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Crosslinked soy protein films and their application as ophthalmic drug delivery system

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ABSTRACT

In this research, the potential of soy protein (SPI) based-films as drug delivery devices for ocular therapy was developed. Hence, crosslinked films with a natural and non-cytotoxic crosslinking agent, genipin (Gen), coated with poly(lactic acid) (PLA), were prepared. Filmogenic solutions were loaded with timolol maleate (TM) as a model drug, to be used as drug delivery devices, a novel application for this material. The mechanical properties of the films were studied, observing that with the presence of PLA coating, more rigid materials with improved properties were obtained. Furthermore, the release behavior of TM was evaluated in aqueous medium, it being influenced by the degree of film crosslinking. Furthermore, it was determined that PLA coating decreased TM release rate compared to that of uncoated films. Similarly, this behavior was observed via indirect estimation of the release by assessing the hypotensive effectiveness of the films by in-vivo assays. Through intraocular pressure (IOP) determination tests in rabbits, it was demonstrated that, through the use of high crosslinked and coated films, a significant decrease in IOP could be achieved for prolonged time periods. These results suggest that the use of soy protein-based films as drug delivery systems is highly suitable.

1. Introduction

Biodegradable films derived from natural products have received considerable research interest during the last years due to ecological concerns aroused by the use of traditional petroleum-based materials. This has led to the use of several renewable agro-products as promising biodegradable materials [1]. Main products studied include polysaccharides and proteins [2]. However, protein-based films showed some advantages due to their excellent film-forming abilities, low cost and barrier properties against oxygen and lipid under low-to-intermediate humidity conditions [3,4]. In particular, soy proteins produce smoother, clearer and more flexible films as compared to those arising from other sources [5]. Novel soy protein based-materials are currently developed in order to be applied to several fields such as industrial coatings, food packaging, agriculture and medicine.

A particular interest in medicine is currently focused in the development of novel drug delivery systems that could improve the disadvantages arising from traditional dosage systems. In ocular therapy, for example, conventional dosage systems are generally based on topical application of eye drops. Most of these pharmaceutical formulations are administered for two main purposes: a) outside eyeball structure treatments, including conjunctivitis, blepharitis and dry keratitis; b) treatment of intraocular disorders such as glaucoma, uveitis and endophthalmitis. In the latter, the bioavailability of topically administered drugs shows significant limitations brought about by the rapid and extensive loss of formulation from the precorneal area due to drainage and lacrimal replacement [6]. In addition, the significant decrease in drug penetration by this route usually derives from the highly efficient barrier properties of the cornea.

With the administration of eye drops, less than 5% of the drug passes through the cornea and reaches intraocular tissues, while most is absorbed systemically via conjunctiva and naso-lacrimal ducts.

Further disadvantages of these dosage systems include discomfort experienced by the patient due to the high-frequency administration of small volumes of medicine (every 3 to 4 h). Hence, efforts are currently addressed to design more efficient drug delivery systems with sustained- or controlled-release properties in order to increase drug

Abbreviations: SPI, soy protein; Gen, genipin; PLA, poly(lactic acid); Gly, glycerol; TM, timolol maleate; IOP, intraocular pressure; RH, relative humidity; FTIR-ATR, Fourier Transform Infrared Spectroscopy in Attenuated Total Reflectance mode; TS, tensile strength; E, elongation at break; ANOVA, analysis of variance.

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concentration in the eye structure for an extended period of time. In addition, efforts are focused on the development of a more convenient and practical delivery system for patient's administration.

Some of the systems under study are related to the use of liposomes [7,8], nano- and microparticles [9–12], in situ gel formation [13–15], and more recently, to the novel use of non-cytotoxic films.

In particular, a method of increasing the residence time of the formulations during application is the use of bioadhesive systems capable of releasing controlled amounts of the desired drug formulation [16].

