Hyaluronic acid - PVA films for the simultaneous delivery of dexamethasone and levofloxacin to ocular tissues

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Highlights

- Polymeric films for simultaneous ocular delivery of lipophilic and hydrophilic drugs.
- Modified release platform for the reduction of administration frequency.
- Film swelling behavior modulation by varying polyvinyl alcohol degree of hydrolysis.
- Possible use of the film for both corneal application and transscleral delivery.

Abstract

Ocular drug delivery is challenging due to the poor drug penetration across ocular barriers and short retention time of the formulation at the application site. Films, applied as inserts or implants, can be used to increase residence time while controlling drug release. In this work, hydrophilic films made of hyaluronic acid and two kinds of PVA were loaded with dexamethasone (included as hydroxypropylcyclodextrin complex) and levofloxacin. This association represents one of the main treatments for the post cataract surgery management, and it is also promising for eye infections whith pain and inflammation. Films were characterized in terms of swelling and drug release and were then applied to porcine eye bulbs and isolated ocular tissues. Film swelling leads to the formation of either a gel (3D swelling) or a larger film (2D swelling) depending on the type of PVA used. Films, prepared in an easy and scalable method, demonstrated high loading capacity, controlled drug release and the capability to deliver dexamethasone and levofloxacin to the cornea and across the sclera, to potentially target also the posterior eye segment. Overall, this device can be considered a multipurpose delivery platform intended for the concomitant release of lipophilic and hydrophilic drugs.

Keywords

Film; dexamethasone; levofloxacin; polyvinyl alcohol (PVA); hyaluronic acid, ocular delivery, insert

1. Introduction

Ocular drug delivery represents a real challenge due to the peculiar structure of the eye, characterized by the presence of numerous static, dynamic and metabolic barriers. Ocular bioavailability is tipically very low, due to insufficient drug permeation across the ocular membranes and short retention time of the drug at the application site (Lanier et al., 2021). For these reasons, this field is one of the most interesting for the research in drug delivery, since the formulation can highly impact on the residence time at the application site and on drug permeability across ocular tissues, dramatically changing the performance of a medication. Among the numerous innovative vehicles, solid formulations, namely inserts and implants, answer predominantly to the need of a reduction of the administration frequency, since they allow for increased residence time in the site of application and a controlled release of the drug (Grassiri et al., 2021; Kompella et al., 2021). To highlight the relevance of a reduced administration frequency, we can cite two emblematic examples: the need of hourly drug application in the conjunctival sac in the treatment of some serious corneal infections (Lin et al., 2019) and the need for lifetime intravitreal injection of corticosteroids and/or anti-VEGF compounds in the treatment of some retinal diseases (Ghanchi et al., 2022). The reduction of the administration frequency is of outmost importance: on one hand, it increases adherence to the treatment while, on the other hand, it

reduces fluctuations in drug tissues concentration and related side effects. Thus, both efficacy and patient's compliance are increased. Despite the number of inserts and implants currently on the market is still not very high, the research in this field is lively and concerns both the anterior and the posterior segment of the eye (Alvarez-Lorenzo et al., 2019; Gaballa et al., 2021; Kim and Woo, 2021; Maulvi et al., 2021; Terreni et al., 2020). As illustrated in Figure 1, controlled-release formulations can be inserted in the anterior segment of the eye, namely in the conjunctival sac, in the lachrymal ducts or on the top of the cornea, as in case of medicated contact lenses or implanted in the vitreous, in the subconjunctival/periocular space.

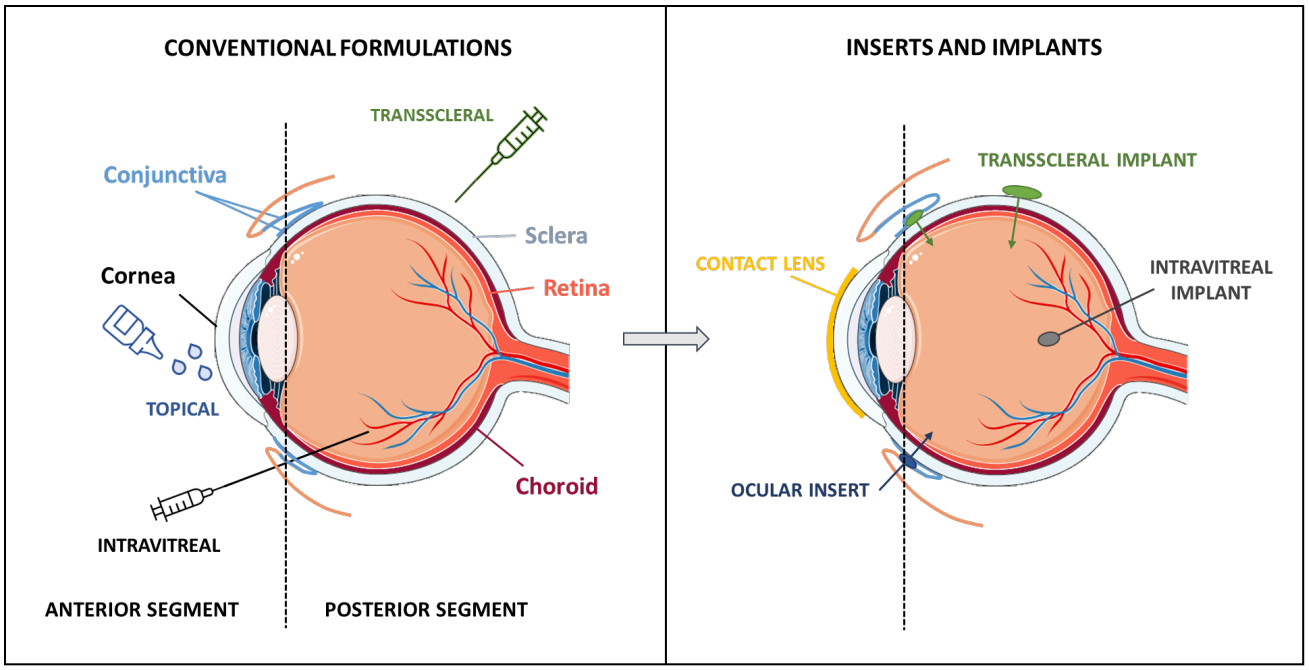

Figure 1. Main routes for drug delivery to the anterior and posterior eye segment and possible location of solid controlled-release formulation. Figure modified with text, arrows and annotation after adaptation of "Eye" from Servier Medical Art by Servier, licensed under a Creative Commons Attribution 3.0 Unported License (https://smart.servier.com, accessed on 24 November 2022).

Dexamethasone (DEX) is one of the most potent corticosteroids used for the treatment of diseases affecting both the anterior and the posterior eye segment (Gaballa et al., 2021). Its association with antibiotics is used to treat eye infections which have pain and inflammation components, or, post-surgery, to prevent infections and to treat the inflammation (Bandello et al., 2020; Rizzo et al., 2022). The most frequent use of this drug combination is after cataract surgery, the most common surgical intervention worldwide. Corticosteroids and antibiotics (sometimes also combined with NSADs) are generally applied 4 times a day, and a 5-minute interval between the administration of different eye drops should be respected. This results in a complex schedule and low adherence to the treatment (Matossian, 2020). To address, at least in part, this problem, a new eye drop formulation containing 1 mg/mL DEX and 5 mg/mL levofloxacin (LVX), a broad-spectrum fluoroquinolone antibiotic (Keating, 2009), was recently authorized in several European countries (EMA, 2022; Rizzo et al., 2022). This combination could simplify the therapy also reducing the danger for antibiotic resistance. However, administration frequency is high (1 drop every 6 h) (Rizzo et al., 2022) and appropriate eye-drop instillation remains a challenge (Matossian, 2020). Recent data suggest that a drop-free approach could improve outcomes and the patient experience (Assil et al., 2021).

The aim of this work was the development, characterization and ex-vivo evaluation of an ocular film for the controlled release of DEX and LVX. This combination could be useful for post-cataract management, but also for posterior segment diseases, in the treatment of inflammatory conditions which can involve bacterial

infections (Gaballa et al., 2021; Prieto et al., 2020; Wang et al., 2013). Excipients used for the preparation were hyaluronic acid (HA) and polyvinyl alcohol (PVA). Polymeric films containing PVA or HA have been previously investigated as ocular drug delivery systems: PVA is well known for its excellent film-forming properties and has been used, for instance, in combination with PVP to prepare films for the ocular release of progesterone (Alambiaga-Caravaca et al., 2021). A timolol-loaded film composed of hyaluronic acid and hydroxypropyl methylcellulose demonstrated biocompatibility and an extended-release profile (Tighsazzadeh et al., 2019); a novel hyaluronic acid membrane showed its usefulness for the treatment of ocular surface diseases by promoting corneal epithelium healing (Kim et al., 2021), whereas a dexamethasone-loaded film made of hyaluronic acid and itraconic acid demonstrated to reduce IL-6 production by TNF-α inflamed corneal epithelial cells (Calles et al., 2016). Recently, Bao et al prepared hydrogel films for the combined delivery of dexamethasone and levofloxacin; the system, made of oxidized hyaluronic acid and dexamethasone covalently conjugated with glycol chitosan, showed to be promising for the treatment of postoperative endophtalmitis [50]. In this work, HA and PVA were combined and PVA hydrolisys degree was exploited to modulate swelling properties (2D or 3D) of the film and tailor its characteristics to the application in different eye compartiments. Additionally, with respect to previous works, a delivery platform for the simultaneous delivery of an hydrophilic and a lipophilic drug was developed, using an easy and potentially scalable method, and excipients approved for ocular administration. After an initial screening phase, two polymeric compositions were selected: both contained HA and differed for the presence of two kinds of PVA, characterized by similar molecular weight (MW), but different hydrolysis grade. As DEX is a poorly water-soluble drug, its loading in the hydrophilic films was possible thanks to the inclusion in cyclodextrins. Films were characterized in terms of swelling and drug release. They were then applied to the porcine ocular tissues (namely cornea, sclera and the trilayer sclera-choiroid Bruch's membrane) using validated ex-vivo models, to evaluate their capability to deliver the two drugs to the ocular structures. Moreover, in order to figure-out if dexamethasone penetrates the tissue alone or included in cyclodextrins, some films loaded with Nile-red-cyclodextrin complex were prepared and applied to ocular tissues, which were then analysed by two-photon microscopy (Helmchen and Denk, 2005).

