International Journal of Pharmaceutics 455 (2013) 48–56

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Novel bioadhesive hyaluronan–itaconic acid crosslinked films for ocular therapy

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ARTICLE INFO

Article history:
Received 15 March 2013
Received in revised form 24 June 2013
Accepted 19 July 2013
Available online 1 August 2013

Keywords:
Hyaluronan
Glaucoma
Bioadhesive
Polymer
Drug delivery

ABSTRACT

New hyaluronic acid (HA)–itaconic acid (IT) films have been previously synthesized and used as potential topical drug delivery systems (DDS) for ocular administration. In this study we explored homogeneous and heterogeneous crosslinking reactions of HA using glutaraldehyde (GTA) and polyethylene glycol diglycidyl ether (PEGDE) in the presence of IT, a naturally occurring compound that is non-toxic and readily biodegradable.

We have studied the morphology, mechanical properties and *in vitro* biocompatibility between these new materials and ocular surface cells (human corneal epithelial cell line) and evaluated the biopharmaceutical performance of the designed formulations. Although all the synthesized materials exhibited good mechanical properties, the PEGDE modified films exhibited the best biocompatibility, with *in vivo* assays showing good adhesive performance and minimal irritation. PEGDE films were also tested for their effects in the treatment of intraocular pressure (IOP) in rabbits using timolol maleate (TM) as the model drug.

These results may be useful for further design of novel bioadhesive matrix containing drugs by topical application in ophthalmology.

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1. Introduction

Traditional topical dosage forms (solutions, suspensions and ointments) have a limited use in solving many eye diseases, mainly due to rapid and extensive drainage loss of the formulation in the pre-corneal area as a result of blinking and tear replacement (Ding, 1998).

The high complexity of the eye anatomy represents an important challenge in the development of new drug delivery systems (DDS). Conventional drug formulations for ocular disorders vary considerably, depending on their nature and the location of the affected tissues (Mitra, 2003; Reddy, 1996). Topical administration is preferred for ocular drug delivery to the structures of the front part of the eye, such as the cornea and the conjunctiva, with the pre-corneal tear film and the corneal epithelium representing the front line where an effective DDS has to interact. The preservation of tissue morphology and tear pH without alterations is the first requirement that a new formulation must fulfill, as well as stability, bioadhesion and good drug delivery properties.

As a consequence of the above requirements, current goals in the design of new drug carrier systems in ophthalmology are oriented to achieve: (a) pre-corneal contact time lengthening; (b) an increase in drug permeability; and (c) a reduction in the rate of drug elimination. An alternative to increasing the residence time of formulations in the area of application is the use of bioadhesive systems capable of releasing controlled amounts of the desired drug formulation.

The use of biodegradable polymers for DDS has been the subject of numerous studies (Lehr and Haas, 2002). The desirable system characteristics of these polymers are: zero or minimal biological effects, no toxicity or contamination due to inappropriate chemical residues, and the rapid degradation or excretion of the polymer used as the platform for the release of the applied medication.

Hyaluronic acid sodium salt (HA), also known as hyaluronan, is a naturally occurring high molecular mass linear biopolymer consisting of alternating units of N-acetyl-β-D-glucosamine

Abbreviations: HA, hyaluronic acid; IT, itaconic acid; DDS, delivery systems; GTA, glutaraldehyde; PEGDE, polyethylene glycol diglycidyl ether; IOP, intraocular pressure; TM, timolol maleate; RT, room temperature; SR, swelling ratio; ESEM, environmental scanning electron microscopy; HCE, human corneal epithelial; FBS, fetal bovine serum; BKC, benzalkonium chloride; PEG, polyethylene glycol.

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http://dx.doi.org/10.1016/j.ijpharm.2013.07.063

and β-D-glucuronic acid with reactive carboxyl groups. It is a biodegradable, biocompatible, non-toxic, non-immunogenic and non-inflammatory biomaterial, being widely used in medical practice in many pathological conditions such as osteoarthritis, wound repair and eye surgery (Ambrosio et al., 1999; Balazs and Denlinger, 1993; Borzacchiello et al., 2005; Brown and Jones, 2005; Chen and Abatangelo, 1999). The excellent water-binding capacity of HA makes it capable of retaining moisture in eyes, joints, and skin tissues (Robert et al., 2010). Also high molecular weight (1.0–2.9 million Dalton) HA solution, being highly viscous, allows its use in orthopedic applications (Witteveen et al., 2010). Many studies have produced HA in an injectable form, which is used to treat osteoarthritis of the knee (Strand et al., 2006). In addition, HA is frequently used in ocular surgery as a viscoelastic agent, and it has experimentally been demonstrated to facilitate wound healing (Nakamura et al., 1997). Although the oral application of HA has been recently suggested, its effectiveness still needs to be demonstrated.

HA has potential as a biodegradable carrier for transdermal drug delivery (Avila et al., 2008), and it has also been used as a novel depot system (Oh et al., 2010). Several HA derivatives have also been developed. In the form of physically and chemically crosslinked hydrogels (Kim and Park, 2002; Leach and Schmidt, 2005; Li et al., 2004), HA has also been developed as nano- and micro particulate systems (Segura et al., 2005) for various protein, drug (He et al., 2009), peptide (Moriyama et al., 1999) or gene (Lee et al., 2007; Luten et al., 2008) deliveries.