By their hydrophilic nature, films formed from natural and biodegradable materials generally have the ability of containing hydrophilic drugs (soy proteins contain 58% polar amino acids that cause hydrophilicity [4]). However, when they are immersed in aqueous environments, rapid drug diffusion is observed through the matrix, generating a fast release. An efficient release results from the presence of effective drug concentration in the environment for a preset time. By using non-sustained systems, several doses have to be applied to produce the same effect, as compared to the use of sustained systems. It can also be noted that, sometimes, by using conventional systems, the concentration yielded does not reach minimum levels to be effective, while in other cases, concentration reaches toxic values for the organism or tissues. In contrast, a sustained release system maintains concentration levels within the therapeutic window for a prolonged period of time.

Films formed by three-dimensional networks of crosslinked materials have the ability to swell in aqueous solvents in a controlled manner (according to crosslinking degree). When the swelling is generated in a drug-containing matrix, this can be dissolved and released to the medium by diffusion. As swelling properties, drug release can be controlled adjusting the crosslinking degree [17]. Therefore, films with correct structural relationship between hydrophilicity and crosslinking degree can release drugs with a sustained behavior [18–21].

Glaucoma is the main cause of blindness worldwide, particularly among elderly people. This eye disease is usually characterized by the pathological increase in intraocular pressure (IOP) due to lack of drainage of aqueous humor, showing a common optic neuropathy characterized by progressive loss of nerve fibers in the optic nerve. This asymptomatic disease causes progressive loss of visual function, accompanied by ocular hypertension. One of the drugs conventionally used for this condition is timolol maleate (TM). This is a non-selective blocking agent of the ß-adrenergic receptor. Due to its high stability and water solubility, this drug is perfectly suitable to be used as a model drug for the possible development of drug delivery systems.

This research work aims at employing a natural polymer such as soy protein, to develop materials to be used in the field of medicine and ophthalmology. For this, soy protein (SPI) films crosslinked with different amounts of genipin (Gen) previously obtained and characterized in our research group [22] were applied as TM delivery devices in ocular therapy.

Genipin is a novel and biocompatible cross-linking agent, about 10,000 times less cytotoxic than glutaraldehyde [23,24]. The colony-forming assay also showed that the proliferative capacity of cells after being exposed to Gen was approximately 5000 times greater than that of cells exposed to glutaraldehyde [23,25]. Genipin-crosslinked soy protein (SPI-Gen) films have the required characteristics as drug delivery devices for ocular therapy due to their hydrophilic and crosslinked structure, the biocompatible properties of their components, their low solubility in water and good mechanical properties and intense color, which would greatly help patients during their correct application. Presumably, due to small size, thickness and absence of sharp edges, these films do not cause inconveniences or discomfort in the eye.

The modification of mechanical properties produced by the addition of the drug was assayed, as well as the in-vitro release behavior in aqueous systems and the effect of the release on the ocular hypotensive effectiveness in in-vivo systems.

2. Experimental

2.1. Materials

The following chemicals were used: isolated soy protein SPI SUPRO E with 90% protein on fat-free, dry-weight basis (donated by The Solae Company, Argentina), glycerol (Gly) (Taurus, Argentina), genipin (Gen) (Wako, Japan), timolol maleate (TM) 99% (Parafarm, Argentina), NaCl (Cicarelli, Argentina), KCl (Cicarelli, Argentina) and $CaCl_2$ (Cicarelli, Argentina).

2.2. Animals

Twelve New Zealand white rabbits weighing 2–2.5 kg were used. These rabbits were provided with food and water ad libitum in a temperature-controlled room (21 ± 5 °C) and exposed to 12 h light:12 h dark cycles. Animal management procedures complied with ARVO (Association for Research in Vision and Ophthalmology) resolution on the use of animals in research from the European Communities Council Directive (86/609/EEC). The Institutional Animal Care and Use Committee of the School of Chemistry from the National University of Córdoba, Córdoba, Argentina reviewed and approved the protocols. After a week of adaptation in the facilities, animals were admitted to the experimental sessions.