2. Material and Methods

2.1. Materials

Dexamethasone (DEX; IUPAC name: (8S,9R,10S,11S,13S,14S,16R,17R)-9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthrene-3-one; MW: 392.5 g/mol; logP 1.83) was purchased from AlfaAesar (Karlsruhe, Germany) while levofloxacin (LVX; IUPAC name: (2S)-7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1-azatricyclo[7.3.1.$0^{5,13}$]trideca-5(13),6,8,11-tetraene-11-carboxylic acid; MW: 361.4 Da; pKa 5.2, 6.2, 8.2) was bought from Sigma-Aldrich (St. Louis, MO, USA). Polyvinyl alcohol (PVA87; EG-25, MW: 83.4 kDa, hydrolysis degree: 86.5–89.0 mol%) was purchased from Gohsei (Japan); polyvinyl alcohol (PVA98; MW: 72 kDa, hydrolysis degree: 97.5–99.5 mol%) was from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Sodium hyaluronate (HA; MW: 1000 kDa and 300 kDa) was from IBSA Farmaceutici S.p.A. (Lodi, Italy). Hydroxypropyl β-cyclodextrin (Kleptose®; HPCD; MW: 1391 g/mol) was kindly provided by Roquette (Lestrem, France). Figure 2 illustrates the structure of HA, PVA and HPCD. PEG400 (poly(ethylene glycol); MW: 380-420 g/mol) was purchased from Eigenmann & Veronelli S.p.A. (Milano, Italy), whereas glycerin was bought from Merck (Darmstadt, Germany). Nile red (NR; 9-(diethylamino)-5H-benzo[a]phenoxazin-5-one; MW 318.4 g/mol) was purchased from Sigma Aldrich (St. Louis, MO, USA).

For analysis, HPLC-grade acetonitrile and high purity water (Arium® comfort, Sartorius, Goettingen, Germany) were used. All other chemicals were of analytical grade.

Simulated tear fluid (STF) was made of 0.06 g/l $CaCl_2$, 2.18 g/l $NaHCO_3$, 6.7 g/l NaCl. The solution was brought to pH 7.4 with HCl. Citrate buffer was made of 1.92 g/l citric acid and 2.94 g/l sodium citrate. The solution was brought to pH 3.5 with HCl. Phosphate Buffered Saline (PBS) was made of 0.19 g/l KH_2PO_4, 8.80 g/l NaCl and 2.37 g/l Na_2HPO_4. The solution was brought to pH 7.4 with H_3PO_4 85%.

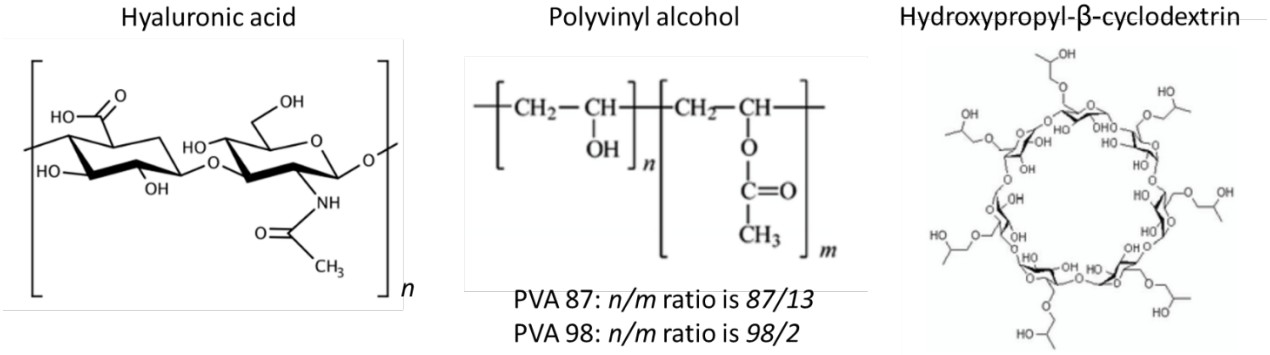

Figure 2. Excipients used for film preparation.

2.2. Analytical methods

2.2.1. HPLC analysis of dexamethasone

DEX was quantified by HPLC-UV (Infinity 1260, Agilent Technologies, Santa Clara, CA, USA) following a previously validated method (Pescina et al., 2019). The instrument was equipped with a reverse-phase C18 column (Nova-Pak C18 4 µm, 60 Å, 3.9 × 150 mm, Waters, Ireland) and a C18 guard column (SecurityGuard C18, Phenomenex, Torrance, CA, USA), thermostated at 45°C. The mobile phase, composed of CH_3CN:H_2O (35:65,v:v), was pumped at 1 mL/min. Injection volume was 10 µL and drug detection was carried at 240 nm. In these conditions, the retention time of the drug was about 3 min. Calibration curves were built both in PBS and in the mixture CH_3CN:H_2O (35:65,v:v), in the concentration interval 0.25 – 5 µg/mL. Accuracy (RE%) and precision (RSD%) of the analytical method were evaluated and resulted lower than 3.3% and 3.7%, respectively.

2.2.2. HPLC analysis of levofloxacin

LVX was measured by HPLC-UV (Infinity 1260, Agilent Technologies, Santa Clara, CA, USA) following a previously validated method (Pescina et al., 2012). The column used was a C18 (Simmetry300®, 5 µm, 4.6 x 250 mm, Waters, Ireland) with a C18 column guard (SecurityGuard C18, Phenomenex, Torrance, CA, USA) thermostated at 30°C. The mobile phased was composed of 0.01 M citrate buffer at pH 3.5:methanol:acetonitrile (70:17:13, v:v:v). Injection volume was 50 µL, the analysis was carried out at 294 nm, with a flow rate of 1 mL/min and a retention time of about 5 min. A calibration curve in PBS was built in the interval 0.5 -50 µg/mL. Accuracy (RE%) and precision (RSD%) resulted < 5.1% and <0.4% respectively. Solutions obtained from the corneal extraction were diluted 1:1 with water prior to injection.

2.3. Solubility studies of dexamethasone

Excess amounts of DEX were separately added to PBS, high purity water or a 3% w/v HPCD water solution. After stirring for at least 24 h at room temperature, the sample was filtered (0.45 µm, Regenerated Cellulose), diluted when necessary, and analyzed by HPLC-UV for drug quantification.

2.4. Films preparation

2.4.1. Blank films

To obtain the polymeric films, 2.5% w/w HA (300 or 1000 kDa) solutions and 15% w/w PVA (with hydrolysis grade of either 87% or 98%) solutions were prepared and mixed in different ratios (see composition details in Table S1). The method was modified from the method previously described by Padula et al (Padula et al., 2007). Briefly, polymeric solutions were prepared separately by adding the polymer to high purity water under magnetic stirring (100 rpm) at room temperature. In the case of PVA98, the dispersion in water was heated at 90°C to get an homogeneous solution; the amount of water lost during heating was restored. The prepared polymer solutions were carefully mixed (100 rpm) and the plasticizer (either glycerin or PEG 400) was added to the final mixture by gently stirring. The obtained mixtures were left to stand for 24 h, then they were spread on a glass plate using a film casting knife (BYK Gardner, Silversprings, MD, USA) with a gap of 2 mm, and oven-dried at 70°C for 1 hour. The composition of the films prepared is illustrated in Table S1. The optimized formulations (87 and 98 in Table 1) were further loaded with drugs.

2.4.2. Films loaded with dexamethasone and levofloxacin

Formulations 87 and 98 were loaded with drugs. To solubilize DEX in the hydrophilic mixture, hydroxypropyl-β-cyclodextrin (HPCD) was used.

Eight mg of DEX were dissolved in 4 mL of water containing 123 mg of HPCD. LVX (40 mg) was subsequently added. The obtained solution was used for HA hydration (final conc. 2.5% w/w). Finally, 1 g of 15% w/w PVA solutions and 28 mg of glycerin were added under gentle stirring until a homogeneous mixture was obtained. The system was left to stand for at least 24 h to eliminate air bubbles, then spread on a glass plate using a film casting knife with adjustable gap (BYK Gardner, Silversprings, MD, USA) that was set at 2 mm, and oven-dried at 70°C for 1 hour.

