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ABSTRACT

The aim of this work was to develop cyclosporine ocular inserts combining sodium hyaluronate (HA) and hydroxypropyl-β-cyclodextrin (HPβCD). The hydrogel inserts were crosslinked with poly(ethylene glycol) diglycidyl ether to improve the mechanical behaviour and achieve controlled drug release. Four different formulations were studied to elucidate the role of the HA:HP-βCD proportion on the physical characteristics and drug release patterns. All the formulations (300 μm thickness) showed porous surfaces and high swelling levels (after 30 min swelling ratio was ~10). Blank inserts were highly cytocompatible as revealed after direct contact with fibroblasts and chorioallantoic membrane (HET-CAM test). Once loaded with cyclosporine, inserts appeared translucent with a drug content of ~0.5% w/w. Cyclosporine release tests carried out under continuous flow of simulated lacrimal fluid revealed a controlled release of the peptide drug during the first 1 h. Conversely, differences among formulations were evidenced when the inserts were immersed in plenty volume of fluid (the vial method); inserts with low content in HP-βCD released the drug faster. These later inserts also facilitated cyclosporine accumulation into sclera (in the 5.6 to 32.7 $\mu g_{drug}/g_{sclera}$ range). Thus, inserts of HA and HPβCD crosslinked with PEGDE appear as a suitable platform for peptide loading and release to the ocular surface.

KEYWORDS: Cyclosporine; Cross-linked hydrogel; Cyclodextrin; Sodium hyaluronan; Ocular insert

1. Introduction

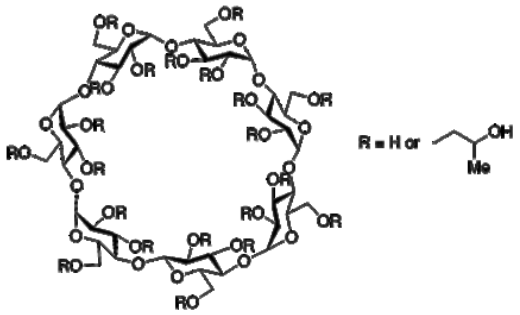
Currently, cyclosporine is one of the most used pharmacological agents for the treatment of several immune-mediated ocular surface disorders (Donnenfeld & Pflugfelder, 2009). Although this drug has been approved only for dry eye treatment, it can be used as an off-label therapeutic alternative for other ocular inflammatory events (Price & Price, 2006; Kim, Lee, Oh et al., 2018). Conventional topical cyclosporine administration is challenging for obtaining therapeutic drug levels in the target tissues, as the drug is poorly soluble in water (Mondon, Zeisser-Labouèbe, Gurny et al., 2011) and shows a partition coefficient of ~3 (el Tayar, Mark, Vallat et al., 1993). Only two topical formulations of cyclosporine have been approved for the treatment of dry eye syndrome, Restasis® (Allergan, USA), i.e. an anionic emulsion (0.5 mg/ml cyclosporine) approved by FDA in 2002 (Sall, Stevenson, Mundorf et al., 2000), and more recently Ikervis® (Santen, Tampere, Finland), a cationic nanoemulsion containing 1 mg/ml cyclosporine launched in Europe (Leonardi, Van Setten, Amrane et al., 2016). Ikervis® was demonstrated to be efficacious and, differently from emulsions and ointments, does not cloud vision thanks to its low viscosity; however stinging and pain have frequently been reported (Leonardi, Van Setten, Amrane et al., 2016). The extensive literature on cyclosporine delivery systems reflects the medical interest in this challenging drug (Lallemand, Schmitt, Bourges al., 2017; Yenice, Mocan, Palaska et al., 2008; Shen, Wang, Ping et al., 2009; Basaran, Yenilmez, Berkman et al., 2014; Karn, Cho, Park et al., 2013; Di Tommaso, Bourges, Valamanesh et al., 2012; Grimaudo, Pescina, Padula et al., 2018). Most of the formulations involve the encapsulation of cyclosporine in nanocarriers that can be instilled as eye drops, but the rapid drainage caused by tear turnover limits the ocular bioavailability. As an alternative, inserts in the form of hydrogel films may provide prolonged drug release on the eye surface to ensure drug levels that may favour ocular site-specific effects (Hermans, Van den Plas, Kerimova et al., 2014).

Hyaluronan (HA) is a suitable excipient for ophthalmic applications because of its high water binding capacity and pseudoplastic behaviour (Guter & Breuning, 2017). Additionally, this polymer can be an adjuvant in the treatment of dry eye syndrome thanks to its similar rheological behaviour to the mucus and its protective role at the cornea/conjunctiva epithelium (Debbasch, De La Salle, Brignole et al., 2002). Indeed, combinations of cyclosporine and HA have been tested as eye drops (Kim, Lee, Oh et al., 2018). Despite the evident advantages of HA for ophthalmic applications, poor biomechanical properties and fast dissolution in water might discourage its employment for the design of sustained release formulations (Guter & Breuning, 2017). During the last decades, chemical crosslinking of HA has been tried to meet the requirements for its applications as biomaterial (Choi, Yoo, Lee et al., 2015). For example, films of hyaluronic acid-itaconic acid cross-linked with poly(ethylene glycol) diglycidyl ether (PEGDE) have demonstrated good ocular safety and controlled release of timolol maleate for treatment of intraocular pressure (Calles, Tártara, Lopez-García et al., 2013).

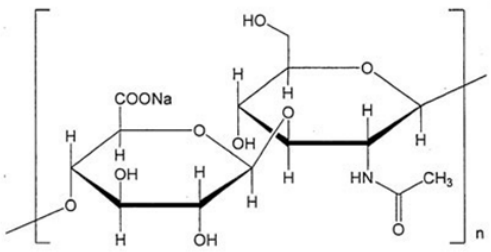
Differently to hydrophilic drugs, the yield and homogeneity of hydrophobic drug loading into hydrogel films is still a challenge. In the case of drugs forming inclusion complexes with cyclodextrins, such is the case of cyclosporine, addition of cyclodextrin to chitosan dispersions before film casting has been tested but no significant effect on drug release control was recorded (Hermans, Van den Plas, Kerimova et al., 2014). As an alternative, cyclodextrins can be chemically grafted to the polymer chains during cross-linking to create binding domains rich in cyclodextrin cavities that can host the drug and regulate the release as a function of the affinity constant (Concheiro & Alvarez-Lorenzo, 2013; Rodriguez-Tenreiro, Alvarez-Lorenzo, Rodriguez-Perez et al., 2007). In ophthalmology, cyclodextrins are commonly explored for increasing apparent solubility of hydrophobic drugs on the lachrymal fluid, creating a favourable concentration gradient for cornea

penetration while improving chemical stability (Johannsdottir, Kristinsson, Fülöp et al., 2017).