2.3. Preparation of TM-containing SPI-Gen films

TM-containing films were prepared by casting method as described in a previous work [22] with the addition of TM in the last step of the preparation. In brief, SPI powder was dispersed under constant stirring in distilled water (8.33 g/100 mL) and Gly was added at 50% (w/w) of SPI while pH was adjusted to 9 with 0.5 M NaOH. The dispersions were stirred for 30 min at room temperature. Different volumes of 0.4% w/v Gen solution were then added to SPI dispersions to obtain the final SPI-Gen mixture with 0; 0.1; 1; 2.5; 5; 7.5 and 10% (w/w of SPI) of Gen. The dispersions were heated at 70 °C for 2 h. Once room temperature was reached, 1.5 mg of TM (equivalent to 6 normal doses) was added to the dispersions and stirred for 2 h. All dispersions were poured into plastic plates (polypropylene) and dried in an oven with air circulation at 40 °C for 12 h. Subsequently, films were removed and conditioned for 48 h at 25 °C and 50% relative humidity (RH) before use. The films were named SPI; SPI-Gen 0.1%; SPI-Gen 1%; SPI-Gen 2.5%; SPI-Gen 5%; SPI-Gen 7.5% and SPI-Gen 10%. The different films were cut in small 4 mm-diameter circles. In addition, PLA covered SPI-Gen-TM films were prepared. For this, the different disks were submerged for 3 min in a solution of PLA in chloroform (0.53% w/v) and dried at room temperature in a vacuum chamber until complete chloroform elimination.

2.4. FTIR-ATR analysis

In order to confirm the presence of PLA covering, disk surfaces were analyzed by Fourier Transform Infrared Spectroscopy in Attenuated Total Reflectance mode (FTIR-ATR) using a ZnSe crystal with an incidence angle of 45 grades. 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 to 400 cm^{-1} range, using air as background.

2.5. Mechanical properties

Stress–strain curves for each film (25 × 100 mm) were recorded; tensile strength (TS) and elongation at break (E) were determined according to ASTM methodology [26]. An Instron Universal Testing Machine (model 3342, Norwood, MA, USA) equipped with a 500 N capacity cell was used with an initial grip separation of 100 mm and

crosshead speed of 0.5 mm/s. Four replicates were tested for each sample. The data obtained were statistically analyzed. The analysis of variance (ANOVA) was used to evaluate the significance in the difference between means according to the Tukey test. Differences between means were considered significant when $P \leq 0.05$.

2.6. Film thickness

The thickness was determined as the average of 10 measurements for each sample with a hand-held micrometer (Schwyz model ESP1-0001PLA, Schwyz, Swiss).

2.7. In-vitro drug release

TM-loaded circular films of 4 mm diameter were immersed in glass vessels with 50 mL of a Ringer's solution (1 L of water; 8.6 g of NaCl; 0.3 g of KCl and 0.3 g of $CaCl_2$). These vessels were incubated at 36 °C (the highest temperature commonly measured on closed eyes [27]) with magnetic stirring at 100 rpm by 54 h. At appropriate time intervals, 2 mL of the solution was withdrawn from glass vessels and the amount of TM released from drug-loaded films was evaluated by UV–Visible spectrophotometry at 295 nm using a Shimadzu UV-1800 spectrophotometer. An equal volume (2 mL) of the same dissolution medium was added back to keep a constant volume after each determination. All determinations for each film were performed in quadruplicate.

2.8. Ocular hypotensive effectiveness tests

Basal IOP of rabbits was measured with an Icare Vet TonoVet J1000 tonometer (Finland), calibrated according to the manufacturer's instructions. 4 mm-diameter disks of loaded films were placed into the conjunctival fornix of twelve rabbits' eyes. Fig. 1 shows the application site. The evolution of the IOP was measured. At each interval, the measurements were repeated six times, and a mean was taken. In all cases, the IOP was measured at 0, 2, 4, 6, 8, 10 h and in some cases at 24 h. The experiments were always carried out at the same hour of the day. The results are expressed as the mean IOP unit change with respect to the basal value. No treatment or experiment was developed in the rabbits for the next week in order to reach normal IOP values. Differences within groups were statistically determined. Values were considered different when $P \leq 0.05$ according to a two-tailed Student t-test.

Furthermore, to assess signs of irritation that could be potentially produced by the films, a determination with ophthalmoscope was performed at each time in order to find signs of irritation, redness and corneal or conjunctival microlesions.

