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Dual drug delivery from hydrophobic and hydrophilic intraocular lenses: *in-vitro* and *in-vivo* studies

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Abstract

Posterior capsule opacification (PCO) still remains the most frequent long term complication after cataract surgery, while endophthalmitis is rare but severe and should be prevented at all cost. Intraocular lenses (IOLs) with different designs (eg. edge and body-haptics angle) and materials (acrylic hydrophobic and acrylic hydrophilic surfaces) have been studied to reduce PCO. For the prevention of endophthalmitis, intracameral injection followed or not by topical treatment with antibiotics and anti-inflammatories are usually prescribed. The objective of this work was to investigate the use of IOLs as controlled release platforms of two drugs, the antibiotic moxifloxacin (MXF) and the anti-inflammatory ketorolac (KTL) that could advantageously substitute the usual treatment. Two types of IOLs were chosen, hydrophobic and hydrophilic. Hydrophobic IOLs have shown better results in the prevention of PCO because they adhere better to the posterior capsular bag, while hydrophilic IOLs are advised in the case of patients with uveitis, glaucoma or diabetes. The IOLs were loaded with MXF+KTL and sterilized by high hydrostatic pressure. Both IOLs reduced the tendency for adhesion of LECs. *In vivo* tests were done to compare the concentration of the drugs in the aqueous humor obtained after eye drops administration and drug-loaded IOLs implantation. The developed IOLs were able to release MXF and KTL at therapeutic levels, in a sustained way, which contrasts with the eye drops prophylaxis. No PCO signs were detected and histological analyses demonstrated biocompatibility of these devices.

Keywords: Intraocular lenses, Moxifloxacin, Ketorolac, Drug release, *In vitro*, *In vivo*

1. Introduction

Cataract is the leading cause of blindness worldwide. According to the World Health Organization, in 2020, 32 million persons will need surgical removal of the cataracts with implantation of intraocular lenses (IOLs) [1]. The huge medical, social and economic impact of this prediction justifies the increasing number of studies on the prevention and treatment of the complications associated to this procedure. In fact, although cataract removal is a commonly performed surgery, it is not free of risks. The posterior capsule opacification (PCO), which may occur months or years after surgery, decreases significantly visual acuity having an incidence that can be as high as 50% [2]. In contrast, post-cataract surgery endophthalmitis is a rare (0.04-0.2%), but severe complication, usually associated with limited visual recovery [3,4].

PCO results from the adhesion of lens epithelial cells (LECs) to the surface of the IOL facing the posterior capsular bag. The reduction of the PCO development has been addressed in three ways: adequate choice of the IOL biomaterial [5–7], design of the lenses [5,7,9] and drug vehiculation using the IOLs as delivery devices [10–13]. Two decades ago, it was already recognized that hydrophobic polyacrylic IOLs were associated with a lower PCO incidence than hydrophilic polymethylmethacrylate and silicone IOLs [5]. Hydrophobic IOLs can adhere more easily to the collagen membrane of the posterior capsular bag, reducing the free space between both surfaces, which minimizes the possibility of LECs to reach the surface of the lens [14]. However, this type of IOLs have a high tendency to adsorb extracellular matrix proteins and inflammatory cells, which may lead to other complications, such as iris posterior synechiae and anterior capsular opacification, mainly in patients with disruption of blood-ocular barriers. For this reason, hydrophobic IOLs, the most commonly used, are not recommended in patients with uveitis, glaucoma and diabetes who receive hydrophilic IOLs instead [15]. Other authors claim that the PCO incidence is independent of the nature of the biomaterial [16], thus the role of the biomaterial remains a controversial issue. Concerning the lenses design, reduced PCO incidence was found in IOLs with sharp edges [5,7,8]. Finally, in recent years, the use of IOLs as drug delivery devices, such as caffeic acid, phenethyl ester and methotrexate [10,11], rapamycin [12] and erlotinib [17] has been identified as a potential strategy for PCO prophylaxis.

Endophthalmitis is mainly caused by gram-positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus pneumonia* and may assume an acute form that occurs in the first 48 h following surgery or appear more than 6 weeks afterwards. Furthermore, post-cataract surgery inflammation is associated with the physical trauma of the surgery, which induces an inflammatory response with the release of inflammatory mediators. Continuous inflammation leads to discomfort, compromised visual outcomes and cystoid macular edema (CME) [18]. To prevent or treat these complications, eye drops of antibiotics and anti-inflammatories are topically administered during a relatively long period of time with a very frequent posology [19,20]. Typically, both antibiotics and anti-inflammatories are administered individually. However, formulations of eye drops containing both drugs are already available in the market, such as the combination of moxifloxacin and ketorolac (Megacom ™). The disadvantages of topical instillation are well known and concern low drug bioavailability [21] and adverse side effects due to absorption at the systemic level [22]. Intracameral injection, which is also an option for antibiotics [23], has not been adopted by many surgeons due to several concerns despite the observed reduction in the endophthalmitis rate [24]. In recent years, the possibility of using IOLs as the vehicle to deliver, in a sustained way, antibiotics and anti-inflammatories to prevent endophthalmitis has been addressed through *in vitro* and *in vivo* studies [25–30]. Some of these works were carried out by our

group. We have been working on the optimisation of *in vitro* release profiles of moxifloxacin (MXF) and ketorolac (KTL) from CI26Y, a commercial hydrophilic IOL material [31–33]. Simultaneous release of both drugs complying with the therapeutic needs was achieved: the loaded lenses were effective against *S. aureus* and *S. epidermidis* up to 15 days, and the amount of released ketorolac was enough to prevent inflammation for a minimum of 16 days [34]. MXF was chosen because it is a fourth-generation fluoroquinolone antibiotic, whose spectrum includes, not only the gram-positive bacteria earlier mentioned as the common causes of endophthalmitis (*S. aureus*, *S. epidermidis* and *S. pneumoniae*), but also Gram-negative rods (*Escherichia coli*, *Proteus*, *Klebsiella*), *Haemophilus influenzae*, atypical bacteria (*Mycoplasma*, *Chlamydia*, *Legionella*), and anaerobic bacteria [35,36]. KTL is a nonsteroidal anti-inflammatory drug (NSAID) used after cataract surgery to control pain, avoid inflammation, and reduce the incidence of CME [37]. MXF is a lipophilic drug and its protonated form predominates in water at pH 5.6 [38], while KTL is anionic and soluble in water as salt, but it is lipid soluble in the nonionized state [39].