Several studies related to biocompatibility and biodegradability (Avitabile et al., 2001) have suggested the use of HA as a promising biomaterial to design modified DDS with ophthalmic applications. However, HA's low stability in living tissues, its poor biomechanical properties and fast water dissolution make it an unsuitable material for the preparation of drug carrier systems (Jinghua et al., 2008) with it being necessary to introduce some chemical modifications on the polysaccharide backbone. Many problems can be solved by applying structural changes (*e.g.* the degree of crosslinking) in order to obtain more robust materials with better mechanical properties and improved bioadhesion without losing biodegradability.

The goal of obtaining stable mucoadhesive films has been a subject of study for both systemic and local drug administration (Jones and Medlicott, 1995; Okamoto et al., 2001; Yoo et al., 2006). In particular, the bioadhesive formulations intended for use in ocular mucosa, have been shown to possess clear advantages over gels or eye drops as they can provide longer residence time, and unlike the inserts, can be easily removed from the area (Anders and Merkle, 1989). Crosslinking reactions have improved the HA stability in aqueous environments, leading to more adhesive and stable materials (Calles et al., 2011). Related to this, several crosslinking methods have been reported for this polymer, with reactions being carried out through diverse chemical processes and reagents, such as, polyethylene glycol–propiondialdehyde and adipic dihydrazide (Luo et al., 2000), bisepoxide and divinylsulfone (Laurent et al., 1964), *via* Ugi's condensation reaction (Crescenzi et al., 2003), crosslinking and surface coating (Vercruysse and Prestwich, 1998), the intra-esterefied method (De Laco et al., 1998), and in previously cast solid HA films or membranes using glutaraldehyde (GTA) and polyethylene glycol diglycidyl ether (PEGDE) as crosslinker agents (Tomihata and Ikada, 1997a,b). Nevertheless, despite the benefits of these new materials, the compounds used to obtain the desired modifications in the base materials may produce toxic or unwanted effects on the target tissues. It is therefore of major importance to evaluate the cytotoxicity, morphology and possible functional changes caused in cells exposed to these new materials.

In the present investigation, we report on the preparation of novel hyaluronic acid–itaconic acid (HA/IT) crosslinked films with GTA and PEGDE, and together with an evaluation of their mechanical and morphological properties, their biocompatibility with human corneal tissues and the *in vivo* pharmaceutical performance of the formulations using timolol maleate (TM) as a model drug.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salt (Mw: 1.560.000 Da) and TM were obtained from Parafarm® (Buenos Aires, Argentina) and IT for synthesis was provided by Merck Schuchardt OHG (Hohenbrunn, Germany). Glutaraldehyde (25% in H_2O), polyethylene glycol diglycidyl ether (average Mn = 500) and 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Phenol red-free RPMI and DMEM/F-12 were provided by Invitrogen-Gibco, Inchinnan, UK and Alamar Blue® reagent was acquired from AbD Serotec, Oxford, UK. The well plates were purchased from Nunc, Roskilde, Denmark, with, all other chemicals being of extra pure reagent grade and used as received.

2.2. Animals

New Zealand white normotensive 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 conformed to the 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 Care and Use Committee of the Chemistry Faculty of the Córdoba University, Córdoba, Argentina, reviewed and approved the protocols. After a week of adaptation in the facilities, animals were admitted to the experimental sessions.

2.3. Synthesis

Three HA systems were synthesized from HA/IT/GTA (GTA-film) and HA/IT/PEGDE (PEGDE-film) solutions, which were prepared using twice distilled water as the solvent. The amount of each reagent was adjusted to produce (1:1:2) molar ratios for HA/IT/GTA and HA/IT/PEGDE. A heterogeneous crosslinking method was also used, consisting of immersion of previously cast HA/IT (1:1) films in a 5% (w/v) PEGDE in H_2O/acetone (20:80) solution for 24 h (PEGDEH-film).

Crosslinking reactions were carried out under acidic conditions for the GTA-films, and the pH value was adjusted near 3.7 with 0.01 M HCl. The HA solution concentration was 2% (w/w). After a 24 h reaction time under slight stirring at room temperature (RT), gels were cast at room temperature under an extraction hood as circular films of 7.1 cm diameter. Then, sample discs of 7.5 mm and 4 mm diameter were cut.

TM-loaded films were synthesized in the same way as non-loaded films, but incorporating 100 mg TM during the solution stage. After casting, the TM concentration was 10% of the obtained film weights.

2.4. Film swelling, medium pH and medium osmolarity

Swelling behavior of cross-linked films was tested in distilled water at RT after sample drying to achieve constant weight ($n = 2$ in triplicates). The swelling ratio (SR) was calculated *via* the equation: $SR = W_s/W_d$; where W_s is the weight of the sample at equilibrium at each temperature and W_d is the weight of the dried sample.

Similar sized samples ($n = 3$ in duplicates) were used to determine whether pH changes occurred in the culture medium after film immersion for 24 h using a micropH meter 2001 from Crison Instruments S.A. (Barcelona, Spain). Culture medium osmolarity was tested after 48 h of sample immersion in a Micro-Osmometer Fiske 210 (Advanced Instruments Inc., Norwood, MA, USA), with all samples being previously sterilized by immersion in 70% ethanol. Measurements were performed in duplicate.