3. Results and discussion

3.1. Preparation and characterization of TM-containing films

As described in Section 2.3, SPI-Gen films were prepared with 0; 0.1; 1; 2.5; 5; 7.5 and 10% of Gen. Their preparation and characterization were previously developed in the research group [22]. In addition, the same films coated with a thin layer of PLA were prepared. The purpose of the coating was to produce a retarding effect on drug release. This effect would be attributed to the hydrophobic characteristic of PLA, creating a barrier to the entry of water within the films leading to a decrease in swelling. This effect causes less drug diffusion to the medium, which would result in a more sustained release. Further examples of soy protein coated-film can be found in the literature where authors developed a polyurethane coating in order to increase surface hydrophobicity [28].

The films were cut into 4 mm-diameter disks. This shape was chosen due to the absence of edges that could produce eye injury; in addition, such a size allows manipulation without causing discomfort when administered under the eyelid. The amount of drug contained in each disk corresponded to 6 doses, using as a dose the amount of drug contained in a drop of commercial timolol solution (Zopirol DM®). Fig. 1 shows the macroscopic appearance of the films prepared.

Thickness from 55 to 65 μm was obtained. The films were homogeneous and flexible and coloration varied from yellowish (films without Gen) to dark blue (films with Gen); color intensity increased with the amount of Gen added [22]. TM-containing films and films without TM are visually indistinguishable. In addition, PLA covered and uncovered films are also indistinguishable since the PLA layer formed is particularly thin and transparent.

To demonstrate the correct formation of PLA coating, FT-IR-ATR spectrophotometry was used. This methodology allows obtaining the

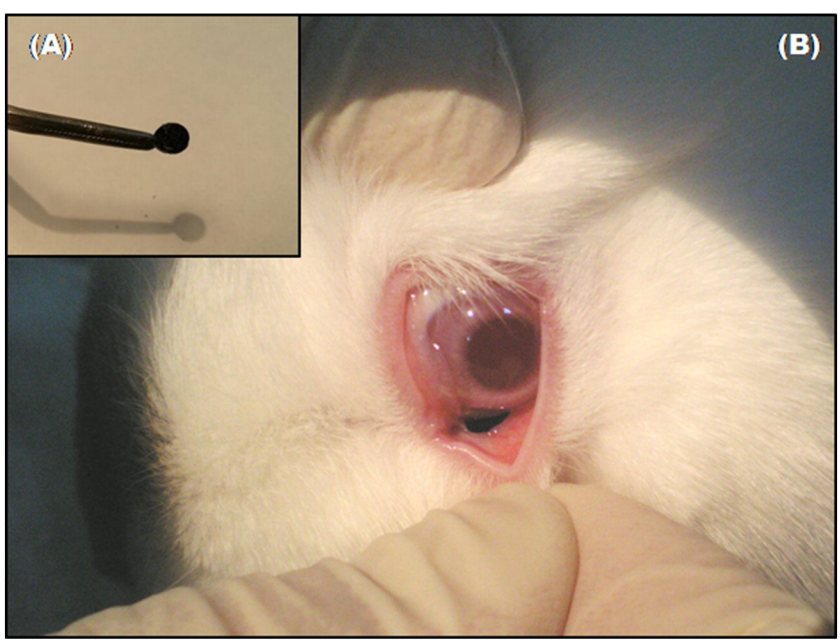

Fig. 1. Macroscopic aspect of TM loaded films and their action site.

infrared spectra of film surfaces. From the spectra, a typical PLA spectrum can be observed with the following characteristic bands: C═O stretching at 1746 cm^{-1} and C–O stretching of the ester group at 1179 and 1079 cm^{-1}. The presence of these bands and the absence of SPI characteristic bands (O–H stretching and N–H bending at 3000–3500 cm^{-1}; C═O stretching at 1668 cm^{-1} and N–H bending at 1548 cm^{-1}) indicate that covering was correctly formed.

Subsequently, mechanical properties of PLA covered films were studied in order to determine whether cover changes film properties. Thus, tensile properties such as tensile strength (TS) and elongation at break (EB) were determined. Table 1 shows the results of these determinations obtained in triplicate.