In the present work, the two main problems associated with IOL implantation (endophthalmitis and PCO) were tackled and the possible solutions were tested with in vitro and in vivo tests. Acrylic based hydrophobic and hydrophilic lenses were used. Hydrophobic lenses, which have a low water content, were modified through the coating of the anterior side with a layer of hydrophilic material to enhance drug loading, while the posterior side remained hydrophobic to better adhere to the capsular bag. For both lenses, drug loading was performed by soaking in solutions containing MXF and KTL, using optimized conditions, as described in a recent paper [34]. Finally, the materials were submitted to sterilization by high hydrostatic pressure (HHP), a technique recently studied by the authors to sterilize intraocular lens materials [40]. The IOLs were characterized with respect to some important physical properties. Adhesion of human lens epithelial cells (HLECs) to the IOL surfaces was investigated. *In vitro* drug release tests were done and the antimicrobial activity of the released antibiotic was determined. Finally, the *in vivo* performance and safety of both hydrophobic and hydrophilic lenses was compared.

2. Experimental

2.1. Materials

The IOL materials tested in this study were G-free® (named from now on GF, proprietary of PhysIOL SA, Belgium) and CI26Y (from Contamac, UK). GF is a hydrophobic acrylic-based material containing ethylene glycol phenyl ether acrylate (EGPEA, 57%), 2-hydroxyethyl methacrylate (HEMA, 34%) and poly (propylene glycol) dimethacrylate (PPGDMA, 8%). CI26Y is a hydrophilic chemically crosslinked copolymer of HEMA (80-90%) and methyl methacrylate (MMA, 10-20%), incorporating a UV and blue-light blocker [41]. PhysIOL SA (Belgium) offered disks of both materials already polymerized, as well as lenses. The mixture of CI26Y monomers used for the coatings was bought from Contamac (UK). Moxifloxacin hydrochloride (MXF, purity ≥ 98%) was acquired from Carbosynth (UK), and ketorolac tris salt (KTL, purity ≥ 98%), from Santa Cruz Biotechnology (Germany). Phosphate buffer solution (PBS), pH 7.0 and AIBN (2,2'-azobis(2-methylpropionitrile), purity ≥ 98%) were purchased from Sigma-Aldrich (USA). Ethanol (96%) was obtained from Carlo Erba (Spain). Distilled and deionised (DD) water obtained from a Millipore system was used. For the antibacterial tests, Mueller-Hinton (MH) agar and antimicrobial susceptibility test discs were purchased from Oxoid (UK). Thioglycollate liquid medium

(Thio medium) from PanReac AppliChem (Spain) and soybean casein digest broth (CASO broth) from Sigma-Aldrich (USA) were used for sterility tests. For the cell adhesion assays the following reagents were used: D-Hanks cell culture grade, from Meilunbio® (China); Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, 1:1) with L-glutamine and 15 mM HEPES from Gibco; 0.05% Trypsin-EDTA (1x) from Gibco (USA); Trypan blue stain (0.4%) and CountessTM Cell Couting Chamber Slides from InvitrogenTM (USA); Fluorescein diacetate (FDA) staining solution from Sigma-Aldrich (USA); HLEC line (HLE B3, CRL-11421TM) from American Type Culture Collection (ATCC, USA). Regarding the *in vivo* experiments, the following commercial eye drops were used in the control group: Vigamox 0.5% (moxifloxacin hydrochloride ophthalmic solution) from Alcon (USA) and Acular 0.5% (ketorolac tromethamine ophthalmic solution) from Allergan (Ireland). For pupil dilatation and local anesthesia, Mydrin®-P (tropicamide and phenylephrine ophthalmic solution) from Santen (Japan) and Alcaine 0.5% (proparacaine hydrochloride ophthalmic solution) from Alcon (USA) were used, respectively. The povidone iodine (PI) solution was from Zhejian Puluo Kangyu Biological Pharmaceutical (China) and the sodium chloride injection 0.9% from Sichuan Kelun Pharmaceutical (China). To anesthetise the animals, pentobarbital sodium 0.2% was from Fujian Mindong Lijiexun Pharmaceutical (China) and Xylazine hydrochloride from Sheng Xin® (China). The medical sodium hyaluronate gel was from Qisheng (China). Formalin 10% (neutral buffered formalin) and glucose (purity ≥ 99.5%) were acquired from Sigma-Aldrich (USA). The optimal cutting temperature compound Tissue-Tek® O.C.T.™ Compound was from Sakura (Japan). All the chemicals used for the hematoxylin and eosin (HE) protocol were of analytical grade and gently provided by the Wenzhou Medical University.

2.2. Methods

2.2.1. Preparation of the samples

Disks with 1 mm of thickness and 20 of mm diameter and prototype IOLs of GF and CI26Y were washed, as recommended by the manufacturer, in a soxhlet extractor for 60 cycles using as solvent, DD water for CI26Y, and ethanol for GF material. Then, the hydrated disks were cut with a puncher in smaller disks, with 5 mm of diameter and ≈ 20 mg of dry mass. The IOLs were provided sterile and packed in saline solution. To remove the salts, the IOLs were washed with DD water under agitation for 5 days. All samples were dried for 3 days, at 36 ºC, and stored inside closed flasks until further use. The small disks were used for the *in vitro* tests, while the IOLs were used for the *in vivo* experiments.