Films containing only DEX were also prepared and, to investigate the possible role of HPCD in drug transport across the cornea, DEX films containing higher HPCD amount (namely 246 mg) were prepared as well. In this case, the film resulted very stiff and a higher amount of glycerin (140 mg) was necessary. Table 1 reports the composition of the different mixtures used for film preparation, together with the codes used to identify the films.

Table 1. Codification of the films and composition of the mixtures used for their preparation (wet basis).

	Amount (mg) of each component in the mixture						
CODE	**PVA**	**HA**	**Glycerin**	**Water**	**DEX**	**LEVO**	**HPCD**
87	150 (PVA87)	105	28	4000	-	-	-
98	150 (PVA98)	105	28	4000	-	-	-
87_βDL	150 (PVA87)	105	28	4000	8	40	123
98_βDL	150 (PVA98)	105	28	4000	8	40	123
87_βD	150 (PVA87)	105	28	4000	8	-	123
98_βD	150 (PVA98)	105	28	4000	8	-	123
87_ββD	150 (PVA87)	105	140	4000	8	-	246

2.5. Films characterization

2.5.1. Film swelling behaviour

Circular samples (0.6 cm^2) were cut from each film, placed on a glass support and wet with 250 µl of STF heated at 37 °C. Pictures were taken with a camera (Nokia Lumia 630) after 0, 5, 10, 15 and 20 minutes together with a reference area (A_R), and analyzed using ImageJ software (ImageJ 1.52q, NIH, USA).

2.5.2. Dexamethasone and levofloxacin content in the films

Three circles (0.6 cm^2) were cut from each loaded film. Each sample was measured for weight and thickness (Absolute Digimatic 547-401, Mitutoyo, Milan, I, resolution 0.001 mm), immersed in 7 mL of high purity water and stirred for 24 h at room temperature until complete dissolution. One mL of the solution was then sampled, centrifuged for 10 minutes at 8000 rpm and analysed by HPLC in order to determine the amount of DEX and LVX contained in the film. The results are expressed as % of drug (w/w) and as $\mu g/cm^2$.

2.5.3. Dexamethasone and levofloxacin release from the films

The experiment was performed at room temperature (19–22 °C). The set-up used, previously published (Pescina et al., 2017), was a slight modification of the method described by Sandri et al (Sandri et al., 2010). Film circles (0.6 cm^2) were applied to a 9 cm diameter glass Petri dish inclined at 45°. Then, simulated tear fluid was flushed onto the film using a syringe pump (Harvard Apparatus, Holliston, MA) set at 0.06 mL/min flow-rate. The solution was collected at predetermined time points up to 4 h and analyzed by HPLC for the determination of DEX and LVX released. Release studies were carried out for the films 87_βD, 87_βDL, 87_ββD, 98_βD and 98_βDL.

2.6. Dexamethasone and levofloxacin permeation across ocular tissues

2.6.1. Tissue preparation

Porcine eyes were obtained from a local slaughterhouse within 3 h from animal death (pig breed, Large White and Landrance; weight, 145-190 kg; age, 10–11 months; sex, male and female). Eye bulbs were transported to the lab in PBS buffer; the adherent muscle and the conjunctiva were removed. Porcine eyes were either used intact, or dissected to isolate the sclera or the trilayer sclera-choroid-Bruch's membrane. For corneal experiments only bulbs with macroscopically intact corneas were employed, whereas eyes showing opaque corneas were discarded. During the dissection to isolate sclera or trilayer, the anterior segment of the eye was circumferentially cut behind the limbus and discarded. The vitreous was removed, the eye cup was cut into two halves and retina and Retinal Pigment Epithelium (RPE) were removed by using a cotton swab to obtain the trilayer composed of sclera, choroid and Bruch's layer. When the isolated sclera was used, the choroid was eliminated by careful removal with tweezers.

2.6.2. Uptake into the cornea

To evaluate DEX and LVX uptake into the cornea, films 87_βD, 87_βDL, 87_ββD, 98_βD and 98_βDL (composition in Table 1) were applied to the corneal surface of intact eye bulbs. The set-up used permits to thermostat the eye at 37°C. Details of the set-up used are shown in the supplementary material (Figure S2). Each film (0.6 cm^2) was applied on the cornea after wetting the surface with STF. 10 µL of STF were further added on the top of the film every hour, to maintain surface hydration. After 4 h of contact, the swollen film/gel was removed, the cornea was carefully washed with high purity water and filter paper, and subsequently isolated and transferred in an Eppendorf tube. The tissue was then extracted by addition of 1 mL of CH_3CN:H_2O (35:65, v:v) for 1.5 h at room temperature. After centrifugation (12000 rpm for 12 min), the supernatant was sampled for the HPLC analysis and the tissue was extracted a second time (1 mL of CH_3CN:H_2O (35:65, v:v); overnight). To calculate the drug accumulated in the tissue, the amount from the first and second extractions were summed. This procedure was validated for DEX recovery, and the extracted % resulted 100 ± 2.1 (approximately 82% in the first extraction and 18% in the second). This procedure was also challenged for levofloxacin extraction; the recovery % obtained was 98 ± 6%. Each condition was replicated 3 to 4 times.

2.6.3. Permeation across the sclera and the trilayer (sclera-choiroid-Bruch's membrane)

Permeation experiments were performed using vertical diffusion cells with an area of 0.6 cm^2. The scleral tissue or the trilayer was clamped between the donor and the receptor compartments with the conjunctival side facing the donor. The receptor compartment contained 4 mL of pH 7.4 PBS kept at 37 °C, and magnetically stirred. Before film application (area 0.6 cm^2), the sclera was wetted with 24 µL of PBS to favour film adhesion to the tissue. The receiving solution was sampled every hour up to 6 (5 h in the case of the trilayer) for the quantification of DEX and LVX permeated. The solubility of DEX in the receptor buffer was determined and resulted enough to guarantee sink conditions (solubility: 55.78 ± 1.36 µg/mL). The films tested were 87_βDL and 98_βDL for trans-scleral transport, while only 87_βDL for trilayer permeation.

The amount of drug permeated, normalized for the permeation area, was plotted against time and the flux J (µg/cm^2 h) was calculated at the as the slope of the regression line after the achievement of the steady state. The lag time is represented by the intercept of the regression line on the time axis. The permeability coefficient P (cm/s) was calculated as $P = J/C_d$, where C_d (µg/mL) is the concentration of DEX or LVX in the film (Table 2).

2.7. Two-photon microscopy and fluorescence spectroscopy

Nile red-loaded films based on PVA87 were prepared for the two-photon imaging: 123 mg of HPCD were solubilized in 4 mL of water and added to 2 µL of a 10 mg/mL Nile red solution in DMSO. The obtained vehicle was used for HA hydration, then the method was the same as in par 2.4.2., except for the absence of the two drugs. The films were evaluated on corneal and scleral tissues, with the method previously described (par 2.6.2). Corneal and scleral tissues were mounted on Franz-type vertical diffusion cells (area 0.6 cm^2 for sclera and 0.2 cm^2 for cornea). The experiment duration was 4 h. The samples were then frozen at -20°C until microscopy analysis.

Corneal and scleral samples were analyzed with a Two-Photon Microscope Nikon A1R MP+ Upright coupled with a femtosecond pulsed laser Coherent Chameleon Discovery (~ 100 fs pulse duration with 80 MHz repetition rate, tunable wavelength output 660 - 1320 nm). A 25x water dipping objective with numerical aperture 1.1 and 2.0 mm working distance was used to focus the excitation beam and to collect the two-photon excited fluorescence (TPEF) and the second harmonic generation (SHG) signals. The outcoming signal was directed by a dichroic mirror to two non-descanned detectors (high sensitivity GaAsP photomultiplier tubes) allowing fast image acquisition. Optical filters preceded the detectors allowing the simultaneous acquisition of two separated channels: green channel (506 - 593 nm) and red channel (604 - 679 nm). The operation software of the microscope performed the overlay and the processing of the two channels images. A third photomultiplier GaAsP detector, connected to the microscope through an optical fiber and preceded by a dispersive element, was used to record emission spectra of the two-photon excited samples (wavelength range 430-650 nm with a bandwidth of 10 nm).

Fluorescence measurements on liquid samples were performed with a FLS1000 Edinburgh Fluorometer. Emission spectra were collected on diluted solutions, with absorbance lower than 0.1 to avoid inner filter effects. All fluorescence spectra were duly corrected for the excitation intensity and the detector sensitivity. For the preparation of NR solution in water, 20 µL of a 400 µM DMSO NR stock solution were added in 3 mL of high purity water (final NR concentration 2.7 µM, total percentage of DMSO <1%) and then filtered (hydrophilic PTFE, AISIMÔ 0.22 µm).

2.8. Statistical analysis

Results are expressed as the means of at least three experiments ± standard deviation. Statistical analysis was performed using Student's t test. The chosen level of significance was $p<0.05$.