Comparing TM loaded films with different amounts of crosslinking agent, a similar behavior can be observed with respect to the results reported in a previous work for SPI-Gen films without TM [22]. In this case, a TS increase was observed for films with 2.5% of Gen with respect to SPI films, while values remained constant for higher Gen amounts. In addition, a rigidity increase was observed in films (determined by greater TS/EB ratio) when the amount of Gen increases. It can also be observed that TS and EB values obtained for films with TM were relatively lower compared to those of films without drug. This effect is probably attributed to the fact that TM entrapped between protein chains could interact with the matrix, reducing the interactions between the protein structures themselves. This is reflected in the significant deterioration of film mechanical properties, although no significant influences were revealed upon the application of the devices.

Comparing loaded films in the presence and absence of PLA coating, it can be observed that the coating conferred more TS to the films. This same effect was observed and described in films coated with PLA by the casting method [29]. Furthermore, the presence of coating did not significantly influence the elongation of the films as no significant decrease was observed in EB as compared to uncoated films. Analyzing the TS/EB ratio, it could be observed that the rigidity is not influenced by the PLA coating in 0 and 2.5% films. In the case of the film with 10% of Gen, the rigidity decreases due to the greater EB values obtained for the covered compared to the uncovered film.

3.2. Study of in-vitro drug release behavior

Release assays were performed in Ringer's saline solution in order to simulate tear fluid. These assays were performed in quadruplicate using 4 mm-diameter disks of SPI-Gen coated and uncoated films containing 6 normal doses of TM. Films were introduced into 50 mL of Ringer's solution and drug concentration versus time was plotted.

Drug quantification was performed by UV–Visible spectrophotometry. By performing a calibration curve, a molar extinction coefficient of 8584.9 M^{-1} cm^{-1} was determined. The results obtained can be seen in Figs. 2 and 3, where drug release rates are shown as a function of time for PLA coated and uncoated films, respectively.

A first analysis of these results allows noticing a very quick release in uncoated films with low crosslinking degree (films with 0; 0.1 and 1% of Gen). These films reach the largest percentages of release in the examined time interval. In addition, it can be clearly seen that the increase in crosslinking degree, produced by a higher amount of Gen added, decreases release rate (showing a more sustained behavior) and a decrease in the total drug released. This effect is observed in both coated and uncoated films. For example, in the case of uncoated films, 80% of drug release is achieved in 1.7 h in the non-crosslinked film (SPI), while release percentage is yielded in 5.4 and 17.6 h in SPI-Gen 2.5% and SPI-Gen 10% films, respectively. The decrease in release rate is ascribed to the minor water entry into the films which is reflected in the reduction in swelling caused by an increase in the crosslinking degree. Minor water inside the matrix allows a decrease in drug solubilization and therefore minor drug diffusion to the medium. In addition, in highly crosslinked films, a portion of drug could be retained into the matrix due to the water inaccessibility into some places of the matrix. The same trend was observed in coated films.

In particular, films with 2.5 and 5% of Gen seemingly show an uncertain release rate behavior at initial times since film with 2.5% presents a slower release than the film with 5% of Gen. However, with statistical analysis it can be seen that the major proportion of measured points of both curves at each time are not significantly different ($P \geq 0.05$) (for example at 1; 2; 3; 4 and 5.5 h). Actually, the P-values determined for the percentage of released drug for these films at initial times are 0.0018; 0.0075; 0.1349; 0.1399; 0.3507 and 0.3017 for 0.5; 1; 2; 3; 4 and 5.5 h, respectively. This analysis allows concluding that the global behavior of these two films is consistent with the overall performance of all films.

On the other hand, a marked effect produced by the presence of PLA coating was determined, observing a very marked decrease in the drug release rate from coated films compared to those from uncoated films. For example, SPI uncoated film reaches 80% of drug release in 1.7 h, while SPI-PLA (coated) film requires 4.5 h. The same applies to SPI-Gen 10% uncoated film, where 80% of drug is released in 17.6 h whereas the coated film requires 39.5 h. Clearly, the hydrophobic characteristic of PLA coating acts as a barrier to water, producing a decrease in the drug diffusion rate.

Table 1
TS, EB and TS/EB ratio of the SPI-Gen covered and uncovered films.