To increase the drug loading capacity of the GF samples, a hydrophilic coating was made on the anterior surface of the disks/IOLs, by deposition of 2 µL of CI26Y polymeric solution with AIBN at 1%wt followed by evaporation at 60ºC. This deposition was repeated 12 times with a minimum interval of 2 h between two consecutive depositions. The coated disks/IOLs were washed by soxhlet extraction with DD water and dried, as previously described. The obtained coating had a dry mass of ≈ 3.5 mg.

Drug loading was done according to optimised conditions described in our previous works [**Error! Bookmark not defined.**,**Error! Bookmark not defined.**]. MXF and KTL were dissolved together in phosphate buffer solution (5 mg/mL of each drug). Each disk/IOL was soaked in 1 mL of the drugs solution at 60°C for 2 weeks and 4 days, respectively, for CI26Y and GF materials. The soaking of the GF disks/IOLs was carried out for a shorter period of time, since the amount of drug loaded did not increase after this period. The disks/IOLs were sterilized in sealed bags containing the loading solution by HHP, at 600 MPa, for 10 min and at 70ºC. Before the sterilization, the disks/IOLs were submitted to a pre-heating

step of 10 min at 70ºC. More details about the sterilization method are given in a previous work [40]. The sterilized disks/IOLs were stored at room temperature until they were used in the various experimental tests.

2.2.2 *In vitro* drug release tests

In vitro drug release tests were performed in static sink conditions, by immersion of the disks in 3 mL of PBS with agitation at 180 rpm and 36 ºC (in an Incubating Mini Shaker from VWR). At specified times, aliquots of 0.3 mL were collected and substituted by the same volume of fresh PBS solution, until a constant value in the release profile was reached. The quantification of the released drugs was done through the analysis of the collected aliquots with a spectrophotometer UV–VIS MultiscanGO from ThermoScientific®. The spectra of the release solutions were acquired between 200 and 700 nm. The concentration of both drugs in the solution was determined using the method described by Kim and Chauhan [42] to deconvolute the spectra, taking into account the spectra of the single drug solutions.

2.2.3 Physical characterization of the lenses materials

The liquid uptake (LU) and the transmittance of CI26Y and GF materials hydrated in PBS and in the final form, i.e. loaded with MXF and KTL and sterilized with HHP (and coated, in the case of GF) were assessed.
The LU was calculated by placing the dried disks in 4 mL of testing liquid (PBS or drugs solution) at 60ºC for 2 weeks or 4 days to replicate the loading conditions of CI26Y and GF, respectively. At least three replicates were done. After removing the disks from the testing liquid, their surface was blotted with absorbent paper, and the disks were weighted. The *LU* percentage was calculated using the following expression:

$$\%LU = \frac{W_t - W_0}{W_0} \times 100 \qquad (4)$$

where W_t is the weight of the swollen hydrogel at time t, and W_o is the weight of the dry hydrogel.
The transmittance of the disks was measured with an UV–vis spectrophotometer (Multiskan GO, ThermoScientific), in the wavelength range 350–650 nm (intervals of 1 nm).

2.2.4 Antibacterial activity of the release solutions

The antibacterial activity of MXF+KTL release solutions, collected after 26 days of release from sterilized disks, against *S. aureus* ATCC 25923 and *S. epidermidis* CECT 231, was assessed by agar diffusion tests. Both microorganisms were striked on a new agar plate and incubated at 37 °C for 24 h. Grown colonies were collected and suspended in a sterile solution of NaCl (0.9 %) until 1 McFarland of optical density was achieved. MH agar was prepared and sterilized according to the manufacturer's instructions. Before using, its temperature was stabilized at 50ºC in a water bath at the same temperature. Next, 350 µL of the prepared bacterial suspension was gently mixed with 50 mL of MH, spread into petri dishes (120x120 mm^2) without forming air bubbles and left to dry. Antimicrobial susceptibility test discs were carefully placed into the petri dishes and 15 µL of the solution to be tested was poured into the disks. As negative control, for each plate, sterile PBS was also poured into one disk. The incubation of the

petri dishes was done at 37 ºC for 24 h. After incubation, the induced inhibition halos were read with an electronic calliper. The same method was used to obtain a calibration curve, using solutions of MXF with known concentrations. This allowed estimate the MXF concentration in the released solutions.
The concentration of MXF of the release solutions was also determined by UV-Vis spectroscopy, to access any possible loss of drug activity, by comparison with the concentration obtained from the inhibition halos of *S. aureus* and *S. epidermidis*.

2.2.5 Sterility tests

The samples used for the sterility tests were only hydrated in PBS, to avoid possible interference of the drugs in the sterility verification. Sterility tests were done following the Portuguese Pharmacopeia 9 [43]. Thio medium and CASO broth were prepared and sterilized according with the fabricant instructions, at least one day before the sterility tests. The thio medium is intended for the culture of anaerobic bacteria, while the CASO broth is intended for the culture of fungi and aerobic bacteria. At the time of the test, the disks were removed from the bags and transferred aseptically into flasks containing the proper media, inside a laminar flow chamber. A triplicate was done for each. The thio medium flasks were incubated at 30 ºC and the CASO broth flasks at 25 ºC, both for 14 days. Positive (medium/broth + microorganisms) and negative (only medium/broth) controls were also made and incubated in the same conditions. For the positive control, *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 15442) were inoculated in two different flasks containing thio medium, and *Candida albicans* (ATCC 10231) was inoculated in a flask containing CASO broth. After 14 days, the eventual growth of microorganisms was assessed through visual observation of each flask. To validate sterility, no growth of microorganisms can occur in the flasks with the samples and in the negative control, while the positive control must present microorganisms' growth.