The aim of this work was to design mucoadhesive cyclosporine inserts using HA and hydroxypropyl-β-cyclodextrin (HPβCD) to be placed in the conjunctival sac (**Fig. 1**). Four different networks were prepared using PEGDE as cross-linker and characterized in terms of microstructure, water uptake and mechanical properties. Cytocompatibility with fibroblasts and HET-CAM tests were performed to assess biocompatibility of blank inserts. Cyclosporine was loaded into inserts by soaking, and then release profiles were investigated using two different setups trying to mimic the tear fluid renovation dynamics. Preliminary *ex vivo* penetration experiments were carried out and cyclosporine levels into the sclera were quantified.



Hydroxypropyl β-cyclodextrin



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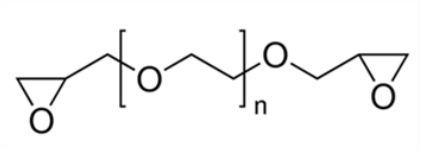

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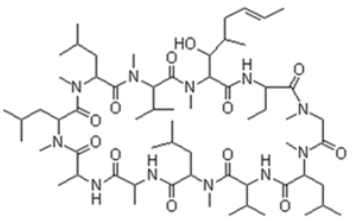


Cyclosporine A

Figure 1. Chemical structure of hydroxypropyl-β-cyclodextrin (HPβCD), sodium hyaluronate (HA), poly(ethylene glycol) diglycidyl ether (PEGDE), and cyclosporine.

2. Materials and Methods

2.1 Materials

Cyclosporine ($C_{62}H_{111}N_{11}O_{12}$, MW 1202.61 Da, crystalline solid) was from ThermoFisher Scientific (Karlsruhe, Germany). Sodium hyaluronate (HA, MW 360,000 Da, glucuronic acid 47.4 %) was purchased from Guinama (La Pobla de Valbona, Spain). Hydroxypropyl β-cyclodextrin (HPβCD, Kleptose® HP oral grade, MW 1399 g/mol) was from Roquette (Lestrem, France). Ethanol absolute AnalaR Normapur® (Reagent Ph Eur, Reagent USP) was from VWR Chemicals (Milano, Italia). Poly(ethylene glycol) diglycidyl ether (PEGDE, MW 526 g/mol, density 1.14 g/ml) and trifluoroacetic acid (TFA, MW 114.02 g/mol), were purchased from Sigma Aldrich® (Saint Louis, MO, USA). Potassium hydroxide (MW 56.10 g/mol) was from Honeywell Fluka™ Chemika (Morris Plains, NJ, USA). Water was purified using reverse osmosis (resistivity>18MΩ·cm, MilliQ, Millipore®, Spain). Buffered solution was simulated lachrymal fluid (SLF; $CaCl_2$ 0.06 mg/ml, $NaHCO_3$ 2.18 mg/ml, NaCl 6.7 mg/ml, pH 7.4 adjusted with HCl 1 N).

2.2 Cyclosporine quantification method

Cyclosporine was analysed using a reverse-phase Nova-Pack C_{18} cartridge (150x3.9 mm, 4 µm, Waters, Milford, MA, USA) and a C_{18} guard column (3.2x0.8 mm, Security Guard™ Cartridge, Phenomenex, Torrance, USA) thermostated at 65°C using a HPLC-UV system (Infinity 1260, Agilent Technologies, Santa Clara, CA, USA). The mobile phase was a mixture CH_3CN:water with 0.1% TFA in 65:35 (v/v) ratio, pumped at 1.6 ml/min. The injection volume was 100 µl and absorbance was monitored at 230 nm. Using these conditions, cyclosporine retention time was ca. 5 min. Two calibration curves were built in the concentration ranges 0.25-5 and 2.5-50 µg/ml. The HPLC method was previously validated for precision and accuracy (Grimaudo, Pescina, Padula et al., 2018).

2.3 Preparation of blank crosslinked inserts

HA-HPβCD crosslinked inserts were prepared by a solvent casting evaporation technique. HA (4.3% w/v) and HPβCD solutions (22.7-113-227-454 mg/ml) were both prepared in KOH 2 mM and mixed to obtain a final HA concentration of 4% w/v (10 ml final volume). Different HPβCD concentrations were tested to assess the role of cyclodextrins concentration on insert characteristics (final HPβCD concentrations, 0.15-0.75-1.5-3% w/v). PEGDE (1 ml) was then added as cross-linker to HA-HPβCD dispersions (10 ml). After 12 h reaction at 25°C, gels were cast on a Petri dish (Ø=5 cm) and dried for 48 h at 37°C. Insert films (coded B1-B2-B3-B4, see **Table 1**) were then cut as 9 mm discs, immersed in 1 ml of absolute ethanol overnight to remove the cross-linker in excess (37°C, 150 osc/min, VWR® Incubating Mini Shaker, Spain), and finally dried for 20 min at room temperature to allow for ethanol evaporation.

2.4 Characterization of blank inserts

The thickness of four dried inserts (0.9 cm in diameter) was measured using a Caliper Digital Electronic (Fowler™, Newton, MA, USA) and the weight was recorded. Blank cross-linked inserts (0.9 cm in diameter) were weighed (W_d) and then immersed in SLF pH 7.4 (1 ml) at room temperature. The weight increase of the swollen films (W_s) was measured every 30 min for 2 h in triplicate. The swelling ratio (SR) was calculated as follows:

$$SR = \frac{(Ws - Wd)}{Wd} \quad \text{(eq. 1)}$$

Mechanical properties were evaluated by estimating the puncture strength (PS) using a TA-TX Plus Texture Analyzer (Stable Micro Systems, Surrey, UK). Blank inserts (0.9 cm in diameter) were fixed into a support rig for avoiding the slippage. A stainless steel spherical ball probe (P/5S; 5 mm) moved downward at rate of 1 mm/s. The PS was estimated by

normalizing the maximum force recorded before rupture by the mean thickness of the inserts.

Scanning electronic microscopy images of blank inserts were obtained using a field emission scanning electron microscopy (FESEM; FESEM Ultra Plus, Zeiss, Oberkochen, Germany). Inserts were placed onto metal plates and then sputter-coated with 10 nm thick iridium film (Q150T-S, Quorum Technologies, Lewes, UK) before viewing.