3. Results and Discussion

Several synthetic and natural polymers have been used for the preparation of inserts and implants and, among them, hyaluronic acid (HA) and polyvynilalcohol (PVA) represent two examples of interest (Figure 2). HA is a natural, biocompatible, biodegradable, hydrophilic and mucoadhesive polymer. It is widely used as excipient, but it also shows lubricating and anti-dehydration properties, it showed to reduce the inflammation caused by dryness and to stimulate the corneal re-epithelialization (Zhang et al., 2021). PVA is a synthetic polymer with very good film-forming properties; it is produced from hydrolysis of polyvinyl acetate and has been widely used in implantable and non-implantable devices demonstrating safety and tolerability towards conjunctiva, cornea (Akbari et al., 2021; Li et al., 2015) and retina (Maruoka et al., 2006). In this paper we combined the two polymers to prepare films for the ophthalmic delivery of two drugs, namely DEX and LVX. Details regarding the screening phase carried out for optimizing the films are reported in the Supplementary material section. At the end of the screening, two different films were selected (87 and 98 in Table 1); both contained a HA:PVA:glycerin ratio of 36:54:10 and differed for the hydrolysis grade of the PVA used, that was either 87% (PVA87) or 98% (PVA98).

3.1. Behavior of the films in contact with simulated tear fluid: swelling studies

Upon ocular application, films will encounter tear fluid or interstitial fluids; thus, we evaluated the behavior of the two selected films in contact with an aqueous buffer. As can be seen in Figure 3A, the film behaviour highly depends upon the PVA used: films containing PVA87 swell three-dimensionally and then tend to dissolve. The swelling behavior was not monitored since the gel is not enough compact to be sampled and weighted. On the contrary, films containing PVA98 are characterized by a two-dimensional swelling (Figure 3A) which determines an increase in the surface of the film that can be monitored. PVA is produced from hydrolysis of polyvinyl acetate and the hydrolysis grade refers to the % of acetate groups that has been removed from the PVA chain (Figure 2); this parameter highly impacts on the physicochemical properties of the polymer. For instance, a higher hydrolysis grade (PVA98) is associated to a higher possibility of inter- and intra-chain H-bond formation, reflected in a lower water solubility (Morita et al., 2000). Analogously, the different hydrolysis grade can also impact on PVA – HA interaction and on swelling behavior.

Figure 3B illustrates the area of the PVA98-based film, reported as A_t/A_0 ratio, as a function of time (A_0=area of the dry film; A_t=area of the film at time t after STF addition). To understand the contribution of all the components, films made of PVA98 alone, PVA98 and HA (without glycerin), films of PVA98, HA and glycerin and film also containing HPCD were evaluated.

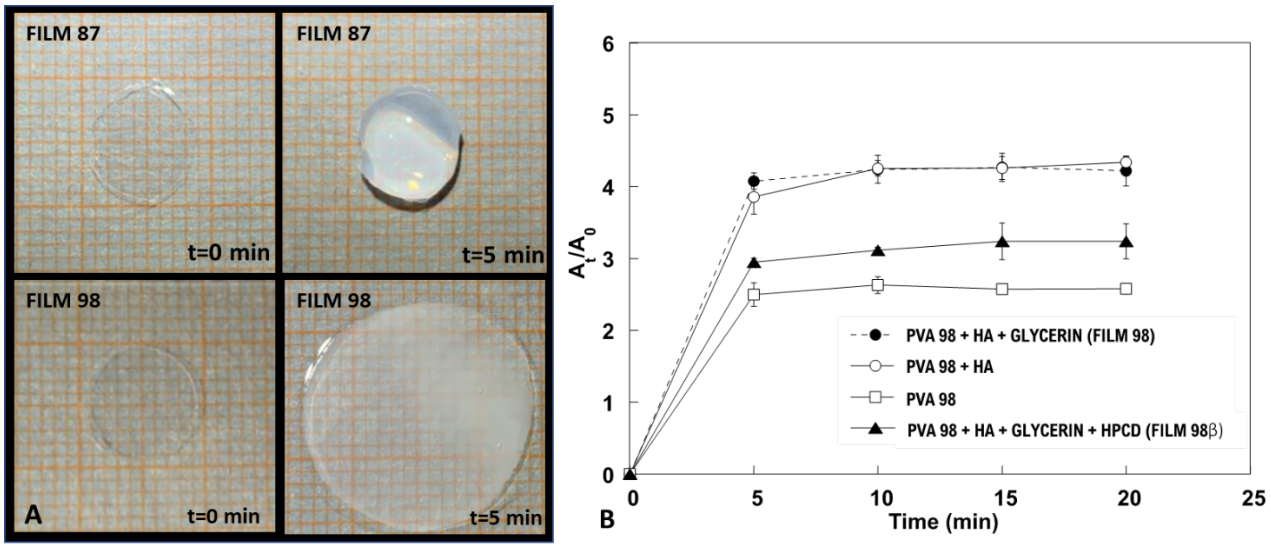

Figure 3. Panel A shows the appearance of the films based on PVA87 and PVA98 (HA:PVA:glycerin ratio of 36:54:10) after 5 minutes contact with simulated tear fluid. Panel B represents the 2D Swelling of PVA98-based films with various compositions.

From Figure 3 it is evident that the swelling is very rapid as it occurs mainly in the first 5 minutes. From 10 minutes onwards, there are no more substantial variations. The film containing only PVA (PVA 98) increases in area of about 2.5 times. By adding HA (PVA 98+HA), the swelling ratio increases to 4. Probably, the presence of HA reduces the PVA inter and intrachain interactions, favouring water uptake. The consequence is a higher relaxation of the PVA chains. Finally, the addition of glycerin (PVA 98+HA+GLYCERIN, FILM 98) does not substantially change the swelling behaviour. The swelling was evaluated also for films containing HPCD (PVA 98+HA+GLYCERIN+HPCD, FILM 98_β), since this compound has been used for DEX solubilization. The result highlights a reduction of the swelling extent, that could be ascribed to the formation of H-bonds between the external hydrophilic hydroxyl groups of CD and the two polymers, causing a reduction of polymer-water interaction and thus a reduction of the swelling.
In principle, the two-dimensional swelling could be useful in the case of corneal application, where the film could adhere to the tissue, protect a damaged epithelium and release the loaded drugs. This film is not supposed to behave as a contact lens but as a corneal bandage material.

3.2. Dexamethasone and levofloxacin-loaded polymeric films

Films 87 and 98 were then loaded with DEX and LVX. In order to load a lipophilic drug (DEX) into a hydrophilic film, a solubilizing strategy is needed. Among other, we decided to use cyclodextrins, cone-shaped oligosaccharides which are well known for their ability to solubilize hydrophobic compounds improving also their delivery to the tissues (Kristinsson et al., 1996; Shulman et al., 2015). Specifically, we used 3% w/w hydroxypropyl-β-cyclodextrin (HPCD) and found that DEX solubility increased 30 times, namely from 0.083 ± 0.005 mg/mL to 2.52 ± 0.08 mg/mL, in agreement with literature data (Loftsson et al., 1994a). For the preparation of the final films, DEX was solubilized in 3% HPCD, the obtained solution was added to levofloxacin and then used to hydrate hyaluronic acid to prepare the final films (par 2.4.2). The exact composition of the mixtures prepared and laminated is reported in Table 1. The characteristics of the drug-loaded films in terms of weight, thickness and actual drug content are illustrated in Table 2.

Table 2. Characteristics of the DEX and LVX-loaded dried films.

	Film properties	DEX content	LVX content

	Weight (mg/cm^2)	Thickness (μm)	% w/w	μg/cm^2	% w/w	μg/cm^2
87_βDL	13.5 ± 0.49	100 ± 0.01	1.99 ± 0.08	262 ± 10	9.79 ± 0.51	1287 ± 66
98_βDL	12.78 ± 0.45	110 ± 0.02	2.01 ± 0.05	265 ± 6	8.90 ± 0.50	1170 ± 68

It is worth mentioning that the presence of cyclodextrin modified the characteristics of PVA98-based films, reducing the 2D-swelling behavior (Figure 3B).

3.2.1. DEX and LVX release from the films

For the study of drug release from the films, a flow-through method where STF flows at 60 μl/min on the formulation has been used. This method allows a slow contact of the formulation with water and, at the same time, the mechanical action of fluid flow on the structure of the film, reproducing some of the phenomena that take place after ocular application. Additionally, the plane is inclined at 45°, giving the opportunity to evaluate the possible displacement of the film from the deposition site, thus inferring some information on film adhesion properties. Indeed, none of the film have moved from the application position. The results of DEX and LVX release from PVA87 and PVA98 based films are shown in Figure 4, panel A and B.