2.2.6 *In vitro* HLEC adhesion tests

The HLECs were cultured in DMEM/F12 using T75 flasks. The cells were grown in an incubator at 37 ºC under a humidified atmosphere with 5 % of CO_2 in air. After confluence, the grown cells were harvested and diluted with DMEM/F12 to obtain a cell density of $1.0x10^4$ cells/well. Sterile disks were inserted into each well of a 96-well tissue culture plate and 200 µL of the diluted cell suspension was carefully added onto the disks avoiding formation of air bubbles. The incubation was done at 37 ºC under a humidified atmosphere with 5 % of CO_2 in air for different incubation periods: 24, 48 and 72 h. Cells incubated into the wells without disks were used as control. For each test, a triplicate was done. After the different incubation times, the cells were stained with FDA for 15 min at 37 ºC under a humidified atmosphere with 5 % of CO_2 in air. The disks and the control were gently washed with sterile PBS several times and observed by fluorescence microscopy (Nikon, Minatu-ku, Tokyo, Japan).

2.2.7 *In vivo* experiments

In vivo experiments were done with Japanese white rabbits (2-3 kg), to test the efficacy of the CI26Y IOLs and of the coated GF IOLs simultaneously loaded with MXF and KTL and sterilized by HHP (test groups). Blank IOLs of both materials were implanted in the control groups. The Laboratory Animal Ethics Committee of Wenzhou Medical University approved this study. The rabbits were obtained from

the Animal Administration Centre of Wenzhou Medical University and were treated under the guidelines of the Association for Research in Vision and Ophthalmology. Five rabbits were used in the test groups and four rabbits, in the control groups.

On the day before the surgery, all rabbits were weighted, the eyes were grossly checked and the intraocular pressure (IOP) was measured in the right eye, after instilling 1-2 drops of Alcaine 0.5%. Before the surgery, 3-4 drops of Mydrin®-P were instilled on the right eye for pupil dilatation and all rabbits were anesthetized by injection of 0.2 % pentobarbital sodium (1 mL/kg) and xylazine hydrochloride (0.2 mL/kg). The area around the eye was disinfected with PI solution. A lid speculum was used to retract the lids. One drop of Alcaine 0.5 % was placed on the right eye. Cataract surgery was carried out on the right eye of each animal. Firstly, a primary incision was done at the corneal limbus on the 11 o'clock direction using a 3.00 mm keratome knife. Then, the anterior chamber was filled with a medical sodium hyaluronate gel. After that, a deputy incision was done on the 3 o'clock direction. A central continuous curvilinear capsulorhexis was performed. The cloudy lens was removed with phacoemulsification technology and the capsular bag was filled with the gel. Finally, the intraocular lens was implanted into the capsular bag and the residual gel was removed.

After the surgery, the usual eye drops therapy were applied to the right eyes of the control group, Vigamox 0.5 % and Acular 0.5 % eye drops, with an interval of 5 minutes. Both eye drops were applied 4 times daily. Vigamox 0.5 % eye drops were instilled until the day 7 and Acular 0.5 % eye drops until the day 14. For the test groups, no eye drops were instilled after the surgery.

Postoperatively, the eyes were examined by slit lamp microscopy on the days 1, 3, 7, 14 and 21. Before each examination the pupils of the eyes were dilated with 3-4 drops of Mydrin®-P eye drops. Photographs were obtained with a digital camera attached to the slit lamp microscope. Samples of aqueous humor were collected in the test groups on days 2, 7, 14 and 21 and in the control groups, only on days 2, 7 and 14, since no eye drops were administrated after the day 14. The aspiration of aqueous humor was always done 3.5 h after the second instillation of eye drops of the day, for the control group. Before this procedure, the rabbits were anesthetized as previously described for the surgery. The area around the eye was disinfected with PI. A lid speculum was placed to retract the lids and one drop of Alcaine 0.5 % was instilled. The eye was washed with a solution of PI mixed with sodium chloride 0.9 % in a 1:1 (v:v) proportion, and then with sodium chloride 0.9% to remove all the remaining PI/sodium chloride solution. Approximately 100-200 µL of aqueous humor was aspirated with an insulin syringe. The aqueous humor samples were stored at -80°C until analysis by Liquid Chromatography–Mass Spectrometry (LC-MS), which was based on the simultaneous determination of moxifloxacin and ketorolac according to the method described by other authors [44].

At day 21, the animals were anesthetized and killed humanely with air embolism. The eyeballs were enucleated and placed in 10% neutral buffered formalin for 24h. The iris and the cornea were separated from the remaining ocular tissues and sections were obtained for histological examination. The sectioned tissues were dehydrated in a glucose solution for circa 24 h. Then, the tissues were embedded in small cups made of aluminium foil filled with optimal cutting temperature compound, frozen with liquid nitrogen and stored in the freezer at -20°C for at least 5 h. The sectioning of the tissues was made in a cryostat at a temperature of -20°C, using a section thickness of 8.0 µm. After sectioning, the tissues placed in slides were stained with the HE staining. Briefly, the slides were sequentially immersed in PBS solution, haematoxylin solution, 1% ethanol-hydrochloride solution, ammonia solution and 0.5 % eosin solution. The slides were rinsed under running tap water between every step. After a final rinsing, the slides were immersed in 80% (v/v) of ethanol-water solution, 95% (v/v) of ethanol-water solution,

absolute ethanol and xylene. Finally, the slides were sealed with neutral balsam avoiding the formation of bubbles, covered with coverslips and left to dry. Pictures of the sections were taken with a microscope.