2.5 Biocompatibility assessment for blank inserts

In vitro cytocompatibility of blank cross-linked inserts was evaluated against murine fibroblasts (CCL-163, ATCC, USA). Cells were seeded in a 12-well plate ($1.5 \cdot 10^5$ cells/plate) and grown for 24 h at 37°C/5% CO_2. Fibroblasts were cultured in 2 ml DMEM medium (Dulbecco's modified Eagle's Medium, 10% v/v fetal bovine serum, 1% v/v penicillin-streptomycin). After 24 h culture, 9-mm inserts (dried) were cut into two halves, sterilised under UV lamp for 20 min each side and placed in contact with cells for 48 h at 37°C/5% CO_2. Negative controls included cells with no contact to inserts. At the end of the culturing period, cell medium and formulations residues were removed, cell viability was determined using Cell Proliferation Reagent WST-1 (Sigma-Aldrich®, Saint Louis, MO, USA). Briefly, 50 µl of reagent and 1 ml of DMEM without serum were added to each well. After 20 min absorbance of final solutions was read at 450 nm (UV Bio-Rad Model 680 microplate reader, USA). Cell viability was calculated as a percentage of living cells with regard to controls.

Fertilized hen's eggs (50-60 g; Coren, Spain) were used to perform an irritation test on the chorioallantoic membrane (HET-CAM) (Steiling, Bracher, Courtellemont et al., 1999). Eggs were incubated in a climatic chamber (Ineltec, model CC SR 0150, Barcelona, Spain) at 37°C and 60% relative humidity for 9 days. Eggs were turned 3 times per day, while the last day they were placed with the wider extreme upward. The eggshell was partially

removed (2 cm in diameter) on the air chamber using a rotary saw (Dremel 300, Breda, Netherlands). The inner membrane was wet with 0.9% NaCl (for 30 min in the climatic chamber) and then carefully removed exposing the CAM. Formulations (9-mm blank inserts) were placed on the CAM of different eggs. 0.9% NaCl and 5 M NaOH solutions were used as negative and positive controls, respectively. The vessels of CAM were observed for 5 min, recording the times at which hemorrhage (Th), vascular lysis (Tl), or coagulation (Tc) occurred. The irritation score (IS) was calculated with the following formula:

$$IS = 5 \times \frac{301-Th}{300} + 7 \times \frac{301-Tl}{300} + 9 \times \frac{301-Tc}{300} \quad \text{(eq. 2)}$$

Photographs of CAM vessels were taken with a digital camera (Canon SX 260HS, without zoom) 5 min after the beginning of the assay and downloaded in the computer in JPEG format. GIMP® software was used to obtain a representative zone of the membrane with the tested formulations.

2.6 Cyclosporine loading and release

For obtaining cyclosporine-loaded inserts, blank formulations (0.9 cm in diameter inserts) were immersed into 1 ml of 5 mg/ml cyclosporine in EtOH:H_20 75:25 vol/vol mixture overnight and then dried for 2 hours at 40°C (formulations coded as F1-F2-F3-F4, **Table 2**). After insert soaking and drying, two different setups were used to study cyclosporine release

Vial method: Drug-loaded inserts were placed in a vial containing 5 ml SLF at 37°C under magnetic stirring and drug release was monitored up to 8 h. 0.3 ml of the release medium were sampled at predetermined time points and immediately replaced with the same volume of fresh SLF. After the last sampling time (8h) the inserts were kept in the release medium under magnetic stirring overnight at room temperature to achieve the complete

disintegration of the formulations. Then, a final sampling was performed for mass balance calculations. Collected samples were analysed by HPLC-UV, as described in section 2.2. The mechanism of cyclosporine release from inserts was further investigated by fitting the experimental data to Ritger-Peppas equation (Ritger & Peppas, 1987).

Inclined plane: Cyclosporine-loaded inserts (0.6 cm in diameter, volume of loading medium 444 µl) were placed on a glass Petri disk (9 cm in diameter) by applying 20 µl of 0.9% NaCl and then the setup was inclined at 45°. After 2 min, SLF was flushed onto the inserts at 60 µl/min flow rate at room temperature, using a syringe pump (Harvard Apparatus, Holliston, MA). Samples were collected every 10 min up to 1 h and then analysed. At the end of the experiment, each insert was soaked in 4 ml of 0.9% NaCl up to complete dissolution; after centrifugation (12 min at 12000 rpm, Scilogex D3014 High Speed Micro-Centrifuge, Rocky Hill, Connecticut, USA) the drug was quantified.

2.7 Ex vivo penetration tests in isolated porcine sclera

Fresh porcine eyes were isolated from Landrace and Large White animals (age 10–11 months, weight 145–190 kg, female and male animals) supplied from a local slaughterhouse (Annoni S.p.A., Parma, Italy). The eyes were kept in PBS at 4°C until the dissection, which occurred within 2 h from the enucleation. After the removal of muscular and connective tissues around the eye-bulb, the anterior segment of the eye was circumferentially cut behind the limbus and removed. The obtained eyecup was then cut and everted. The neural retina and the choroid-Bruch's layer were discarded and the obtained sclera was frozen at -80°C.

Penetration experiments were performed using Franz-type diffusion cells (area 0.6 cm^2). The scleral tissue was mounted with the choroidal side facing the receptor compartment. The receptor medium consisted of 4 ml of NaCl 0.9% solution at 37°C, magnetically stirred. Drug-loaded (dried) inserts were applied on the sclera after applying 25 µl of 0.9%

NaCl solution on the tissue to assure insert adhesion to the sclera. After 8 hours (n=3), the receptor fluid was sampled, the formulation was removed, the contact area was isolated and washed with 0.9% NaCl solution. Cyclosporine was then extracted by adding 1 ml of mixture CH_3CN:1% CH_3COOH (87:13) overnight at room temperature. Extraction solutions were filtered (0.45 µm regenerated cellulose filters, Phenomenex, Torrance, USA) before HPLC-UV analysis. The extraction method was previously validated (Grimaudo, Pescina, Padula et al., 2018)

2.8 Statistical analysis

Differences were analyzed using ANOVA and multiple range test (Statgraphics Centurion XVI 1.15, StatPoint Technologies Inc., Warrenton VA). Differences were considered statistically significant when $p<0.05$. In the text, all data are reported as mean value±sd.