Films	TS (MPa)	EB (%)	TS/EB
SPI	1.7 ± 0.3^{A}	19.8 ± 2.5^{A}	0.08 ± 0.01^{A}
SPI-Gen 2.5%	2.6 ± 0.1^{BC}	18.3 ± 4.9^{A}	0.15 ± 0.05^{AB}
SPI-Gen 10%	2.7 ± 0.2^{C}	4.5 ± 0.3^{B}	0.59 ± 0.09^{D}
SPI-PLA	2.1 ± 0.2^{AB}	21.5 ± 4.0^{A}	0.09 ± 0.01^{A}
SPI-Gen 2.5%-PLA	3.7 ± 0.3^{D}	17.8 ± 1.7^{A}	0.21 ± 0.03^{B}
SPI-Gen 10%-PLA	3.3 ± 0.1^{D}	8.8 ± 0.7^{B}	0.37 ± 0.03^{C}
SPI (unloaded film)*	3.2 ± 0.1	22.5 ± 5.0	0.14 ± 0.07
SPI-Gen 2.5% (unloaded film)*	4.46 ± 0.04	36.9 ± 0.5	0.12 ± 0.06
SPI-Gen 10% (unloaded film)*	4.6 ± 0.1	2.8 ± 1.5	1.6 ± 0.2

Any two means in the same column followed by the same letter are not significantly ($P \geq 0.05$) different according to Tukey test.

* TS and EB values showed for unloaded films were extracted from González et al. [22].

3.3. Ocular hypotensive effectiveness tests

To determine the effectiveness of the delivery device in ocular therapy, in-vivo tests in rabbits were conducted. In these assays, determination of IOP decrease as an indirect assessment of drug release was performed.

Although all film components are biocompatible [23,25], signs of irritation, redness or corneal or conjunctival microlesions potentially produced by the films were evaluated at each time. From these measurements, it was possible to observe absence of redness, microlesions or other factors that could suggest irritation produced by the films to eye tissues during this period.

Drug release behavior in in-vivo systems was assessed by determining their hypotensive properties. The concentration of timolol on tear or plasma could be determined analytically [30] or estimated by simulations from the values obtained in-vitro [31]. In this work, an indirect estimation by determining the effect of drug released was performed. Since TM has hypotensive properties, IOP determination of rabbits at various times after film application was determined, checking the magnitude and duration of hypotensive effects.

Although rabbits' eyes are physiologically different from humans in terms of eyelid physiognomy, blinking and tear parts, the provision of timolol in tear fluid in these animals provides a good estimate of its behavior in humans [32,33].

The IOP of a group of 6 rabbits was determined. Therefore, film disks loaded with 6 doses of TM were applied in both eyes ($n = 12$). IOP determination was repeated every 2 h and this procedure was repeated for each film sample. Fig. 4 shows the results found. The values shown