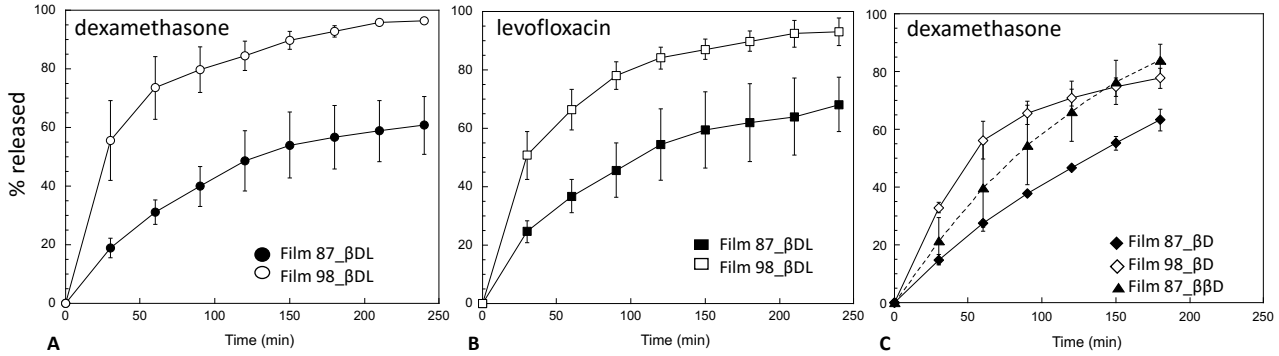

Figure 4. Release profiles obtained by using the inclined-plane set-up. Panel A and B report DEX and LVX release from films 87_βDL and 98_βDL. Panel C shows the release from films containing only DEX (87_βD and 98_βD) also in the presence of a doubled amount of HPCD (87_ββD).

The film containing PVA98 is characterized by a marked burst effect and a faster release than that containing PVA87. For example, after 2 hours the film containing PVA87 released about 48% of DEX and 54% of LVX, while the one containing PVA 98 released the 84% of both drugs. These differences are due to the different degree of hydrolysis of the PVA which affects the structural characteristics of the film (Cascone et al., 2004; Hdidar et al., 2017; Limpan et al., 2012). We can hypothesize that the presence of a higher proportion of acetate groups in PVA87 limits the interactions with hyaluronic acid. Being scarcely involved in these bonds, hyaluronic acid can better interact with water molecules, favoring a three-dimensional swelling of the film and thus ensuring a slower and more controlled release of the active ingredients. On the other hand, in PVA98 the higher degree of hydrolysis involves the presence of a greater number of free OH groups in the polymer chains. This allows the polymer to interact more with hyaluronic acid which, being engaged in these bonds, tends to have fewer interactions with water. This prevents the formation of the gel which is the phenomenon that controls dexamethasone and levofloxacin release. An attempt to support this hypothesis was done by quantifying hyaluronic acid released from the film; a published turbidimetric method (Oueslati et al., 2014) was tried and validated in the presence of all other film components (Supplementary material,

Figure S1). Unfortunately, interactions between components taking place during film drying prevented an accurate quantification of HA release, as detailed in the Supplementary material section.

In principle, these films could also be used for inflammatory diseases which do not need an antibiotic; for this reason, the release of dexamethasone was studied also from films without levofloxacin. The result obtained showed comparable profiles, even if film containing PVA98 showed lower variability and slightly lower release (Figure 4, panel C). Finally, the impact of a higher cyclodextrin content was also evaluated (Table 1) on PVA87 containing films, since cyclodextrin can have penetration enhancing properties and, for this reason, this film was further evaluated on the cornea. In this case, the higher cyclodextrin content reflects in a significantly faster drug release. We can hypothesize that in this film, that contains also a higher amount of glycerin (see par 2.4.2), these two hydrophilic compounds interact with hydroxyl groups of HA, reducing its interaction with water and thus the gel formation.
Overall, the release of the two drugs, obtained with the inclined plane set-up, is relatively fast. Nevertheless, it should be highlighted that this method cannot closely reproduce the in vivo situation, characterized by the presence of much lower fluid volumes. For instance, the volume contained in the conjunctival sac is about 7 µl and tears are produced with an average flow rate of 1–2 µl/mL (Pepić et al., 2014; Van Haeringen, 1981); even lower volumes and fluid movement are involved in case of subconjunctival space. The study of drug release for ocular administration represents an important challenge and non-compendial apparatus and conditions are often used (Adrianto et al., 2022; Pereira-da-Mota et al., 2022; Subrizi et al., 2019).

3.3. Ocular applications of the prepared films

Other authors previously demonstrated the interesting potential of solid platforms as films or contact lenses for the delivery of dexamethasone (Balla et al., 2022; Bengani et al., 2020; Calles et al., 2016; Kim et al., 2010), levofloxacin (Chang et al., 2022; Noori et al., 2021) or dexamethasone associated to one antibiotic (Baeyens et al., 1998; Gade et al., 2020; Peng et al., 2010). Recently, Bao et al prepared hydrogel films for the combined delivery of dexamethasone and levofloxacin; the system, made of oxidized hyaluronic acid and dexamethasone covalently conjugated with glycol chitosan, showed to be promising for the treatment of postoperative endophtalmitis (Bao et al., 2021).

3.3.1. Targeting the anterior segment: drugs retention inside the cornea (intact eye-bulb model)

To evaluate drug uptake into the cornea, the films were applied to the cornea surface of intact eye bulbs (Figure S2). Given the mucoadhesive properties of the films, that can be attributed mainly to the presence of hyaluronic acid, they adhered to the cornea throughout experiment duration, despite the regular addition (every hour) of STF on their surface. The amount of drugs retained in the tissue is reported in Table 3 and show no difference beween the two films, that are able to accumulate approximately 10 µg of DEX and 90 µg of LVX per gram of cornea. This means that differences in the release kinetic do not reflect in a different permeation probably because diffusion across corneal epthelium represents the limiting step to the permeation. Despite this similarity, the behavior of the two films in contact with STF is very different (see Figure 3A), and for this reason, the film containing PVA98 is more suitable for cornea application, while the one with PVA87 could be better applied in the conjunctival sac. The accumulation of LVX (17-folds higher with respect to DEX) cannot be simply explained by its higher concentration in the film. Probably, despite its hydrophilicity the good penetration properties of LVX can be attributed to the presence of a carrier-mediated transport in the epithelium (Kawazu et al., 1999).

Table 3. Amount of DEX and LVX accumulated in the cornea after 4 hours of application of the prepared film. The composition of the formulations is detailed in Table 1; the statistical analysis is summarized in Table S2.

Film	DEX		LVX	
	DEX (μg/cornea)	DEX (μg/g)[a]	LVX (μg/cornea)	LVX (μg/g)[a]
87_βDL	2.48 ± 1.55	10.77 ± 6.75	42.94 ± 2.48	186.80 ± 89.78
98_βDL	2.22 ± 0.79	9.65 ± 3.44	40.70 ± 2.22	176.97 ± 31.31
98_βD	3.18 ± 0.84	13.80 ± 3.64	-	-
87_βD	1.43 ± 0.23	6.23 ± 1.00	-	-
87_ββD	1.04 ± 0.35	4.52 ± 1.52	-	-

[a] Calculate by considering the average cornea weight : 230 ± 36 mg

As far as we know there are not literature data on therapeutic concentration of DEX in the cornea for the treatment of inflammatory diseases. Even though, Djalilian et al. showed that cytokines production in human corneal epithelial cells and fibroblasts cell lines can be inhibited with a concentration of DEX 1 μM (0.392 μg/mL) (Djalilian et al., 2006). As the amount of drug retained in the cornea from both films is at least 5-folds higher than the one required in vitro for cytokines inhibition, the formulations used could be considered promising. Also in case of levofloxacin, the cornea concentrations found largely exceed the MIC for *S. aureus* strains and for other frequent Gram-positive and Gram-negative eye pathogens (Dajcs Joseph et al., 2004; Figus et al., 2020; Yamaguchi et al., 2016). It is however worth mentioning that the experimental set-up used does not reproduce neither blinking nor the real rate of tear fluid production and drainage. These factors can be only studied in vivo, together with the evaluation of the actual mucoadhesive properties of the devices.
Films containing DEX only were also evaluated. DEX-loaded films can be useful for the treatment of dry-eye disease (DED) since they could combine the anti-inflammatory action of DEX with HA, which in clinical studies showed to play a key role in reducing Dry Eye Disease (DED) symptoms (Yang et al., 2021). The amount of DEX accumulated resulted slightly higher (PVA98) or lower (PVA87) with respect to films containing also LVX, however, due to high data variability, differences are not statistically significant. Finally, since cyclodextrins are reported to promote drug transport across epithelia (Másson et al., 1999; Xu et al., 2021) and in particular across the cornea (Pescina et al., 2016), we prepared a film with a higher HPCD content (Table 1): DEX amount in the film was not increased in order to evaluate the ability of free HPCD (not involved in drug solubilisation) to interact with the corneal epithelium and increase its permeability. However, DEX accumulation did not improve (Table 3), resulting on the contrary in a small decrease, even if not statistically significant. The lack of enhancing activity of HPCD has been reported also by other authors (Babu et al., 2008; Loftsson et al., 1994b) and can be linked to the absence of free (i.e. not included in HPCD) drug in the film. Finally, it is worth highlighting that film 87_ββD is characterized by the lowest accumulation and the highest release rate (Figure 4C), again indicating that the penetration into the tissue - and not the release from the film - accounts for the limiting step to the corneal accumulation.
Table S2 summarizes the result (p values) of the statistical analysis among the different conditions tested.

3.3.2. Targeting the posterior segment: permeation of dexamethasone and levofloxacin across the sclera

In principle, the prepared film could be applied below the conjunctiva (on the sclera surface) for the delivery of DEX to the posterior segment of the eye, for instance in the treatment of inflammatory conditions of the sclera, choroid, or retina (Beardsley et al., 2013; Gaballa et al., 2021; Nascimento et al., 2013; Prieto et al., 2020). The additional loading of LVX should be considered a plus in the management of some of these conditions, which can involve bacterial infections, usually resulting from antecedent traumas or surgical interventions (Beardsley et al., 2013; Tittler et al., 2012; Wang et al., 2013).