3. Results and Discussion

Cyclosporine shows unfavourable physicochemical properties with a consequent very low permeability through biological tissues (Lallemand, Schmitt, Bourges al., 2017). Efficient topical delivery of cyclosporine for the treatment of ocular diseases affecting the anterior segment is a real challenge (Di Tommaso, Behar-Cohen, Gurny et al., 2011). Cyclosporine may be effective also for the treatment of severe intraocular inflammations affecting the posterior segment of the eye when administered intravenously (Nussenblatt, Palestine, Chan et al., 1991), but serious adverse events such as nephrotoxicity and/or hypertension may occur (Mihatsch, Kyo, Morozumi et al., 1998).

The present study was aimed at designing an ophthalmic delivery system able to increase cyclosporine residence time on the ocular surface. Ophthalmic inserts could offer an increased ocular residence, controlled drug release and accurate dosing. The improved

residence time of the drug in the conjunctival sac enhances its ocular availability and thus leads to less side effects, with less frequent administration (Saettone & Salminen, 1995).

HA was selected as main component for developing crosslinked inserts. PEGDE, composed of two epoxy terminal groups with a long polyethylene glycol chain between them, was selected as homobifunctional cross-linker (Calles, Tártara, Lopez-García et al., 2013). Chemical modification of HA typically involves the carboxylic acid and/or the alcohol groups of its backbone (Segura, Anderson, Chung et al., 2005) and particularly the alcohol groups can be modified using diglycidyl ethers (Choi, Yoo, Lee et al., 2015; Tomihata & Ikada, 1997). Cross-linkers containing diglycidyl functional groups can be used also for crosslinking cyclodextrins, creating hydrophilic networks (Rodriguez-Tenreiro, Alvarez-Lorenzo, Rodriguez-Perez et al., 2007; Moya-Ortega, Alvarez-Lorenzo, Sigurdsson et al., 2012). HPβCD was added as a functional moiety to the hydrogel structure for enhancing cyclosporine ocular penetration by increasing cyclosporine apparent solubility at the ocular surface. Mild alkaline conditions were employed for cross-linking as hydroxide ions catalyse the ring opening of the oxacyclopropane of the EGDE to react with hydroxyl groups (Concheiro & Alvarez-Lorenzo, 2013). Compared to other covalent cross-linking approaches that involved previous functionalization of both HA (e.g. with amino groups) and HPβCD (e.g. divinyl sulfone derivative) (Fiorica, Palumbo, Pitarresi et al., 2017), PEGDE can directly induce binding of both components through ether bonds (Tomihata & Ikada, 1997).

PEGDE proportion was fixed at 10% vol/vol after various first trials; less amount did not provide gels and higher proportions resulted in brittle networks. Taking into account the molecular weight of the repeating disaccharide units of HA (N-acetyl-D-glucosamine and D-glucuronic acid) of about 410 Da, in the reaction mixture the HPβCD:HA repeating unit:PEGDE mole ratio was approx. 1:9:18. This means that the reactive groups of the bifunctional cross-linker (18x2) are sufficient to react with at least half of the total hydroxyl

groups of HPβCD and HA (21+36 = 57). This finding agrees well with previous studies reporting on the proportion of EGDE suitable to provide hydrogel networks of HPβCD (Rodriguez-Tenreiro, Alvarez-Lorenzo, Rodriguez-Perez et al., 2006) and also with those involving cross-linking of HA and itaconic acid with PEGDE under acidic conditions (Calles, Tártara, Lopez-García et al., 2013).

3.1 Blank inserts

After crosslinking reaction overnight, dried insert films showed an oily appearance because of excess of cross-linker. Thus, 9 mm discs were cut and soaked overnight in ethanol to remove unreacted cross-linker. After this step, inserts became white and opaque (**Figure 2a**), with a mean thickness of 300 μm (**Table 1**).

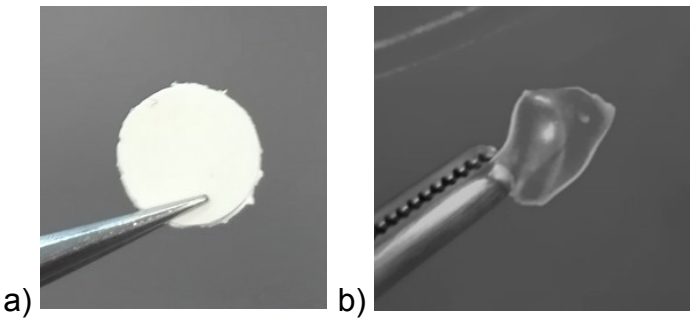


Figure 2. Photographs of inserts (dried after soaking in ethanol) before drug loading (blank insert) (**a**) and after soaking in cyclosporine solution in EtOH:H_20 75:25 vol/vol and dried (drug-loaded insert) (**b**).

Four formulations with different content in HPβCD (**Table 1)** were prepared to elucidate the effect of the HA:HPβCD ratio on the physical properties and drug release patterns. No different results were obtained in terms of thickness as a function of HPβCD concentration.

Table 1. Composition (relative contents in HA and HPβCD) and characteristics of blank inserts (9 mm in diameter) after cross-linking and drying (n=4). PEGDE was added at a fix concentration of 10% vol/vol with respect to the HA/HPβCD solution.

Insert	HA: HPβCD ratio (% w/w)	Thickness (μm)	Weight (mg/cm^2)
B1	96.4:3.6	275±66	12.54±2.64
B2	84.2:15.8	295±30	12.85±1.77
B3	72.7:27.3	283±78	13.84±1.42
B4	57.1:42.9	320±80	19.18±1.90

Given the high hydrophilicity of HA, a change in its content can translate into a different swelling and softness, which are critical to avoid mechanical irritation to the conjunctiva. All blank cross-linked inserts presented a high capability of absorbing water (**Figure 3**). Formulations rapidly reach similarly high swelling levels, but they showed different disintegration time. In fact B1 and B2, with the lower content in HPβCD, broke down after 1 h, while B3 and B4 structures remained intact at least up to 2 h. These results suggest a reinforcement of the HA entanglement in the presence of HPβCD. The cyclodextrin can promote the cross-linking acting as multifunctional tie-junction on the polymeric network. This hypothesis relies on the fact that the epoxy groups of PEGDE, used as cross-linker, are prone to react with the hydroxyl groups of cyclodextrins under alkaline conditions (Rodriguez-Tenreiro, Alvarez-Lorenzo, Rodriguez-Perez et al., 2006; Rodriguez-Tenreiro, Alvarez-Lorenzo, Rodriguez-Perez et al., 2007). In any case, the high degree of swelling

showed by all the inserts can be advantageous to ensure patient comfort (Foureaux, Franca, Nogueira et al., 2015).