2.2.8 Statistical analysis

To perform statistical analysis, the IBM SPSS Statistics software was used. First, normality was checked by performing Shapiro-Wilk tests. An independent-samples t-Test was used if normality was followed. If not, non-parametric tests were done using Mann-Whitney U tests. The level of significance used was 0.05.

3. Results

3.1. *In vitro* drug release profiles

The *in vitro* drug release profiles for MXF and KTL obtained from dual drug loaded CI26Y and coated GF disks are presented in Figures 1 and 2, respectively. An extended and controlled release of both drugs for more than 26 days was achieved with the CI26Y disks (Figure 1). HHP slightly increased the released amount of MXF (15.7%, *p-value* of 0.025) and KTL (14.3%, *p-value* of 0.044). Figure 2 demonstrates the effect of the coating on the drug release amount for GF disks: the released amount of MXF and KTL increased, respectively, 42.1% (*p-value* of 0.001) and 44% (*p-value* of 0.001). HHP did not change significantly the final drug released amount (*p-value* of 0.410 for MXF and of 0.408 for KTL).

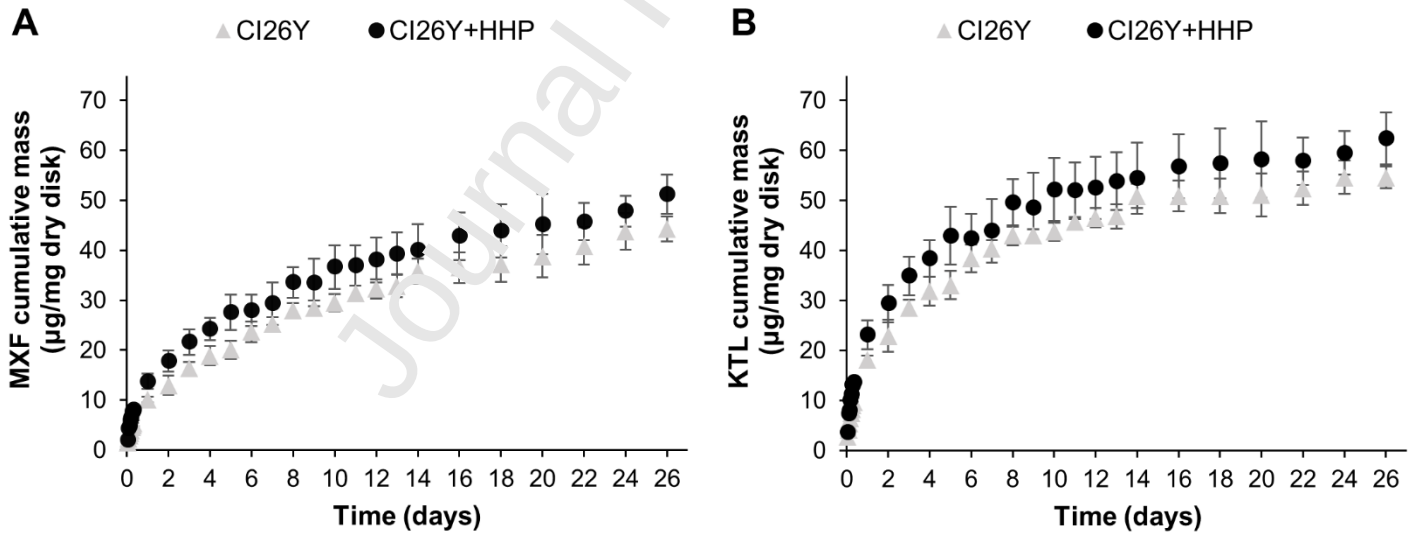

Figure 1. Drug release profiles obtained from CI26Y disks loaded with MXF (**A**) and KTL (**B**). The release data of CI26Y without HHP sterilization were taken from a previous work [34].

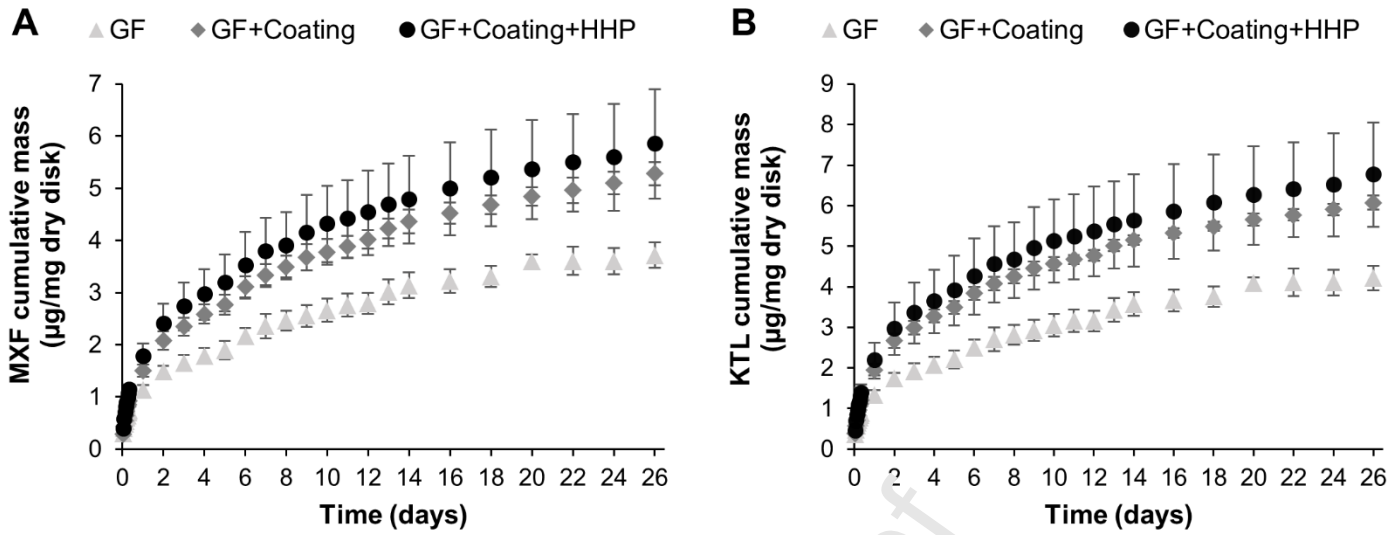

Figure 2. Drug release profiles obtained from GF disks loaded with MXF (**A**) and KTL (**B**).