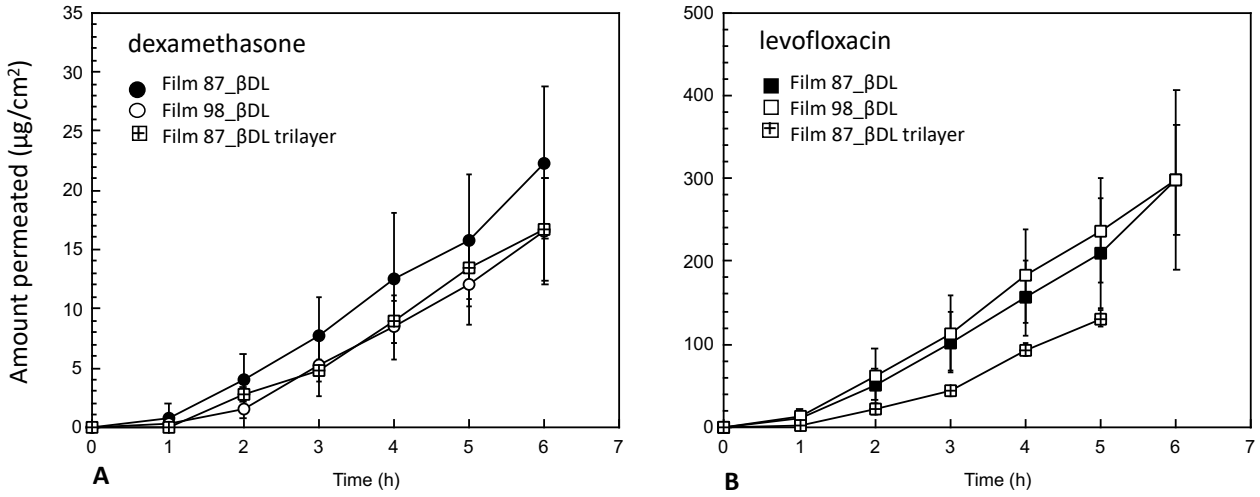

Figure 5. Permeation profiles of DEX (panel A) and LVX (panel B) from films of PVA87 and PVA98 across the sclera. The permeation across the trilayer sclera-choroid-Bruch's membrane from films of PVA87 is also shown.

The permeation profiles obtained for DEX and LVX from the two films are reported in Figure 5. In this case only combined films were evaluated, since the presence of LVX previously demonstrated a limited, if any, effect on DEX release and penetration. Permeation from both PVA87 and PVA98 based films have superimposable profiles for both drugs, although the release from the two films was considerably different. It is however important to underline that the set-up used in the two cases is different since the applied STF volume is different and thus drug release taking place in Franz cell can vary from the incline plane set-up.

Table 4 reports the permeation parameters obtained and highlights a 3-folds higher permeability coefficient of levofloxacin than dexamethasone. This is in agreement with the different nature of the two molecules: LVX is a hydrophilic amphoteric substance and diffusion across the hydrophilic interfibrillar matrix of the sclera is favoured, for comparison with DEX. Additionally, despite the similar MW, we cannot exclude that DEX is released from the film and penetrates inside the scleral pores as HPCD complex, i.e. the diffusion moiety will have a significantly higher MW. If compared with literature data, transscleral permeability coefficient of levofloxacin obtained in previous studies starting from a solution resulted 30-folds higher (approximately $5*10^{-6}$ cm/s; (Pescina et al., 2012)) with respect to the permeation from the film, and the same can be said for dexamethasone: literature reports a permeability coefficient across porcine sclera of $11.1 \pm 2.1 *10^{-6}$ cm/s (Loch et al., 2012). This result supports a controlled release of the dugs from the formulations; indeed the % delivered through the sclera after 6 h was approx. 8% for DEX and 25% for LVX.

Table 4. Permeation parameters of DEX and LVX across sclera and trilayer.

Film	**Tissue**	**Dexamethasone**			**Levofloxacin**		
		J (µg/cm²h)	P*10⁻⁷ (cm/s)	Lag time (min)	J (µg/cm²h)	P*10⁻⁷ (cm/s)	Lag time (min)
87_βDL	Sclera	4.5 ± 1.1	0.63 ± 0.15 [a]	76 ± 20	55.1 ± 17.0	1.56 ± 0.48 [a]	68 ± 13
98_βDL	Sclera	4.0 ± 0.9	0.52 ± 0.13 [a]	117 ± 21	59.8 ± 9.0	1.86 ± 0.28 [a]	63 ± 27
87_βDL	Sclera-CHB	4.3 ± 0.9	0.61 ± 0.13 [a]	115 ± 5	36.7 ± 4.4	1.04 ± 0.12 [a]	87 ± 17

[a] Calculated considering a drug concentration as in Table 2

Given the superimposable results obtained with the two different PVA across the sclera, only the film composed of PVA87 (87_βDL), was evaluated across the trilayer (composed of sclera, choiroid and Bruch's layer), in order to assess a possible contribution of melanin in the barrier properties of the tissue (Pescina et al., 2012). However, as illustrated in Figure 5 no difference is present in comparison with permeation across the sclera alone, suggesting that melanin does not represent a relevant barrier for DEX permeation. Indeed, literature data suggest that DEX tendency to bind melanin is rather low (Rimpelä et al., 2020). On the contrary, even if the differences are not statistically significant, a lower flux and longer lag time were seen in case of levofloxacin, a compound characterized by a high affinity for melanin (Pescina et al., 2012). Overall, the data collected support the use of this platform for the controlled delivery of DEX and LVX to the posterior segment of the eye.

3.4. Two-photon microscopy and fluorescence spectroscopy

From the obtained data it is not possible to speculate if dexamethasone is released from the film as such or included in the cyclodextrin and if the complex or dexamethasone alone penetrate into the tissues.

To figure out the possible penetration of the HPCD in the ocular tissues, Nile Red (NR) was solubilized in HPCD and loaded in PVA87-based films. NR was selected since it is a fluorescent probe which shares with DEX a low water solubility and a similar size (MW: 392.5 vs 318.4 Da). Additionally, the formation of an inclusion complex between NR and HPCD has been reported in the literature (Hazra et al., 2004; Ray et al., 2019). NR-loaded films were applied to cornea and sclera for 4 hours. Then, the tissues were imaged by two-photon microscopy.

Figure 6 reports the images of the cornea (epithelium and stroma) and sclera upon excitation at 1100 nm. The green signal presented in column A of Figure 6 can be mainly attributed to cell autofluorescence (Teng et al., 2006) and Second Harmonic Generation (SHG) signal generated by collagen fibers (Teng et al., 2006; Zyablitskaya et al., 2018), while the red signal (culumn B) corresponds to NR fluorescence; column C show the image overlay. Overall, from Figure 6 we can assess that in the cornea, NR is clearly localized in cells, both epithelial cells and fibroblasts of the stroma, while in the sclera, an inter-fiber localization can be envisaged. It is not possible to extract quantitative information by comparing red intensity signal in the different tissues, for two reasons: 1) the characterisitcs of the tissues are very different in terms of polarity, and NR emission is affected by polarity of the environment (Kucherak et al., 2010); 2) the images were taken in slightly different experimental conditions (see figure caption for details) to better visualize NR emission.