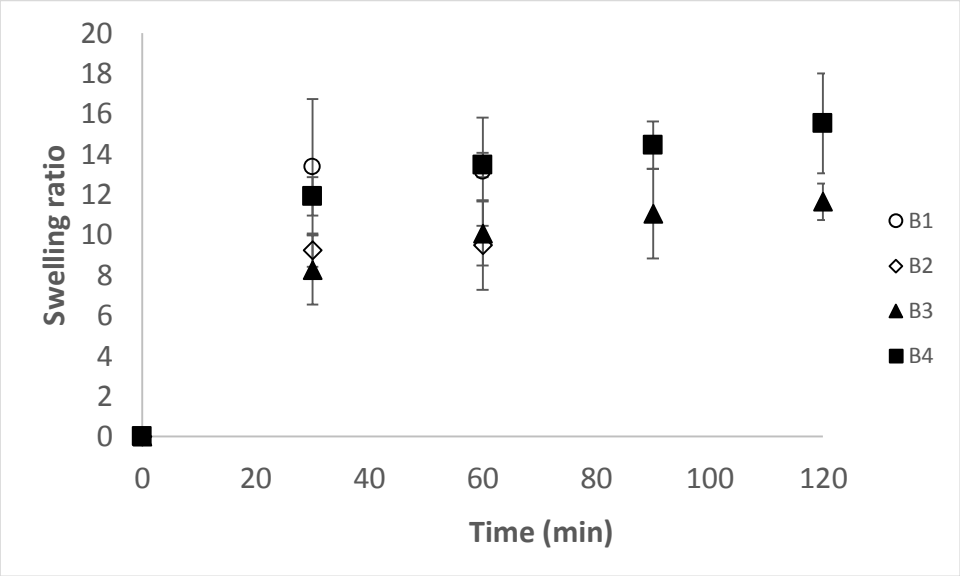


Figure 3. Swelling behaviour in SLF at room temperature of blank crosslinked inserts (mean±sd, n=3)

SEM images of blank cross-linked inserts (**Figure 4**) revealed porous surfaces that should facilitate the entry of water. B4 inserts showed particles on the surface which may be due to HPβCD in excess that was not incorporated inside the hydrogel structure during the cross-linking reaction but aggregate on the surface, as observed for other cross-linked networks prepared with other components (Prabhu, Dubey, Parth et al., 2010).

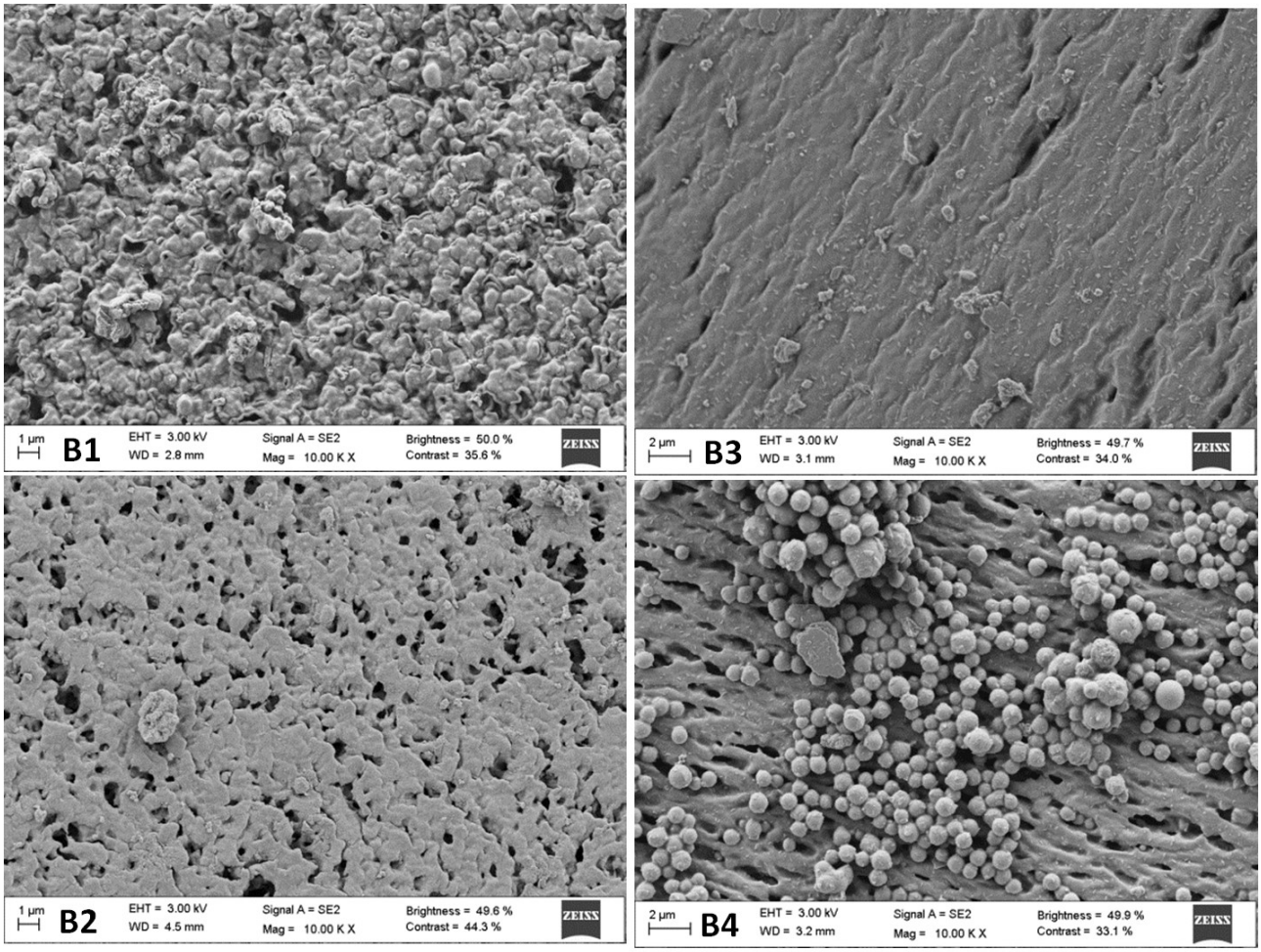
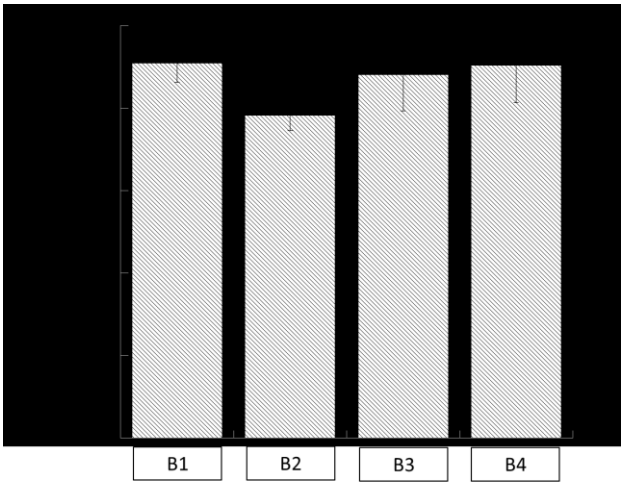


Figure 4. SEM micrographs of surface of blank cross-linked inserts (after soaking in ethanol and drying).