2.5. Stress-strain and morphological characteristics of films

Film thickness (mm) was measured by using a digimatic caliper MDC-1″ SFB (Mitutoyo Corporation, Kanagawa, Japan). Five measurements were made on each of three different films in the central and peripheral areas. The stress-strain properties of the films were studied using an Instron 3369 tester (Norwood, USA) in traction mode at 2 mm/min at RT (23 °C).

Characterization of the film structure and surface morphology was carried out using environmental scanning electron microscopy (ESEM). Films were first frozen in liquid nitrogen and manually fractured, before being immersed in twice distilled water for 2 hours, fixed in 70% glutaraldehyde, and immediately observed in a FEI Quanta™ 200 FEG microscope (Hillsboro, OR, USA).

2.6. Sterilization

In order to assess the feasibility of the film sterilization, two sterilization methods, including UV exposure and immersion in 70% ethanol, were tested. After sterilization, films were immersed in culture medium and maintained at 37 °C for 72 h in a CO_2 incubator (Galaxy®, New Brunswick Scientific Inc., Enfield, CT, USA). Possible bacterial contamination was excluded by visual examination at 24, 48, and 72 h.

2.7. Human corneal epithelial cell line

The SV40-immortalized human corneal epithelial (HCE) cell line was used (Araki-Sasaki et al., 1995) at passages 46 to 52. Cells were cultured in DMEM/F-12 supplemented with 15% fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 ng/ml EGF, 0.5% DMSO, 5 μg/ml insulin and 0.1 μg/ml cholera toxin (all from Invitrogen-Gibco), at 37 °C in a 5% CO2/95% air atmosphere. The media were changed every other day, and daily observations were made by phase contrast microscopy. HCE cells were used to determine whether films were biocompatible after a 24 h exposure in terms of: (a) cell viability, (b) cell proliferation, and (c) cell inflammation.

2.8. Cell viability assay

To evaluate the potential cytotoxic side effects of films, the XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt) test was used. HCE cells were plated in 96-well plates (10,000 cells/well) and grown until achieving 75% confluence (24 h). The culture medium was replaced with fresh phenol red-free RPMI, 48 h after film exposure, with films being previously sterilized overnight in 70% ethanol and rinsed in sterile PBS solution. XTT solution was then added immediately after film removal and the cells were incubated at 37 °C for 15 h. Plates were read in a SpectraMAXRM5 multidetection microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm (reference wavelength: 620 nm), and cell viability was measured immediately after film removal and after an additional 24 h recovery period in culture medium. Controls included cells without treatment and cells exposed to 0.001% benzalkonium chloride (BKC). The cell viability was calculated as a percentage with regard to control cells. Each test was performed three times in sextuplicate.

Cells exposed to crosslinked films were fixed in cold methanol and stained with hematoxylin and eosin in order to evaluate the possible morphological alterations. The slides were examined with a microscope Nikon Eclipse TS100 (Tokyo, Japan).

2.9. Cell proliferation assay

To measure cell proliferation rate alterations induced by film exposure, the Alamar Blue® assay was used, which incorporates a fluorescent dye that can be quantified. The HCE cells were plated in 24-well plates (40,000 cells/well) and grown for 24 h, and sterilized films were placed in the wells for 24 h. Then, films and medium were removed and the cells were incubated with Alamar Blue 10% solution in culture medium at 37 °C for 4 h. The medium with Alamar Blue was removed and 100 ml aliquots were transferred to a 96-well plate, with fluorescence being measured at 560 nm excitation and 590 nm emission wavelengths using a SpectraMax® M5 fluorescence microplate reader. Cells were allowed to grow for an additional 48 hours, and the above mentioned procedure was repeated every 24 h. Cells unexposed to films were used as controls. The proliferation index was then calculated after 24, 48 and 72 h as a percentage by referring to measurements in control cells. Three independent experiments were performed in duplicate.

2.10. Cell inflammation assay

To determine whether films induced an inflammatory response *in vitro*, an *in vitro* inflammation model was used as previously described (Enríquez-de-salamanca et al., 2008). Briefly, HCE cells were plated in 24-well plates (80,000 cells/well) and grown for 48 h. Cells were then maintained for 24 h in a serum-free, non-supplemented medium, before treating them with TNFα (25 ng/ml) (PeproTech, London, UK) for 24 h. Cell culture supernatants were collected and maintained at −80 °C until use. Secretion of IL-6 by HCE cells in response to TNFα stimulation was quantified using the human IL-6 ELISA Kit (Gen-Probe Incorporated, San Diego, CA, USA), as positive control of inflammation. HCE cells were exposed to HA-, PEGDE-, and PEGDEH-films for 24 h and then cell culture supernatants were collected in order to measure the IL-6 production as described above. Supernatants from unexposed control cells were also collected. At least three separate experiments were performed for each film and control in duplicates.