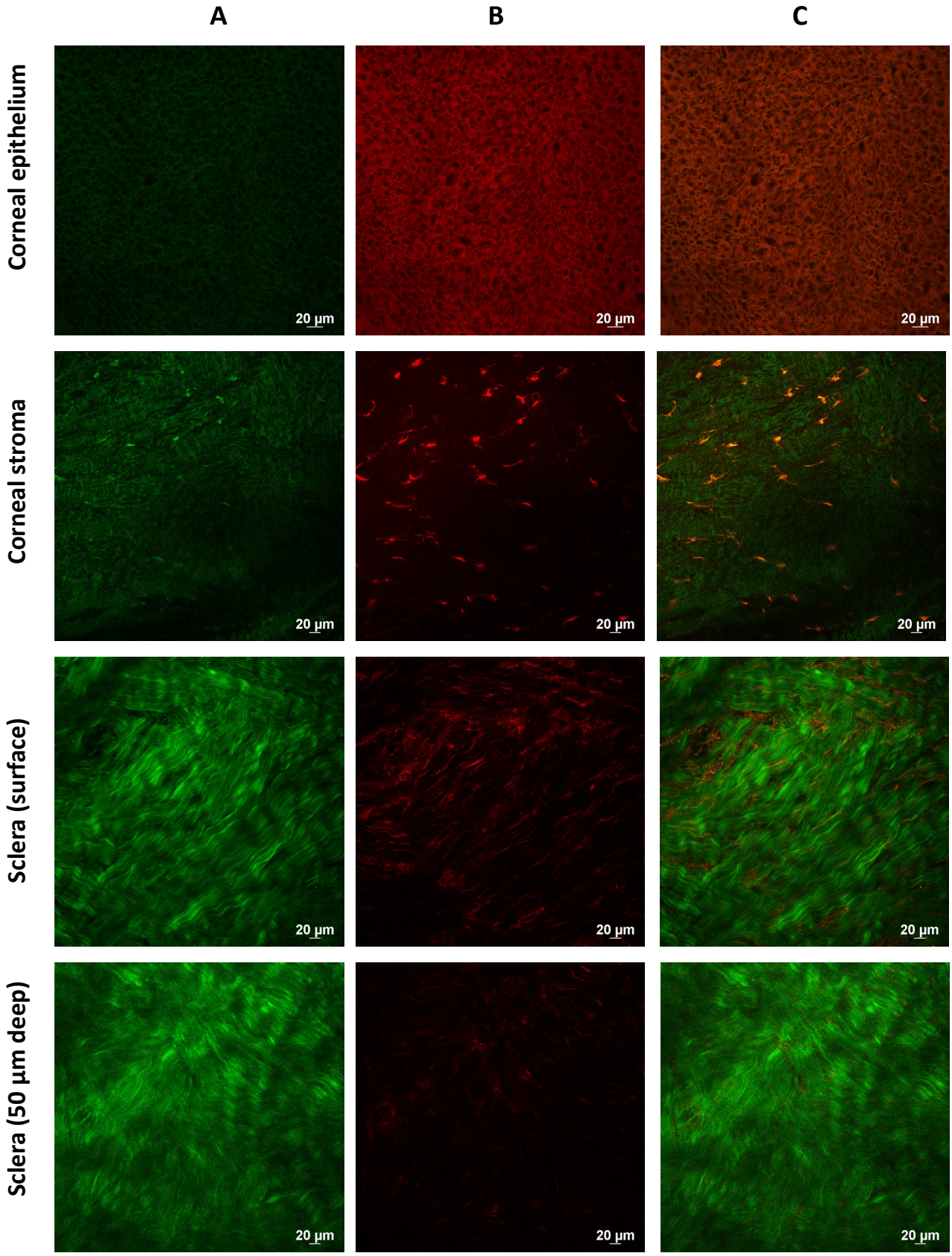

Figure 6. Two-photon microscopy images obtained after tissue irradiation at 1100 nm. Tissues (cornea and sclera) were previously treated for 4 hours with a NR-loaded film (PVA87). Images show the corneal epithelium (Power laser: 1.5%. Green gain: 100. Red gain: 100), corneal stroma (150 µm depth from the endothelial surface; Power laser: 5%. Green gain: 150. Red gain: 150), sclera surface (Power laser: 1%. Green gain: 100. Red gain: 200) and sclera 50 µm deep from the episcleral surface (Power laser: 1%. Green gain: 100. Red gain: 200. Intensity of both channels increased with LUTs.).

Column A report the green signal, collected in the spectral region 506-593 nm. Green signal is due to cell autofluorescence and SHG signal from collagen fibers. Column B report the red signal, collected in the spectral region 604 - 679 nm, which corresponds to NR fluorescence. Column C show overlay of images A and B. Holes visible in the corneal epithelium are an artefact of the experimental procedure (i.e. tissue freezing after the permeation experiment).

Emission spectra were aquired to investigate the presence of HPCD complex in the tissue (Figure 7). In fact, being NR a solvatochromic probe (Boldrini et al., 2002), the spectral position of its emission changes with the polarity of the environment, allowing for the differentiation between NR included in the HPCD and NR released from the HPCD. Figure 7 compares emission spectra of NR collected in different environment. Emission of NR-HPCD complex in water (NR-HPDC, black dashed line in Figure 7) was collected with a fluorometer (see section 2.7 for the instrument description), in order to detect the whole spectrum (the spectral detector coupled with the microscope works in a limited spectral range from 400 nm to 650 nm): the maximum of the emission band is detected at 650nm. The maximum of emission of NR in water is at 665 nm. The spectrum recorded from the corneal epithelium (Figure 7, pink line) is sizeably blue-shifted (25 nm) compared to the spectrum of NR-HPDC complex, suggesting that NR has been released from HPCD and is located in a less polar environment (Ghezzi et al., 2022). This is compatible with the probe localization inside the lipophilic domains of the epithelial cells. A similar spectrum profile of NR is obtained also from the corneal stroma (Supplementary Material, Figure S3), confirming that the NR-HPCD complex dissociates in contact with the corneal surface or in the epithelial cells.

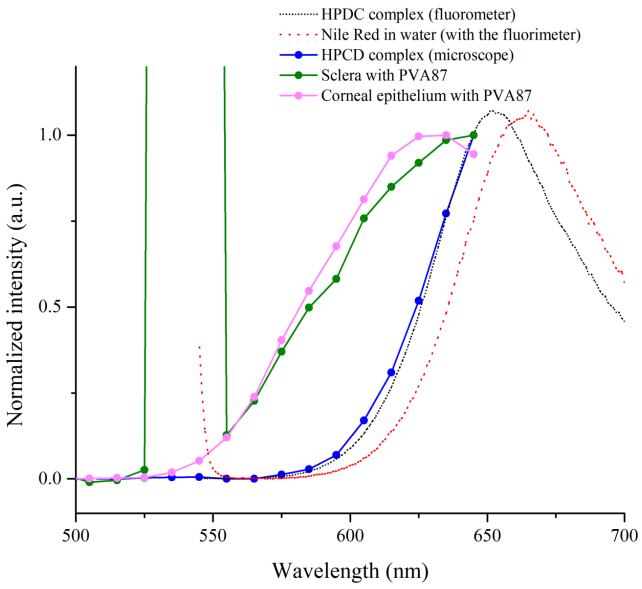

Figure 7. Emission spectra recorded in tissue samples and in solution. Spectra acquired with the fluorometer were obtained exciting the sample at 540 nm, while spectra collected with the two-photon microscope were obtained exciting the sample at 1080 nm, in order to minimize the tissue autofluorescence and maximize at the same time the NR signal. Moreover, spectra collected with the two-photon microscope are collected up to 650 nm, since higher wavelengths fall out of the detectable range of the instrument.

The spectral profile obtained from the sclera (50 µm in depth from the surface) is slightly red-shifted compared to corneal tissue and the maximum of its emission cannot be clearly detected (the same happens for the NR-HPCD complex, when emission is detected with the spectral detector of the microscope, blue line in Figure 7). Sclera is a strongly hydrophilic environment, and in case of release of NR from the HPDC, we would expect an emission spectrum resembling emission in water (Ghezzi et al., 2022) . Actually, emission of

NR appears to be blue-shifted with respect to NR emission in water: this result is in line with the hypothesis that NR-HPCD complex diffuses (at least in part) intact in the interfibrillar matrix of the sclera. However, it is difficult to give a definitive answer on the fate of the NR-HPDC complex within the scleral tissue, since the spectral position and the shape of the emission band could to be influenced by the possible presence of the other film components (HA, PVA and glycerin) that can likewise diffuse across the scleral pores affecting the hydrophilicity of the environment in which NR is found.

4. Conclusion

In this work, a new delivery platform for the simultaneous delivery of hydrophilic and lipophilic drugs to eye tissues was developed. The films, made of biocompatible polymers such as PVA and hyaluronic acid, demonstrated high loading capacity with respect to both levofloxacin and dexamethasone. By changing the hydrolysis grade of the PVA it was possible to modify the swelling behavior, thus adjusting the film characteristics to the need of the specific application site in the eye: films made with PVA 98 and charactezied by a 2D swelling are more suitable for a corneal application, whereas in case of application in the conjunctival sac or under the conjunctiva, film with a 3D swelling can be more easily used.
Films were prepared with an easy and potentially scalable method which did not involve the use of organic solvents, furthermore all the excipients used are approved for ocular administration. Further investigations are needed for a deeper comprehension of these systems concering their behavior in an *in-vivo* condition and to approach relevant issues such as mechanical properties, sterilization and stability. Preliminary data indicate that both drugs are stable in the formulation for at least 6 months, but this aspect need to be investigated for longer time periods.

5. Authors contributions

All authors have read and agreed to the published version of the manuscript.

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7. CRediT authorship contribution statement

Martina Ghezzi: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Ilaria Ferraboschi:** Investigation, Writing – review & editing. **Adriana Fantini:** Methodology, Investigation. **Silvia Pescina:** Methodology, Validation, Writing – review & editing. **Cristina Padula:** Validation, Writing – review & editing. **Patrizia Santi:** Writing – review & editing, Funding acquisition. **Cristina Sissa:** Validation, Writing – review & editing, Funding acquisition. **Sara**

Nicoli: Conceptualization, Methodology, Writing – original draft, Writing – review & editing, Funding acquisition.

8. Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Materials

1. Composition of the films: screening phase

In the screening phase, two different types of hyaluronic acid were considered (300 vs 1000 kDa), as well as two PVAs, characterized by a similar MW, but with a different hydrolysis degree (87% vs 98%), that confers peculiar properties to this polymer. As plasticizer, PEG 400 and glycerin were tested. At first, films flexibility and films texture/handling were evaluated. The evaluation was carried out through simple visual inspection and manipulation, to figure out if the film was easily handled and , at the same time, if it had adequate flexibility to be applied to ocular tissues (too rigid materials can cause mechanical damage).
The film compositions evaluated in the screening phase are summarized in Table S1.
Briefly, films made by PVA only (both 87 and 98) were very stiff. The addition of HA (50:50 weight ratio) increased their flexibility, regardless of the MW of the hyaluronan used (300 vs 1000 kDa). HA with a MW of 1000 kDa was selected for further studies, due to the better texture of the film and the higher performance reported in the literature for ocular surface hydration and lubrication, as a result of an effective maintenance of secreted mucin of the ocular surface [1, 2]. To further increase film flexibility, glycerin and PEG 400, both plasticizers approved for ocular application [3], were tested. The best result in terms of flexibility was obtained with 10% glycerin. Finally, to improve the film texture and handling, the HA:PVA ratio was brought to 40:60.
After the screening phase, two different films were selected for further experiments (#12 and #13, Table S1): they both contained a HA:PVA:glycerin in ratio 36:54:10 and differed for PVA grade (98 vs 87).