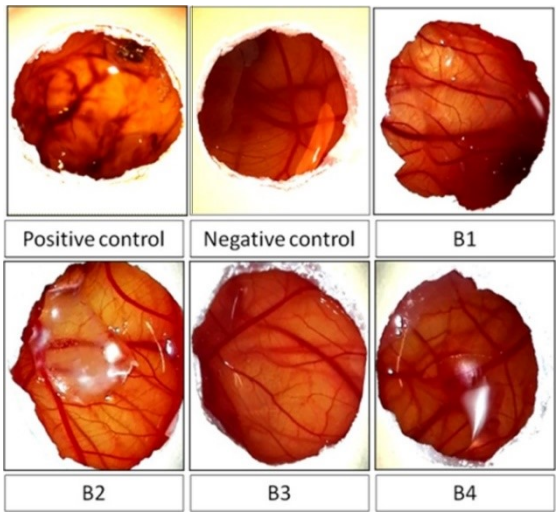
Regarding mechanical properties, all blank dried inserts were quite brittle and showed similar puncture strength (PS ~80 N/mm) at break. The highest PS values were recorded for the intermediate contents in HPβCD (**Figure S1**); the differences were statistically significant (ANOVA $F_{3,8\ d.f.}$= 4.30; p=0.0439). These data could be justified by insertion of cyclodextrins on the hydrogel backbone, as networks with large contents in cyclodextrin and cross-linking agents have been found to be prone to fragment (Rodríguez-Tenreiro, Alvarez-Lorenzo, Rodriguez-Perez et al., 2007).

Ocular compatibility of the inserts was evaluated in terms of cytocompatibility with fibroblasts and absence of irritation of the chorioallantoic membrane. As shown in **Figure**

5A, cell viability reduction was lower than 20% after contact with murine fibroblasts for 48 h, thus showing a good biocompatibility of the formulations and that no toxic substances are being leaked from the inserts (Kronek, Kroneková, Lustoň et al., 2011). No statistically significant differences were observed among the 4 formulations (ANOVA $F_{3,8\ d.f.}$= 2.12; p=0.1757). On the other hand, the HET-CAM test may provide preliminary information about ocular irritancy of formulations due to the similarity of the chorioallantoic membrane of an embryonated hen's egg to the vascular conjunctiva of the eye. Irritating effects after conjunctiva exposure of inserts can be predicted from changes in the chorioallantoic membrane (Kishore, Surekha, Sekhar et al., 2008). In our case, none of the inserts caused haemorrhage, vessels lysis or coagulation, similarly to the control saline solution (**Figure 5B**). These events occurred only in case of the positive control (NaOH 5 M, IS equal to 20), resulting in a rosette-like coagulation. Overall, these results indicate that the blank cross-linked inserts do not leach residual toxic secondary products of the crosslinking reaction and/or cross-linker in excess.



a)



b)

Figure 5. Murine fibroblasts viability after direct contact with blank crosslinked inserts for 48 h (error bars correspond to standard deviation, n=3) (**A**). HET-CAM photographs of blank inserts

after 5 min form the beginning of the test, negative and positive control were 0.9% NaCl and 5 M NaOH, respectively (**B**).

3.2 Cyclosporine-loaded inserts

One of the most important drawbacks of hydrogels relates to the difficulty of loading hydrophobic drugs, which dislike the aqueous phase of the network and may also find difficult the diffusion if the drug molecular size is large. Indeed, it was not possible to load cyclosporine into the blank insert by using an EtOH solution because of the limited swelling of the networks (data not shown). Differently, using EtOH:H_20 75:25 vol/vol mixture as loading medium we reached a compromise between drug solubility, insert swelling and insert stability. It was expected that cyclosporine penetrated into the inserts and formed inclusion complexes with the HPβCD moieties, which should contribute to an enhanced network/solvent partition coefficient (Rodriguez-Tenreiro, Alvarez-Lorenzo, Rodriguez-Perez et al., 2007; Concheiro & Alvarez-Lorenzo, 2013).

Various cyclodextrins have been reported to form inclusion complexes with cyclosporine (Johannsdottir, Jansook, Stefánsson et al., 2015). Particularly, HPβCD renders A_L-type phase diagrams and can linearly increase cyclosporine apparent aqueous solubility (Aksungur, Demirbilek, Denkbaş et al., 2012). Although in case of macromolecules, such as cyclosporine, the cavity dimension is too small for the entire accommodation of the guest (Morrison, Connon & Khutoryanskiy 2013), the lipophilic side chains of cyclosporine are expected to accommodate in the hydrophobic cavity of the cyclodextrin (Hermans, Weyenberg & Ludwig, 2010).

The EtOH:H_20 75:25 vol/vol medium allowed an excellent solubilisation capability for cyclosporine, while the low proportion of water led to a controlled swelling of the inserts (about 70±25%). Relevantly, this water proportion also caused the inserts to become transparent as the medium turned out to be a better solvent for HA (Bicudo & Santana,

2012). The entry of solvent into the insert caused partial polymer chains relaxation and facilitated the penetration of the peptide inside the hydrogel structure. After cyclosporine loading, inserts appeared traslucent (**Figure 2b**). Cyclosporine-loaded inserts F1-2-3-4 (obtained by loading B1-2-3-4, respectively) had a final content in cyclosporine of 0.19±0.12, 0.13±0.08, 0.12±0.07, and 0.11±0.07 mg_{drug}/cm^2, respectively.