3.2. Physical properties

The liquid uptake and the transmittance of CI26Y and GF materials hydrated in PBS and in final form, i.e. loaded with MXF and KTL and sterilized with HHP (and coated, in the case of GF) were assessed, and are compared in Figures 3 and 4, respectively.

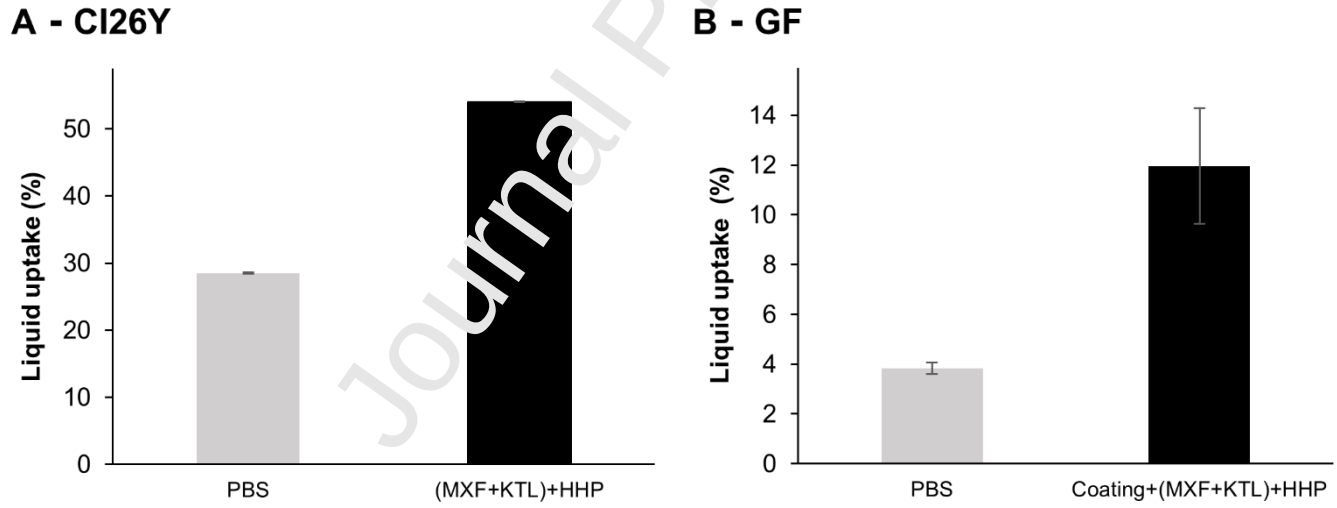

Figure 3. Liquid uptake (%) of: **A)** CI26Y hydrogel hydrated in PBS, and loaded with MXF+KTL and treated by HHP. **B)** GF hydrogel hydrated in PBS, and coated, loaded with MXF+ KTL, and treated by HHP.

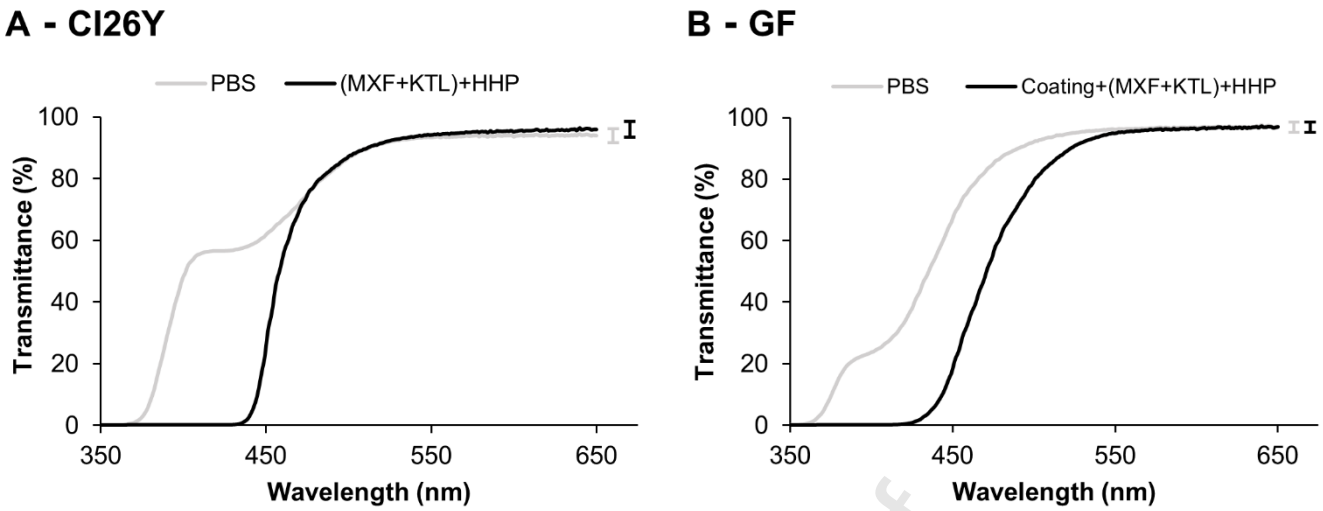

Figure 4. Transmittance (%) of: **A)** CI26Y hydrogel hydrated in PBS, and loaded with MXF+KTL and treated by HHP. **B)** GF hydrogel hydrated in PBS, and coated, loaded with MXF+ KTL, and treated by HHP.