2.11. In vivo irritation and mucoadhesion tests

The potential ocular irritancy and/or damaging effects of the samples under test were evaluated using a slightly modified version of the Draize test (Draize et al., 1944). This was carried out on six eyes of New Zealand male rabbits, with a piece of each film of 4 mm in diameter being placed in the conjunctival fornix of the right eye, and the left eye being used as control. Sodium lauryl sulfate (LS) was utilized as the positive control of irritation.

Pre- and post-exposure evaluations of the eyelids, conjunctiva, cornea and iris were performed by external observation under adequate illumination, and additional information was provided using a binocular indirect ophthalmoscope (Neitz IO-α smoll pupils Tokyo, Japan) and 20 diopter lens (Nikon, Tokyo, Japan). For each observation, one drop of fluoresceine salt (0.25%) was instilled to contrast the potential corneal injury. The rating of ocular irritation or damage was scored by successive inspections at 0.5, 4, 8, 24, 48 and 72 h according to the outcomes listed in Table 1.

To determine the intensity of mucoadhesion, we used a method designed in our laboratory. Films were cut in 4 mm diameter discs and were placed in the inferior conjunctival fornix of 6 rabbit

Table 1
Rating of *In vivo* ocular irritation, according to Drazie test.

Score value	Formulation effects
0–8	No irritation
9–20	Mild irritation
21–40	Mild to moderate irritation
41–60	Moderate irritation
61–80	Serious injury
81–110	Very serious injury

Table 2
Score rating proposed to evaluate *In vivo* bioadhesion intensity.

Score value	Film behavior
0	Moves spontaneously out of the eye
1	Is maintained in the pouch, but does not adhere to the palpebral or bulbar conjunctiva permanently
2	Remains in the pouch and attaches to a particular conjunctive even when eyelid movement maneuvers are carried out
3	As 2, but in this case the film remains attached although rubbing maneuvers are made on the eyelid.
4	It remains attached even if an attempt is made to move it with a spatula

right eyes. Changes in film size were evaluated and the behavior of the films was scored (Table 2), evaluating modifications in size and adhesion with a binocular indirect ophthalmoscope (Neitz IO-α small pupils, Tokyo, Japan) and 20 diopter lens (Nikon, Tokyo, Japan).

2.12. In vitro timolol maleate (TM) release

The kinetics of TM release from films was assayed in a specially designed equipment which is currently in the process of being patented (Argentinean INPI patent pending, expedient: 20130100220) (Calles et al., 2013). Two samples (in duplicate) of TM-loaded PEGDE-films were placed in 10 ml of previously degasified Ringer's solution (Roux Ocefa, Buenos Aires, Argentina) which was used as the medium. The experiment was performed at 36 °C which is the highest temperature commonly measured on closed eyes (Saona Santos, 2006) under stirring, and 2 ml samples were collected at 30 min, 1, 2, 3, 4, 6 and 8 h, with fresh medium reposition. Samples were immediately diluted and filtered, and then the TM concentration was quantified by absorbance determination at 294 nm in a UV-160A UV-visible spectrophotometer (Shimadzu Corp., Kyoto, Japan).

The remaining load of TM was also determined in recovered films after 10 h of an *in vivo* IOP experiment. Groups of recovered films were placed in 10 ml of previously degasified Ringer's solution at 36 °C with stirring performed for 24 h. After medium filtration, the TM concentration was quantified by absorbance determination at 294 nm.

2.13. IOP measurements

Taking into account results of previous experiments, this test was performed with the PEGDE-films, and 4 mm diameter disk samples of loaded films were placed into the conjunctival fornix of twelve rabbits. The evolution of the intraocular pressure was measured with a Perkins MK2 tonometer (HS Clement Clarke, England), which was calibrated according to the manufacturer's instructions. Infant blepharostate was used to maintain the eyelids open during the measurements. A 50 μl mixture of topical anesthetic (0.5% solution of Proparacaine HCl) and fluoresceine salt (0.25% Solucion de GrantMR, AlconMR Montevideo- Uruguay) was applied to the cornea in order to improve the animal welfare during the test, and to achieve the necessary contrast for the IOP recordings

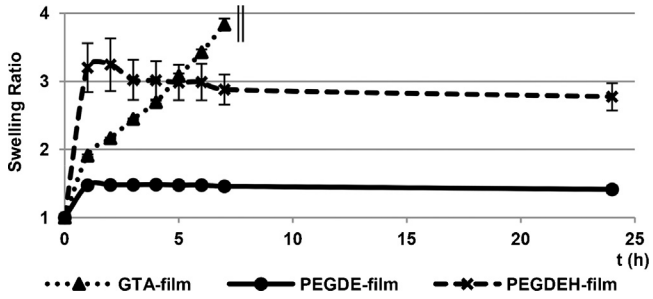

Fig. 1. Swelling behavior of crosslinked films in distilled water. The experiment was interrupted at the time indicated by the double bars.

(fluoresceine salt) before each measurement of intraocular pressure. Fluoresceine salt was used to outline and make clearly visible to the observer the area of cornea flattened by the split prism tonometer head (as the thickness and brightness of the resultant fluorescent meniscus tears govern the accuracy of IOP measurement). After allowing the rabbits to get accustomed to the experimental procedure, twelve animals were used in the experiments. At each interval, the measurements were repeated four times, and a mean of these was taken. In all cases, the IOP was measured at 0, 2, 4, 6, 8, and 10 h. The resting IOP was measured two or three times a day, until two days before drug application. In this way, the normal baseline of each animal was established before the subsequent treatment. The experiments were always carried out at the same time of the day. First, commercial eye drop was applied, and them 50 μl of Zopirol® (Timolol maleate, 0.5%, w/v) were administered to the rabbits according to the schedule described above for making comparisons. The results are expressed as the mean percentage change in IOP from the baseline.