Surprisingly, the amount of drug loaded was not related to the theoretical amount of HPβCD. The inserts sorbed ca. 1/100 of the cyclosporine dispersed in the loading medium, which led to a mean drug content of 0.5 % w/w. This concentration is comparable to the loading solution (5 mg/ml), indicating that the drug is weakly interacting with HPβCD. In previous reports, chitosan-based films containing HPβCD-cyclosporine complexes were prepared dispersing 0.26% w/w drug in the polymer solution resulting in the physical trapping of drug nanoparticles, attaining a maximum content in drug of 8.3% (Hermans, Van den Plas, Kerimova et al., 2014). Compared to commercially available Restasis® (0.5 mg/ml) and Ikervis® (1 mg/ml) that deliver 25 or 50 µg dose per eye drop, insert pieces of 10 mg (area 0.636 cm^2) could provide the same amount as 2 or 1 drop, respectively. It should be noticed that clearance from the eye surface is faster for ophthalmic drops than for the drug being released from the insert and thus higher ocular bioavailability could be obtained with the inserts.

Ocular inserts are intended to undergo gradual erosion while releasing the drug after being placed in the fornix of the conjunctival sac of the lower eyelid, where they are exposed to tears (Saettone, & Salminen, 1995; Foureaux, Franca, Nogueira et al., 2015). The entry of lachrymal fluid causes the relaxation of polymer chains leading to the formation of a gel layer. While the thickness of the layer increases as more and more the fluid enters into the insert, matrix erosion at the surface occurs. The erosion of the matrix following the swelling process depends on the polymer structure and polymer chain relaxation (Kumari, Sharma,

Garg et al., 2010). Drug release depends on the hydrophilicity of the drug, interaction of the drug and polymer, swelling and polymer network degradation. As larger molecules might experience more resistance to diffusion from the polymeric network, release of cyclosporine may depend more on erosion of the matrix than on diffusion.

Different tailored methods have been proposed in literature to evaluate *in vitro* release of ophthalmic dosage forms as no specific methods are reported in Pharmacopoeias (Baranowski, Karolewicz, Gajda et al., 2014). Although it is difficult to mimic the *in vivo* conditions, *in vitro* studies may allow comparing the behaviour of different ophthalmic drug delivery systems (Pescina, Macaluso, Gioia et al., 2017).

Firstly, cyclosporine release from inserts was tested using an experimental setup that involved the flow-through of simulated lacrimal fluid on the formulations, resembling to some extent the physiological conditions. As shown in **Figure 6**, all insert formulations profiles provided almost linear release rate in the first 1 h. No statistically different results were obtained in relation to the different composition of the inserts.

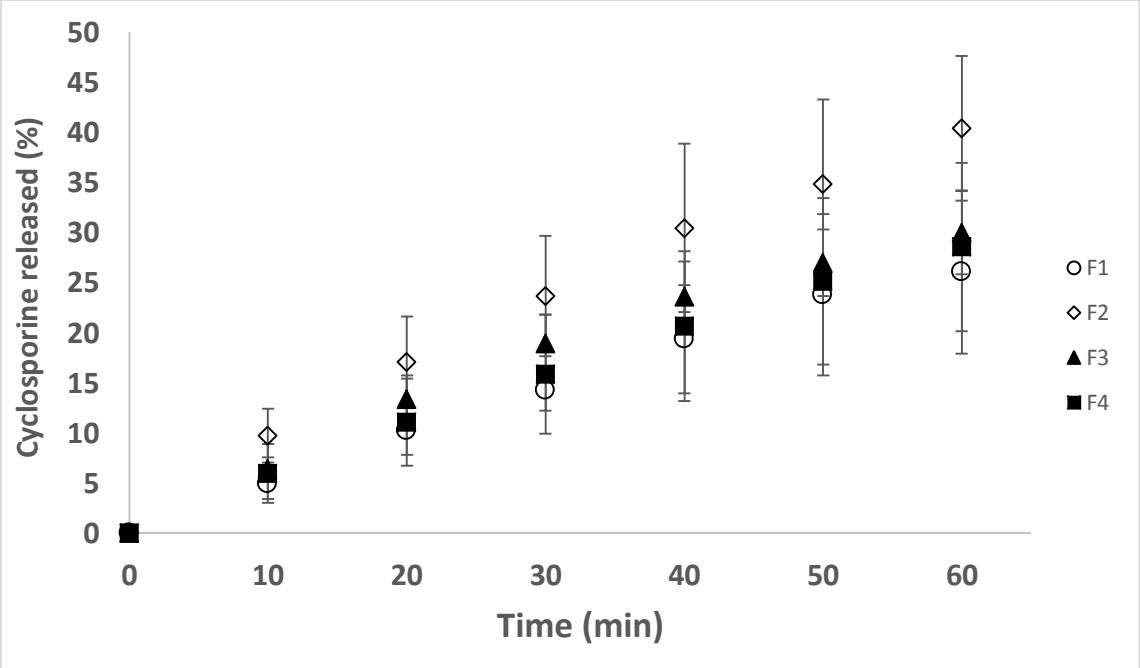


Figure 6. Cyclosporine release profiles from drug-loaded inserts recorded using the inclined plane setup (n=3, mean±sd).

The vial method was used to study mechanisms of cyclosporine release from the inserts. In this method, formulations were immersed in a fixed volume of buffer (Pescina, Macaluso, Gioia et al., 2017). The complete immersion of the inserts into plenty of volume of lacrimal fluid caused rapid swelling, drug release and matrix erosion. Cyclosporine release profiles from crosslinked inserts are shown in **Figure 7**. Drug release from F1 and F2 was controlled only in the first 40 min, and then a fast and almost complete release occurred between 70 and 90 min. Thus, F1 and F2 were not efficient in controlling cyclosporine release under these experimental conditions, because of the rapid disentanglement of polymer chains and consequent disintegration of the matrices, in agreement with the results recorded in the swelling study (**Figure 3**). However, it has to be mentioned that in physiological conditions the volume of tears is limited and thus the drug may be released more slowly than *in vitro* (Foureaux, Franca, Nogueira et al., 2015; Kumari, Sharma, Garg et al., 2010), in accordance with the results obtained with the inclined plane set-up (**Figure 6**).

Differently to F1 and F2, F3 and F4 formulations released cyclosporine in a controlled manner for at least 4 h (**Figure 7**). Experiments were performed up to 8 h to assure the complete release of the peptide from the inserts. As shown in **Figure 7**, in the first hours the release profiles showed a semi-plateau probably due to the formation to a swollen gel layer that acts as a diffusion barrier for cyclosporine release. Between 240 and 300 min a quantitative release of cyclosporine occurred due to the disentanglement of the polymeric structure caused by the matrix swelling and polymer chains erosion.