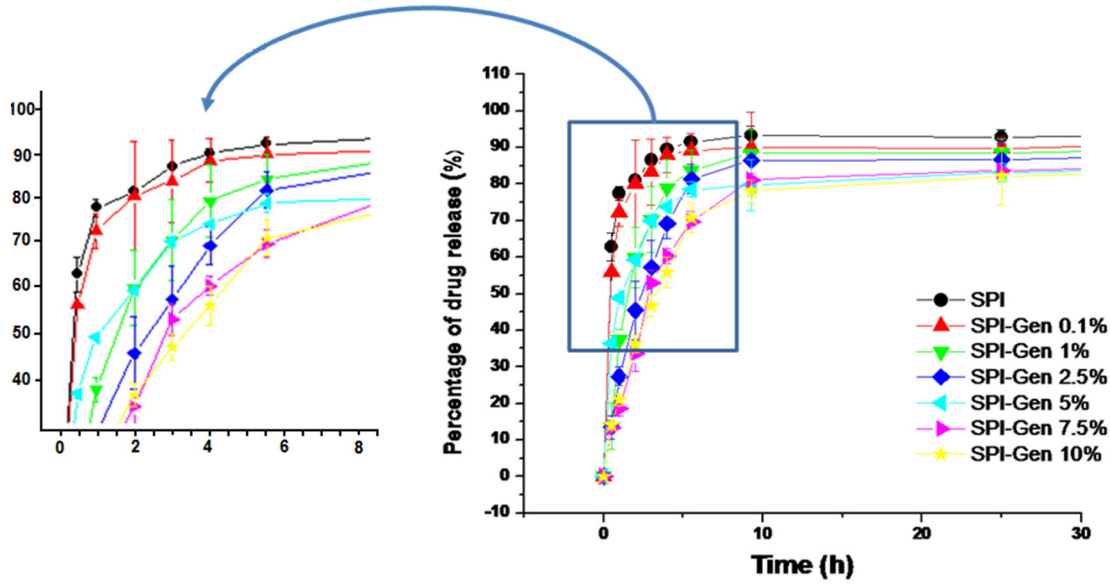

Fig. 2. Percentage of TM released by uncovered SPI-Gen films as a function of time.

by the commercial drops (DM Zopirol ELEA Laboratories) were extracted from a previous work [16] for comparison.

From the results shown in Fig. 4, we can see a significant decrease in IOP between 2 and 4 h after applying the films ($P < 0.0001$ for all films). The same effect is observed with commercial drops; yet, the decrease achieved is smaller than that yielded by the films. This effect is probably ascribed to a higher amount of drug present in the eye, resulting from film release as compared to the amount of drug that would be present in one dose of the commercial drops. An increase in the concentration of eye drops will not achieve greater IOP lowering, since an increase in the dose is not effective to achieve sustained drug levels, as ocular drug bioavailability decreases at higher concentrations [30].

Upon completing this comparison, it was determined that all films were able to maintain a minor IOP with respect to the commercial drops in a period ranging from 0 to 6 h. A similar behavior is described in the literature for biodegradable crosslinked films from hyaluronic and itaconic acids [16] and for nanoparticles supported on contact lenses [34]. It could be determined that at 8 h, the values obtained for IOP were similar to basal values for uncoated films ($P = 0.1777$, 0.7173 and 0.0548 for films with 0, 2.5 and 10% of Gen, respectively). However, at this time, coated films still show a significant IOP decrease ($P = 0.0036$ for SPI-Gen 0%-PLA and $P < 0.0001$ for SPI-Gen 2.5%-PLA and SPI-Gen 10%-PLA, respectively), confirming that films coated with PLA maintained a lower IOP for longer times as compared to that of uncoated films. In addition, the same behavior was observed for most crosslinked films compared to those with a lower amount of Gen. Presumably, this effect is produced by a lower drug release as demonstrated in the in-vitro determinations. Comparing these two assays, the same release behavior was observed for the different films. Thus, by examining Figs. 2 and 3 it can be verified that the lower crosslinked and uncoated films have almost completely released the drug after 8 h. At this time, it can be seen that the decrease in IOP is markedly lower than those determined using films with high crosslinking or coated degree, ranging from 1 to 2 points below baseline. This effect is also found at 24 h,