2.14. Statistical analysis

When applicable, the results of the experiments were expressed as mean ± standard error of the mean (SEM). Differences were considered to be significant when $p \leq 0.05$, as shown in figures. Significant differences were determined by the Student's *t*-test.

3. Results and discussion

3.1. Film swelling, medium pH and medium osmolarity

The swelling assay (Fig. 1) revealed a high stability for both, PEGDE- and PEGDEH-films at least for two days, while GTA-films became unstable. They rapidly reach high levels of swelling and becoming fragile breaking the swollen structure. Although both GTA and PEGDE were found to be effective as crosslinking agent/s leading to insoluble materials, PEGDE and PEGDEH-films were more stable under water immersion.

The culture medium pH remained constant throughout the experiments, with the pH recorded never being outside the cell tolerance limits (pH: 6.5–8.5), and the culture medium osmolarity remained lower than 25% of the non-exposed control medium.

3.2. Stress-strain and morphological characteristics of the films

The film thickness for the stress-strain experiments was approximately 300 μm for PEGDE-films, 50 μm for PEGDEH-films and 100 μm for GTA-films. Stress strain studies (Fig. 2) revealed a marked change in the tensile properties of crosslinked films, with the GTA-film performance changing from ductile to brittle and all samples breaking before reaching the yield point. In contrast, films modified with PEGDE as a crosslinker agent exhibited plastic

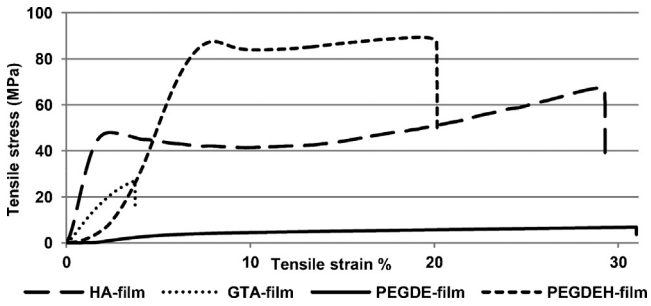

Fig. 2. Stress strain assay of crosslinked films at RT.

deformation. In fact, PEGDE is a homobifunctional crosslinker composed of two epoxy terminal groups with a long polyethylene glycol (PEG) chain between them. Although the crosslinking processes leads to increased polymer rigidity as a result of the restrained movements of the polymer molecules, the particular structure of PEGDE with long flexible PEG chains can mitigate these effects. Thus, the molecular structure of PEGDE gives the possibility of crosslinking HA without losing flexibility.

All swollen films showed homogeneous and smooth surfaces by ESEM (Fig. 3A and B), and pores of different sizes could be identified inside films after freeze-fracture, regardless of cross-linking method used.

3.3. Film sterilization

Both sterilization methods (UV radiation and 70% ethanol immersion) were efficient in preventing bacterial contamination. No bacterial proliferation was observed in the culture medium during the experiments using sterilized materials. This result is important from the experimental point of view considering that sterility is a key requirement for the production of ophthalmic medicines.

3.4. Viability, proliferation and inflammation of film-exposed corneal epithelial cells

The information from cell-material interactions could facilitate the design of the appropriate crosslinked HA film as delivery systems. Several authors have reported similar techniques and alternatives for HA crosslinking. However, depth studies of potential ocular toxicity have not been discussed on these studies for PEGDE (Collins and Birkinshaw, 2007; Tomihata and Ikada, 1997b).

In our studies, corneal epithelial cells exposed to sterilized PEGDE-films for 24 h exhibited viabilities superior to 90%, while sterilized GTA cross-linked formulations led to significant toxicity, as expected (Fig. 4). The cell viability after a 24-h recovery period in the culture medium of both PEGDE films exposed cells remained high, indicating no permanent noxious effects of these films in the cell physiology. Moreover, the morphological details of PEGDE-films exposed corneal epithelial cells remained intact (Fig. 5).

The corneal epithelial cell proliferation rate was slightly increased after exposure to the different films for 24 h (Fig. 6) whereas GTA-film exposed cells showed a significant reduction in proliferation. For the inflammation assay (Fig. 7), no increment in IL-6 expression, was revealed for PEGDE- or PEGDEH-films exposed cells when compared to non-inflamed control cells, indicating that film exposure did not induce an inflammatory-like *in vitro* response in corneal epithelial cells. Similar findings on eye cells