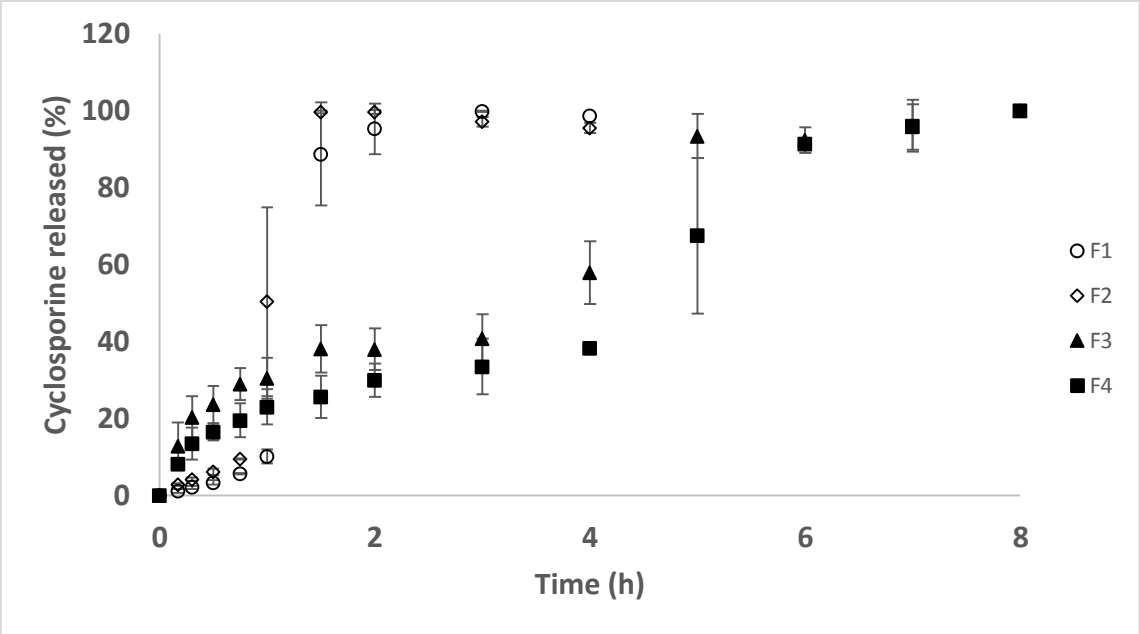


Figure 7. Cyclosporine release profiles from drug-loaded inserts recorded using the vial method (n=2 for F1 and F2, n=3 for F3 and F4, mean±sd).

Experimental data were fitted (first 60% of the total amount released) to the Ritger-Peppas equation to understand the influence of diffusion mechanism and polymer relaxation on drug release:

$$\frac{Mt}{M\infty} = k1 \cdot t^n \quad \text{(eq. 3)}$$

where Mt is the cumulative amount released at time t, M∞ is the total amount of drug loaded in the system, k1 is the kinetic constant and n is the diffusion exponent (Ritger & Peppas, 1987). The n value was calculated to be equal to 0.34 and 0.42 for F3 and F4, respectively, suggesting that the mechanism of release is Fickian diffusion. In fact, as reported for Korsmeyer-Peppas models, the release exponent n ≤ 0.45 is reported for Fickian diffusion release, while 0.45<n<0.89 is for anomalous release, depending both on matrix swelling and erosion (Amarachi, Onunkwo & Onyishi, 2013). Presumably, swelling matrix occurs in the first minutes and then diffusion phenomena allows cyclosporine release with a very slow kinetic due to the large size of the peptide, which can offer resistance to the passage through matrix pores. The very slow release rate due to the

restricted diffusion of the peptide through the hydrophilic matrix could also justify the lack of differences in terms of percentage of released peptide by comparing the two release studies performed.

Differences in drug release rate may in part justify the subsequent results of sclera penetration. Neither permeation nor penetration into isolated sclera occurred in 8 h for F3 and F4 formulations. Differently, detectable drug amounts were found into the sclera by testing the other two formulations, with a ~6 fold times higher cyclosporine accumulation found for F1 in comparison to F2 (32.7±5.1 and 5.6±4.9 $\mu g_{drug}/g_{sclera}$, respectively). These amounts of cyclosporine penetrated are well above the therapeutic levels of cyclosporine reported by other authors for the treatment of diseases affecting the eye (300 ng_{drug}/g_{sclera}, Kaswan, 1988). However, it has to be mentioned that using this experimental setup (Franz diffusion cell) only F1 inserts were disintegrated at the end of the experiment, whereas the other formulations partially swelled but they were still entire. Thus, we hypothesized that the absorption of the fluids from the sclera by the inserts was sufficient to permit inserts swelling and erosion only in the case of F1, while in other cases degradation did not occur. This means that during the sclera penetration experiment, peptide diffusion from inserts was hindered in case of F3 and F4 by the limited amount of fluid available. Thus, taking into account the very slow diffusion rate of cyclosporine across the fully hydrated matrices, minor penetration in sclera could be justified.

To the best of our knowledge, no experimental setup for studying high molecular weight compounds penetration in eye structures from hydrophilic matrices has been reported in literature. On the other hand, the results highlight the limitations of the static method for studying swellable ophthalmic formulations performance in terms of drug accumulation into ocular tissues. The modification of the classical penetration setup using Franz diffusion cells for penetration studies with the insertion of a fluid-through of simulated buffer onto the solid formulations could be beneficial to mimic the conditions occurring on the ocular

surface. This could be useful especially in case of peptides and proteins as the release rate from polymeric entangled structures is known to be very slow. However, it has to be mentioned that *in vivo* enzymatic systems located in the conjunctiva and in the sclera can have an impact on hydrogel structure (HA degradation), accelerating the release of cyclosporine from the inserts (Duvvuri, Majumdar & Mitra, 2004).

4. Conclusions

Inserts of HA and HPβCD cross-linked with PEGDE appear as a suitable platform for peptide loading and release to the ocular surface. All blank formulations showed good biocompatibility, suitable thickness and swelling properties for ophthalmic applications. Cyclosporine was loaded into inserts by soaking to avoid degradation during cross-linking reaction. Interestingly modifying HA:HPβCD weight ratio in the inserts it is possible to tune the rates of swelling and drug release, which play a key role in drug penetration through sclera and thus in the feasibility of accumulating into the ocular tissues. The obtained results point out that formulations with intermediate contents in HPβCD (F2 and F3) could be suitable for achieving therapeutic effects in posterior (F2) or anterior (F3) eye segments.

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