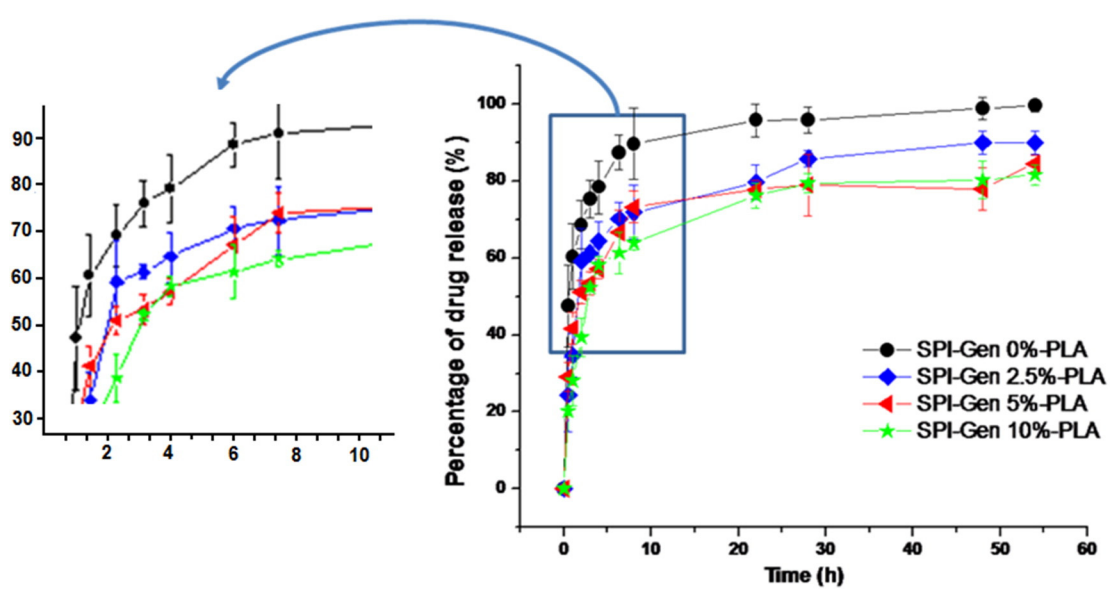

Fig. 3. Percentage of TM released by covered SPI-Gen-PLA films as a function of time.

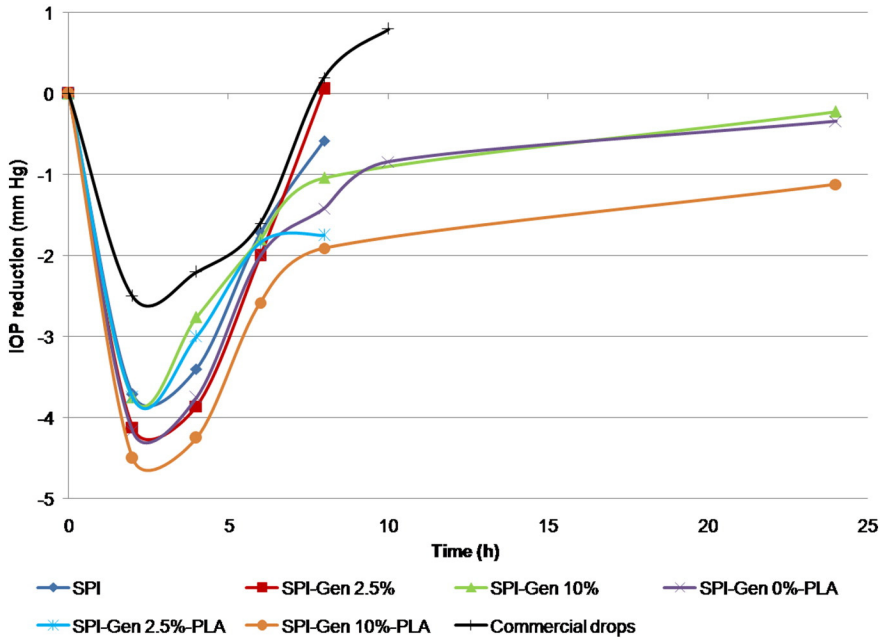

Fig. 4. IOP reduction caused by different TM-loaded films at different times.

since the coated film with a higher crosslinking degree (SPI-Gen 10%-PLA) still maintains a significant IOP reduction ($P = 0.0115$), as compared to the other films.

4. Conclusions

In this research work, we developed TM-containing crosslinked SPI films in both forms, coated and uncoated with PLA, as drug delivery devices for eye therapy. The mechanical properties of the films were found to be optimized by the presence of PLA coating. The release of TM was also evaluated in aqueous medium, concluding that this property was markedly influenced by the crosslinking degree of the films and the presence of their coating. Similarly, this behavior was also observed by the indirect release estimation which determined the effectiveness of hypotensive films. Through IOP determination tests in rabbits, it could be proved that, by using highly crosslinked and PLA coated films, a significant decrease in IOP was achieved for a prolonged period of time (24 h).

On the basis of these results, it can be concluded that this delivery system would provide numerous benefits as compared to those of traditional delivery systems such as eye drops, as it would be more convenient in patients, providing stronger effects for longer time periods.

Such promising results provide a basis for the potential implementation of these agro-derived films as drug delivery systems in domestic animals and even humans.

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