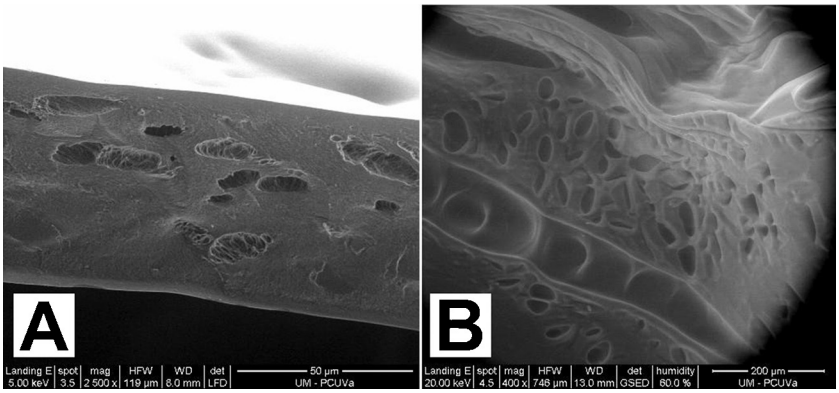

Fig. 3. ESEM photomicrographs of cross-linked swollen (A) PEGDE-film (2500×) and (B) GTA-film (400×).

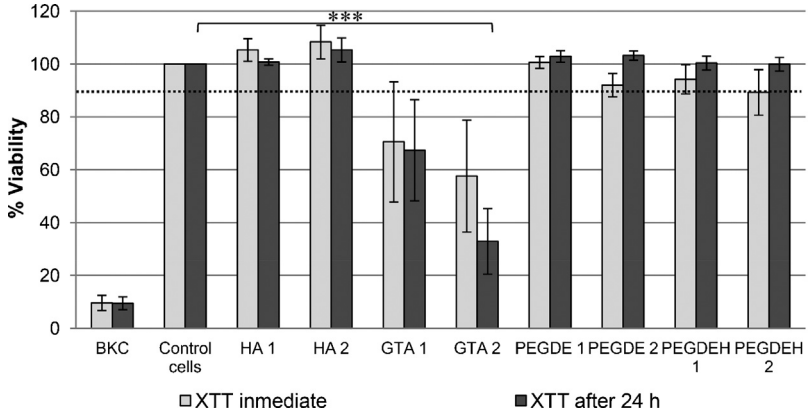

Fig. 4. Cell viability of HCE cells after film exposure for 24 h, measured either immediately or after a 24 h recovery period. (1, 2: one or two film discs). $^{***}p \leq 0.005$.

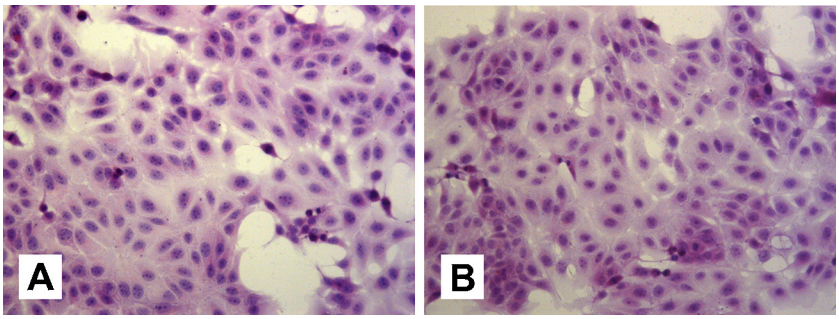

Fig. 5. Hematoxylin-eosin staining: (A) unexposed HCE control, 20× and (B) PEGDE-film exposed cells, 20×.

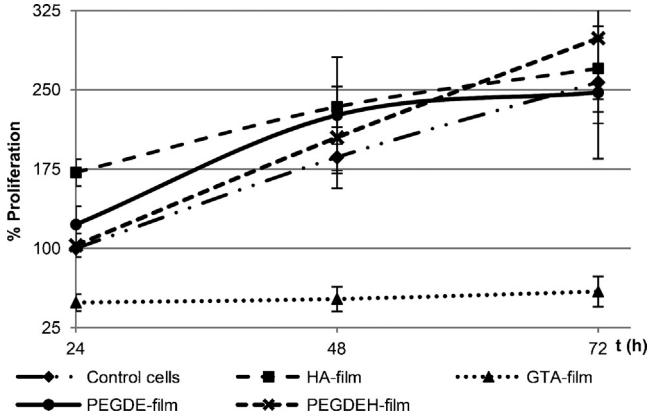

Fig. 6. Cell proliferation rate of HCE cells after film exposure for 24 h.

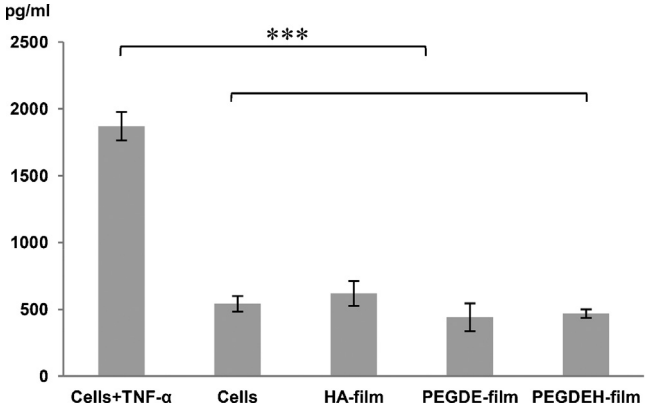

Fig. 7. IL-6 expression in culture medium of film-exposed corneal epithelial cells. $^{***}p \leq 0.005$.

compatibility with PEGDE-films were reported for carbodiimide crosslinked HA hydrogels in primary rabbit corneal endothelial cell cultures (Lu et al., 2008).