Table S1. Composition of the blank films prepared with their codification and main features.

	Components			Weight ratio HA/PVA/PLAST	Flexibility	Texture/ handling		
#	HA[a]	PVA[b]	Plast[c]					
1	-	87	-	-	-	++++		
2	-	98	-	-	-	++++		
3	300	87	-	50/50	+	++		
4	300	98	-	50/50	+	++		
5	1000	87	-	50/50	+	++		
6	1000	98	-	50/50	+	++		
7	1000	87	Glycerin	47.5/47.5/5	++	++		
8	1000	98	Glycerin	47.5/47.5/5	++	++		
9	1000	87	Glycerin	45/45/10	+++	++		
10	1000	98	Glycerin	45/45/10	+++	++		
11	1000	98	PEG 400	45/45/10	+/-	++		**CODE** (further used in the paper)
12	**1000**	**87**	**Glycerin**	**36/54/10**	**+++**	**+++**	***Selected formula***	**87**
13	**1000**	**98**	**Glycerin**	**36/54/10**	**+++**	**+++**	***Selected formula***	**98**

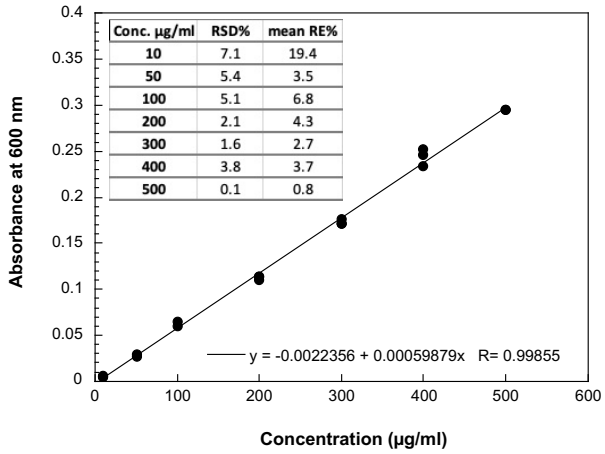
[a] polymer MW
[b] % of hydrolysis
[c] plasticizer

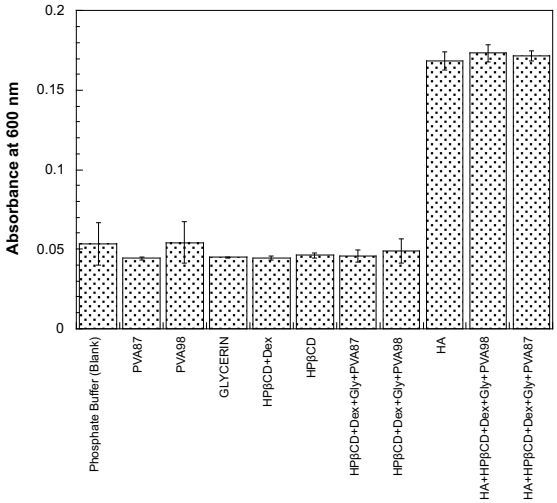
2. Hyaluronic Acid quantification

The method was adapted from a previous work [4]. Briefly, CTAB reagent (1.25 g) was dissolved in 50 ml of 2% (w/v) NaOH solution. A solution of HA at concentration of 1 mg/ml was prepared in 0.1 M phosphate buffer pH 7.4 and then diluted to obtain the standard concentrations used for the preparation of the calibration curve (see Figure S2).
100 µl of HA standard solution were introduced in a 96-well plate and incubated for 15 minutes at 37°C. Afterwards, 100 µl of CTAB were added and the plate was incubated again for 10 minutes at 37°C. After 20

seconds shaking, absorbance was measured at 600 nm wavelength against blank (0.1 M phosphate buffer pH 7.4) and plotted against HA concentrations for the construction of the calibration curve. Selectivity of the method was evaluated by preparing solutions containing the different components of the films, alone or combined (Figure S2).

For the quantification of HA in the film, the punched films (0.6 cm^2 of area) were dissolved in 10 ml of 0.1 M phosphate buffer pH 7.4. Afterwards, 1 ml of solution was withdrawn, centrifuged (12 minutes at 12000 rpm) and/or filtered (0.22 µm) and analyzed with the method described above.





Film	HA % in the film	Calculated %
# 12 beta (PVA87)	~25	50.94±14.05
# 13 beta (PVA 98)	~25	65.19±4.92

Figure S1. Analytical method for hyaluronic acid quantification. Panel A: Calibration curve and related parameters. The concentration range has been selected on the basis of the expected concentrations in a release study. Panel B. Study of the possible interference of the film components on the absorbance of an HA sample (conc about 200 µg/ml). The concentration of the other components has been selected based on HA/component ratio in the film. The method is selective for HA, the presence of PVA, glycerin and cyclodextrins do not interfere with the analysis. Panel C. Quantification of HA in the film using the previously validated method. The film (0.6 cm^2) was dissolved in 10 ml of phosphate buffer (PB). The result suggests that, after drying, an interaction between HA and the other components takes place, preventing the use of this method for quantification of HA release from the film.

3. Drug retention inside the cornea

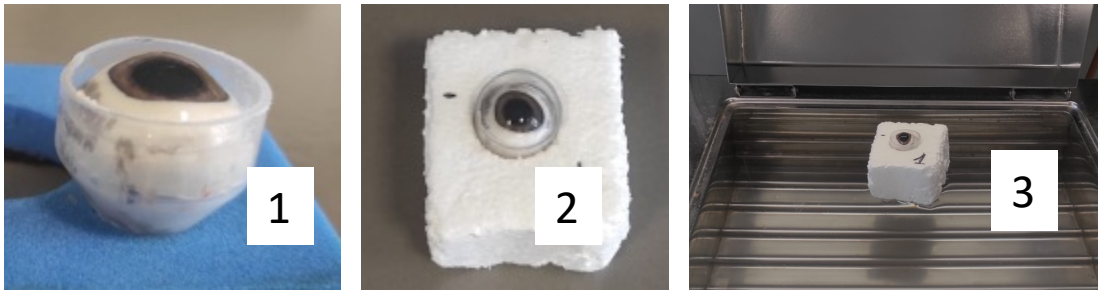


Figure S2. Set-up for the corneal retention experiment using intact eye bulbs. 1) Conical plastic falcons were cut at the bottom and filled with 1 ml of saline solution. The eye bulb was inserted in the falcon with the cornea facing up 2) The

falcon was placed into a floating polystyrene support. 3) Then, the film was applied and the system was inserted inside a bath at 37°C for 4 h, covered with a lid to keep a relative high RH% on eye surface.

Table S2. Comparison between DEX corneal accumulation starting from different films. The table reports the *p* values obtained (*t*-test) from the comparison of the values reported in Table 3 of the main text.

	87_βDL	98_βDL	98_βD	87_βD	87_ββD
87_βDL		0.8098	0.5313	0.2294	0.1241
98_βDL	0.8098		0.2242	0.1109	0.0422
98_βD	0.5313	0.2242		0.0096	0.0053
87_βD	0.2294	0.1109	0.0096		0.1087
87_ββD	0.1241	0.0422	0.0053	0.1087	

4. Two-photon microscopy spectra

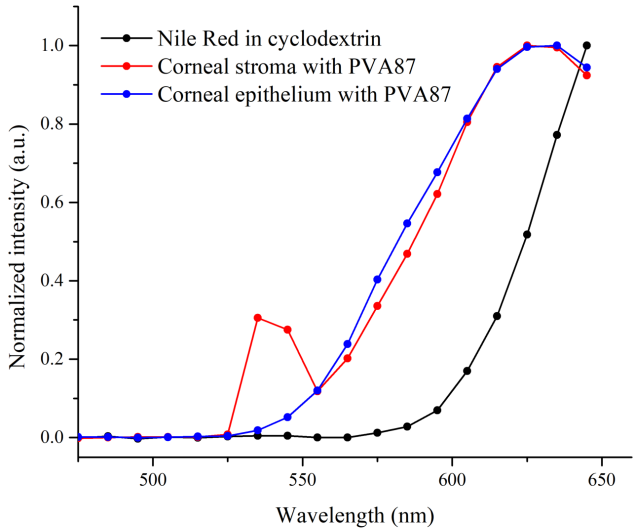


Figure S3. Emission spectra recorded in tissue samples and in solution. Spectra were collected exciting the sample at 1080 nm, in order to minimize the tissue autofluorescence and maximize at the same time the NR signal. Spectra collected with the multiphoton microscope are limited to 650 nm, since higher wavelengths fall out of the detectable range of the instrument.

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