The incorporation of MXF and KTL combined with the HHP treatment increased 90 % (*p-value* of 0.000) the liquid uptake of the CI26Y and 213 % (*p-value* of 0.026) that of GF.
The transmittance of both materials decreased for the lower wavelengths of the visible spectra after drug loading and sterilization, but above 520 nm, values > 90 % were kept.

3.3. Antibacterial activity of the release solutions

Aliquots collected after 26 days of release from the CI26Y disks and from the coated GF disks, both loaded with MXF and KTL, and sterilized by HHP, were tested against *S. aureus* and *S. epidermidis*. The presence of inhibition halos confirmed the activity of MXF against these two bacteria. In a previous study [34], it was demonstrated that KTL does not have antibacterial effects against the two tested microorganisms, so the inhibition halos are resultant only from the presence of MXF. The concentration of MXF was calculated from the inhibition halos and compared with the concentration obtained from UV-Vis spectroscopy. Figure 5 shows the concentration obtained from the inhibition halos normalized to the concentration obtained from UV-Vis and the photographs of the respective inhibition halos. For the CI26Y samples (Figure 5A), the concentration of MXF obtained from the halos against *S. aureus* was 18 % higher than the concentration calculated from UV-Vis (*p-value* of 0.015), while no significant differences were found with the inhibition halos against *S. epidermidis* (*p-value* of 0.440). Regarding GF samples (Figure 5B), no significant differences were found when comparing the concentration of MXF from UV-Vis with the concentration from the inhibition halos for *S. aureus* (*p-value* of 0.619) or *S. epidermidis* (*p-value* of 0.346).

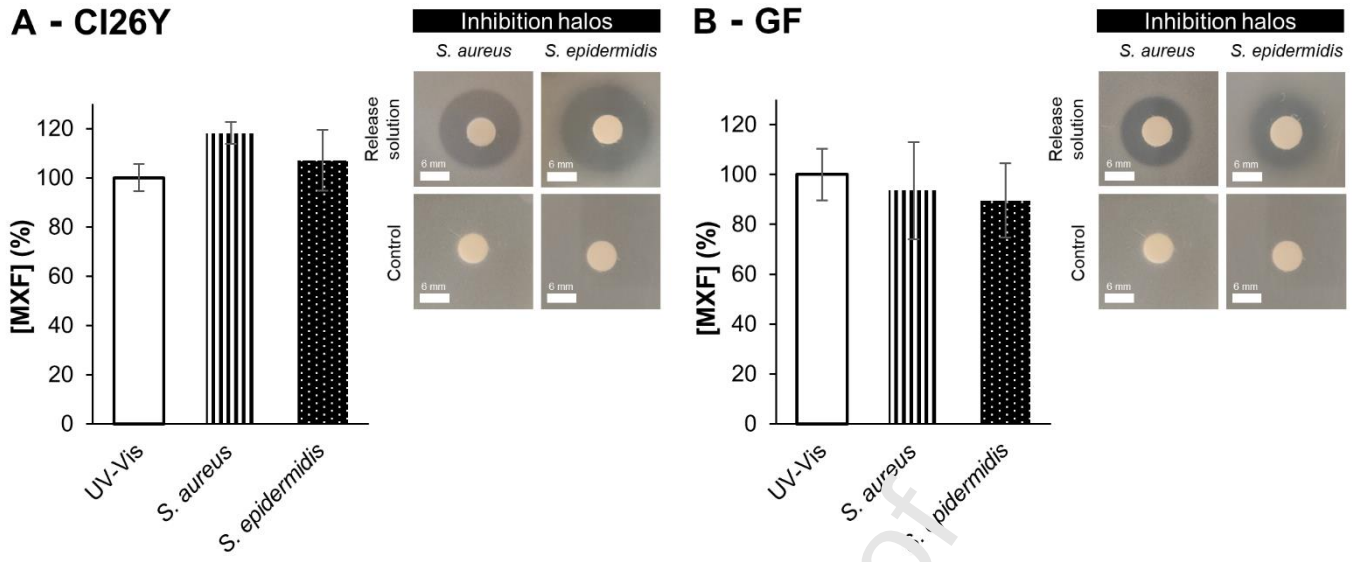

Figure 5. MXF relative concentration obtained from the inhibition halos against *S. aureus* and *S. epidermidis* normalized to the concentration obtained from UV-Vis spectroscopy, using the release solution after 26 days of *in vitro* release test from the final system of CI26Y (A) and of GF (B). Photographs of the inhibition halos are also presented including negative controls (sterile PBS). The inhibition halos presented for CI26Y (A) correspond to 10-fold dilution, while for GF (B) the dilution used was 1-fold.

3.4. Sterility tests

CI26Y and GF blank disks were submitted to HHP and sterility tests were performed. The presence of microorganisms was not detected in the flasks containing CI26Y or GF disks, confirming sterilization after HHP treatment. Validation of the results was based on the analysis of microorganism's growth in the positive control, and absence of microorganisms in the negative control.

3.5. *In vitro* HLEC adhesion test

HLECs were incubated with CI26Y and GF disks for 24, 48 and 72 h to evaluate the adhesion of cells to their surface. In Figure 6 it is possible to identify intense cell adhesion to the surface of both CI26Y and GF disks, hydrated in PBS and sterilized by HHP, which increases with the incubation time. However, CI26Y disks seem to have fewer cells adhered onto the surface than the control or the GF material. The drug-loaded CI26Y disks do not present any cells on their surface for all incubation times. The number of cells adhered on the surface of the coated and drug-loaded GF disks, after 24 h of incubation, is small but increases with the incubation time.