3.5. *In vivo* irritation and adhesion tests

As the graphical information shows (Table 3), the films crosslinked with GTA had a marked conjunctival and discreet corneal irritation, but no intraocular irritation. The inflammation appeared suddenly and was apparently accompanied by pain, as shown by the behavior of the rabbits seconds after placing the film in the conjunctival sac. Throughout the experiment, the inflammation increased and presented more secretions, thereby weakening the bioadhesion of the film. Although the film was removed after 24 h, the irritation remained for 72 h.

The results obtained using GTA were expected. Lai y cols (Lai et al., 2010), studied the biocompatibility of carbodiimide and GTA cross-linked hyaluronic acid hydrogels. Significant inflammatory cell infiltration and foreign body reaction were found in the eyes implanted with GTA–HA discs. These findings clearly indicate that GTA cross-linking greatly affects the biocompatibility between HA systems and ocular tissues.

After rinsing for 1 h in distilled water the GTA-films presented moderate conjunctival irritation, and minimal corneal irritation, but no intraocular irritation during the observed period. However, the rinsed GTA-films (R) were discarded, due to poor adhesion after 4 h. In addition the rinsing processes would produce a total loss of TM from the film. Encouragingly, the film made with PEGDE, showed minimal conjunctival and corneal irritation, with no intraocular irritation being observed during 72 h of study. These results are consistent with those described in Section 3.4.

Therefore, films prepared using PEGDE as crosslinking agent could be placed in the eye without causing irritating effects or tear turnover, maximizing the potential utility of these devices as drug delivery systems.

Table 3
In vivo bioadhesion irritation experiment. (R): Rinsed.

Sample						
Bioadhesion score (see Table 2)						
PEGDE-film	2	2	2.25	1.5	1.5	0.75
PEGDEH-film	1.75	1.75	1.75	1.25	0.5	0.25
GTA-film (R)	1.5	2	1	0	0	0
Irritation score (see Table 1)						
PEGDE-film	0	4	4	4	4	4
PEGDEH-film	0	4	4	4	6	4
GTA-film	10	34	33	24	–	–
GTA-film (R)	4	13	13	13	11	11
LS	58	32	–	–	–	–
Time (h)	0.5	4	8	24	48	72

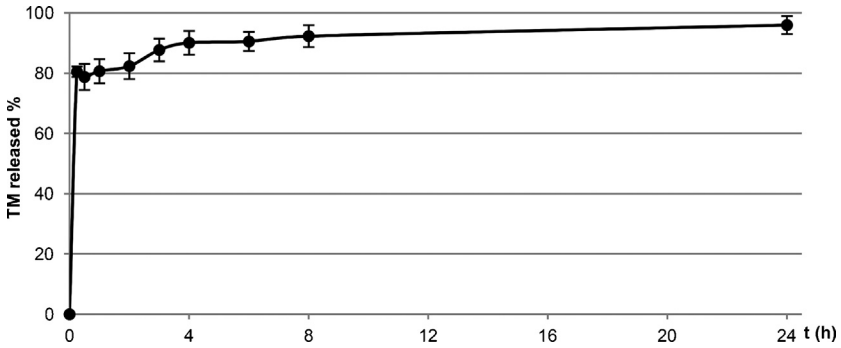

Fig. 8. TM release from PEGDE-film in Ringer's solution at 36 °C.

Following this line, bioadhesion is a key phenomenon in obtaining these types of system. In our case we must take into account the total adhesion time, the degree of adhesion and the effective releasing time.

Bioadhesion tests revealed excellent bioadhesive properties for the PEGDE and PEGDEH films, with both materials permitting adhesion for three days on the rabbits eyes (Table 3). As the PEGDE-films showed the best bioadhesion during the first 8 h of the experiment (colored area of the table), these were selected for the IOP assays with timolol.

3.6. *In vitro drug release*

Timolol maleate, a β-adrenergic receptor antagonist, provides an average IOP reduction of 20–35%. Since its approval (1979), timolol maleate has become the US Food and Drug Administration's (FDA) 'gold standard' drug for IOP reduction. Timolol, however, has significant cardiac side effects and usually requires dosing twice per day to maintain a well-controlled IOP. The molecule is extremely stable and highly water soluble, which makes it attractive for several methods of delivery, including novel drop formulations, implants, and injectables (Lavik et al., 2011). For these reasons, TM was used as model drug and used to load films.

As shown in Fig. 8, TM was released quickly from the PEGDE-film, and it is important to note that after 8 h TM was still being released from the formulation. A second experiment to determine whether loaded films contained TM after an *in vivo* IOP experiment demonstrated that the recovered films had a remaining load of 3.13 ± 0.566%.

Despite of the high release rate of TM because of its hydrophilic nature, the film has a certain ability to retain the drug within the polymeric system maintaining therapeutic concentrations over 8 hours (see Section 3.7). For exploratory purposes, high release rate drugs, as TM, are good models to demonstrate system's ability to achieve extended release patterns.

