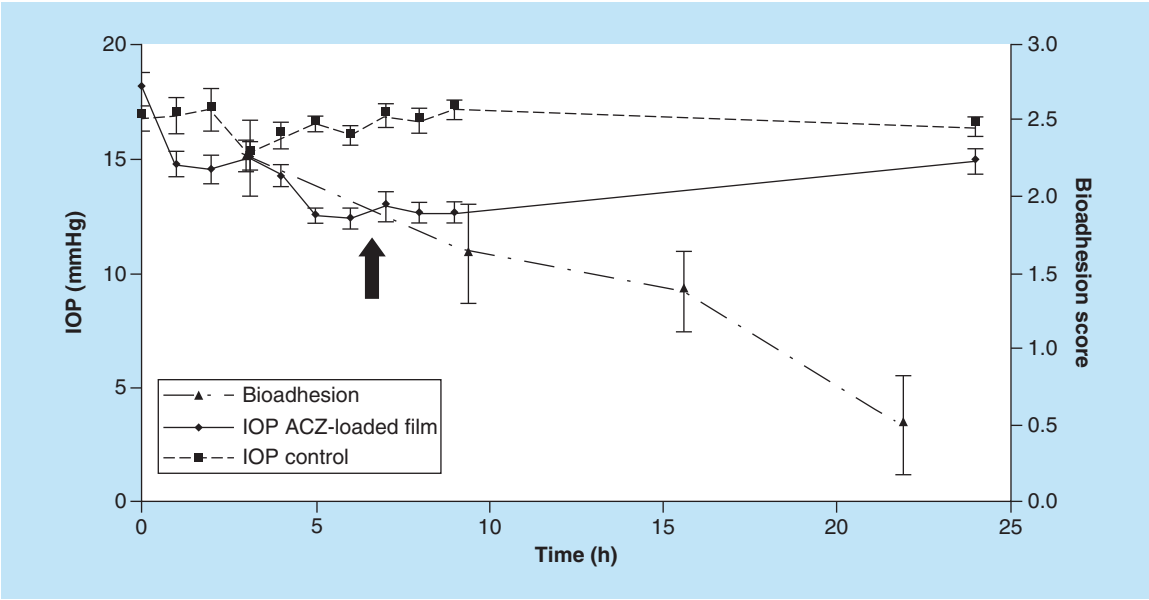

Figure 8. *In vivo* determination of drug efficacy and bioadhesion (n = 20). Application of ACZ loaded film decreased IOP in normotensive rabbits (lowest point 6 h after administration). The maximal hypotensive effect is maintained for 4 h. Then, slowly returns to normal with low IOP values for near 20 h. Bioadhesiveness reduction is related to less hypotensive effect.
ACZ: Acetazolamide; IOP: Intraocular pressure.

such as HA with a natural oligosaccharide such as HPβCD. The presence of HPβCD complexes enabled ACZ incorporation into the aqueous reacting solution of HA, HA provided bioadhesiveness and biocompatibility; and its mild chemical modification enabled to obtaining a sterilizable solid platform to avoid preservative presence. Experimental set showed promising performance and encouraged future studies to optimize formulation.

Future perspective

In our opinion, the future challenges related to the ocular therapy through strategies based on the development of new polymeric materials and devices that are more secure, reliable and efficient and designed to prolong the therapeutic action by increasing the time of permanence of the formulation on the ocular surface, will be the next technological tools. Especially in the case of films or inserts, the development should focus on obtaining biocompatible, mucoadhesive and safe materials that having the property of releasing the drug progressively and thus, maintaining its concentration at the site of action at therapeutic and nontoxic levels. Therefore, future steps will be focused to increase biopharmaceutical performance. Specially, properties related to improve bioadhesiveness and drug retention, and its comparison with other reported systems.

Financial & competing interests disclosure

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Ethical conduct of research

Animal management procedures were conformed to the Association for Research in Vision and Ophthalmology resolution on the use of animals in research, the European Communities Council Directive (86/609/EEC), and the Institutional Care and Use Committee of the Chemistry Faculty of National University of Córdoba, reviewed and approved the protocols.

Summary points

- Glaucoma includes a collection of neurodegenerative ophthalmic diseases with diverse clinical presentation.
- The reduction of intraocular pressure by pharmaceutical treatments or surgical procedures has long been the standard treatment for glaucoma.
- acetazolamide is a potent carbonic anhydrase reversible inhibitor, effective in controlling fluid secretion in the treatment of glaucoma.
- Some of the drawbacks related to ophthalmic topical administration are closely related to liquid dosage formulations and could be overcome by using new drug delivery systems.
- Polymer-based solid systems are capable of keeping the therapeutic agent attached to the mucosal surfaces.
- The presence of HPβCD complexes enabled acetazolamide incorporation into the aqueous reacting solution.
- HA provided bioadhesiveness and biocompatibility; and its mild chemical modification enabled to obtaining a sterilisable solid platform to avoid preservative presence
- The *in vivo* experiments showed an improved antihypertensive performance.

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