3.7. *IOP experiments*

The samples placed in the conjunctival fornix remained adhered to the bulbar conjunctiva (Fig. 9) throughout the experiment. The effect of the TM-loaded PEGDE-films on IOP is presented in Fig. 10. These results show that the application of the TM loaded film produced a remarkable decrease of IOP in normotensive rabbits, which reached the lowest point 2 h after administration (point "a"). Importantly, this reduction, at this time, was comparable with the commercial eye drops. Eight hours after administration (point "b"), the commercial formulation presented no hypotensive effects, while the film continued to reduce the IOP by approximately 15%. Also, in the case of the film, the hypotensive effect lasted for more than 10 hours (point "c"). The results point out that polymeric films had better sustained effect than the traditional ophthalmic drops, reducing the eye drainage of the active principle occurring normally in liquid or semi-liquid forms and preventing its rapid elimination from the eye surface.

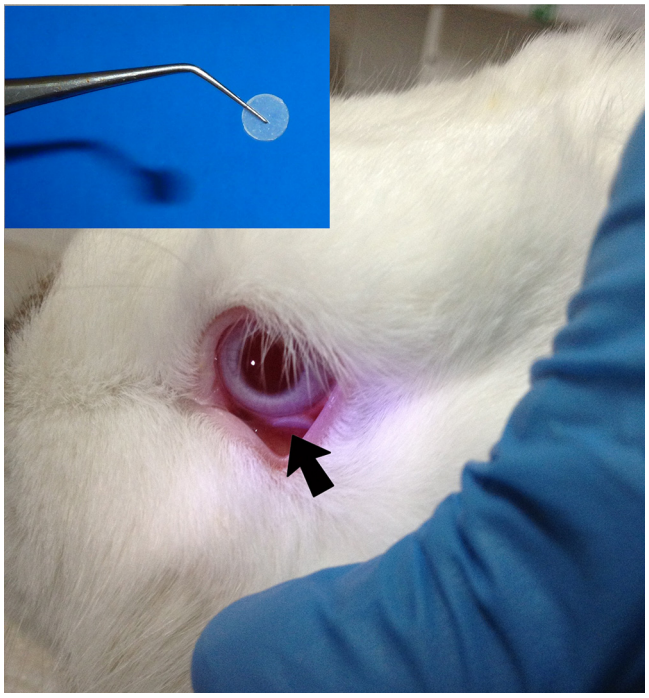

Fig. 9. TM loaded PEGDE-film sample disk in a rabbit's eye.

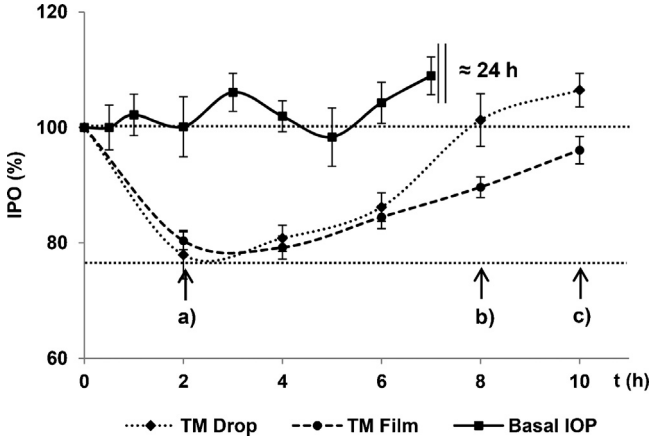

Fig. 10. IOP measurement in normotensive rabbits after TM commercial drops and TM-loaded PEGDE-film treatment.

4. Conclusions

Although both GTA and PEGDE were found to be effective as crosslinking agents leading to insoluble and water stable materials, the PEGDE-films and PEGDEH- films exhibited better mechanical performance. They show higher plasticity and better elongational properties, similar to those of unmodified HA-films. Also PEGDEH-films were more stable under water immersion.

For the *in vitro* biocompatibility assays good cell viability and proliferation rates were shown for PEGDE-films exposed corneal epithelial cells. However, cell exposure to PEGDE and PEGDEH crosslinked films did not increase the IL-6 production after 24 h, which demonstrated that these films did not induce *in vitro* inflammation.

As expected, the TM release was rapid and practically complete for the selected film. Although the permeability of TM through the cornea logically produced a marked decrease of IOP compared with the commercial eye drops, the adhesion of the film (anchored on the bulbar conjunctiva) caused a prolongation of this effect.

This new film can be considered a viable alternative to the conventional ophthalmic dosage form, due to its non-irritation performance and its ability to prolong the effect of the drug. However, further studies are required to evaluate its clinical efficacy and ability to load other drugs.

Acknowledgements

Te authors thank Laura Soriano Romaní, M.Sc. for her help with the cell proliferation experiments and Manuel Avella Romero, Ph.D. (University of Valladolid) for ESEM technical advice.

This work was supported by CONICET-Universidad Nacional del Sur Grant (Argentina) and an EADIC II-Erasmus Mundus Scholarship. Additional financial support was provided by a FEDER-CICYT Grant MAT-2010-20452-C03-01 (Spanish Ministry of Science and Technology) and Regional JCyL Grant VA132A11-2. We thank Dr. Paul Hobson, native speaker, for revision of the manuscripts.

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