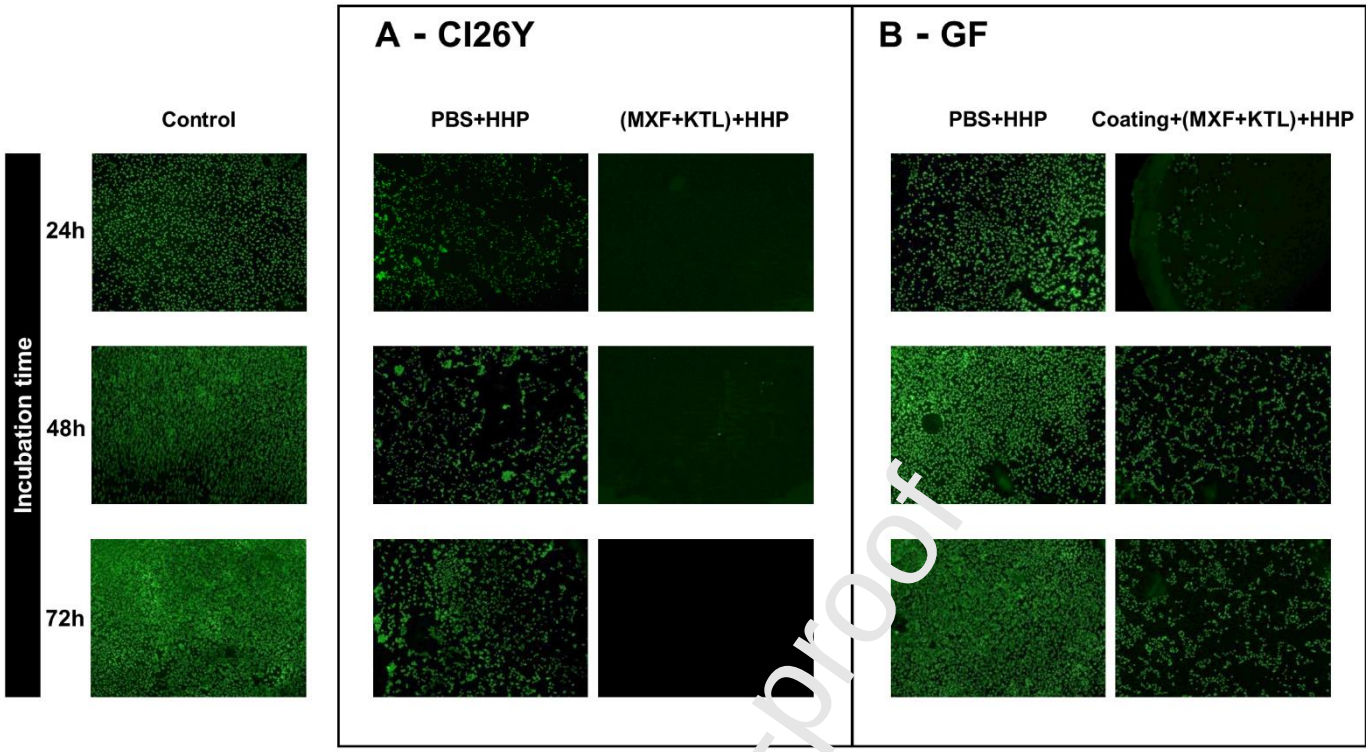

Figure 6. Fluorescent images of the HLECs adhering to the surface of the materials for different incubation times: **A**) CI26Y disks hydrated in PBS and loaded with MXF+ KTL, both sterilized by HHP; **B**) GF disks hydrated in PBS and coated, loaded with MXF+ KTL, both sterilized by HHP. The control showing the cells adhering to the bottom of the wells, for the different incubation times, is also presented. The green spots correspond to live cells.

3.6. *In vivo* experiments

3.6.1. Concentration of MXF and KTL in the aqueous humor

CI26Y and coated GF IOLs loaded with MXF and KTL and sterilized by HHP were implanted in the right eyes of the rabbits of the test groups. In the control groups, blank IOLs of CI26Y or GF, hydrated in PBS and sterilized by HHP were implanted, followed by a therapy with commercial eye drops of MXF and KTL after the surgery. MXF was administered until day 7 and KTL until day 14. The concentrations of MXF and KTL in the aqueous humor of the eyes of the rabbits of both groups are plotted as a function of time in Figures 7 and 8, respectively.

Regarding the tests with CI26Y IOLs, the concentration of MXF (Figure 7A) is always higher than the one obtained with the eye drops therapy and above the range of the minimum inhibitory concentrations (MICs) reported in the literature for *S. aureus* and *S. epidermidis* [45–47]. For KTL (Figure 7B), the concentration obtained with the drug-loaded IOLs is higher than the one obtained with the eye drops therapy during at least 7 days. However, at day 14, the concentration of KTL from the drug-loaded IOLs is lower than the one from eye drops therapy, but it remains above the range of the half maximal inhibitory concentration (IC50) for two enzymes that are responsible for inflammation, cyclooxygenase 1 (COX-1) and 2 (COX-2) [37]. At day 21, the KTL concentration is below the IC50 for COX-2, but above the IC50 for COX-1.

The results obtained with the GF IOLs show that the MXF concentration (Figure 8A) obtained from the drug loaded IOLs is higher or similar to the one obtained with the eye drops therapy until day 7. The concentration obtained from drug-loaded IOLs is within the known range for both MICs of *S. aureus* and *S. epidermidis* for all the tested times. The concentration of KTL (Figure 8B) for the drug-loaded IOLs is higher than the one obtained with the eye drops therapy for the first two days of release, but it stays above the ranges of both COX IC50 for 21 days.

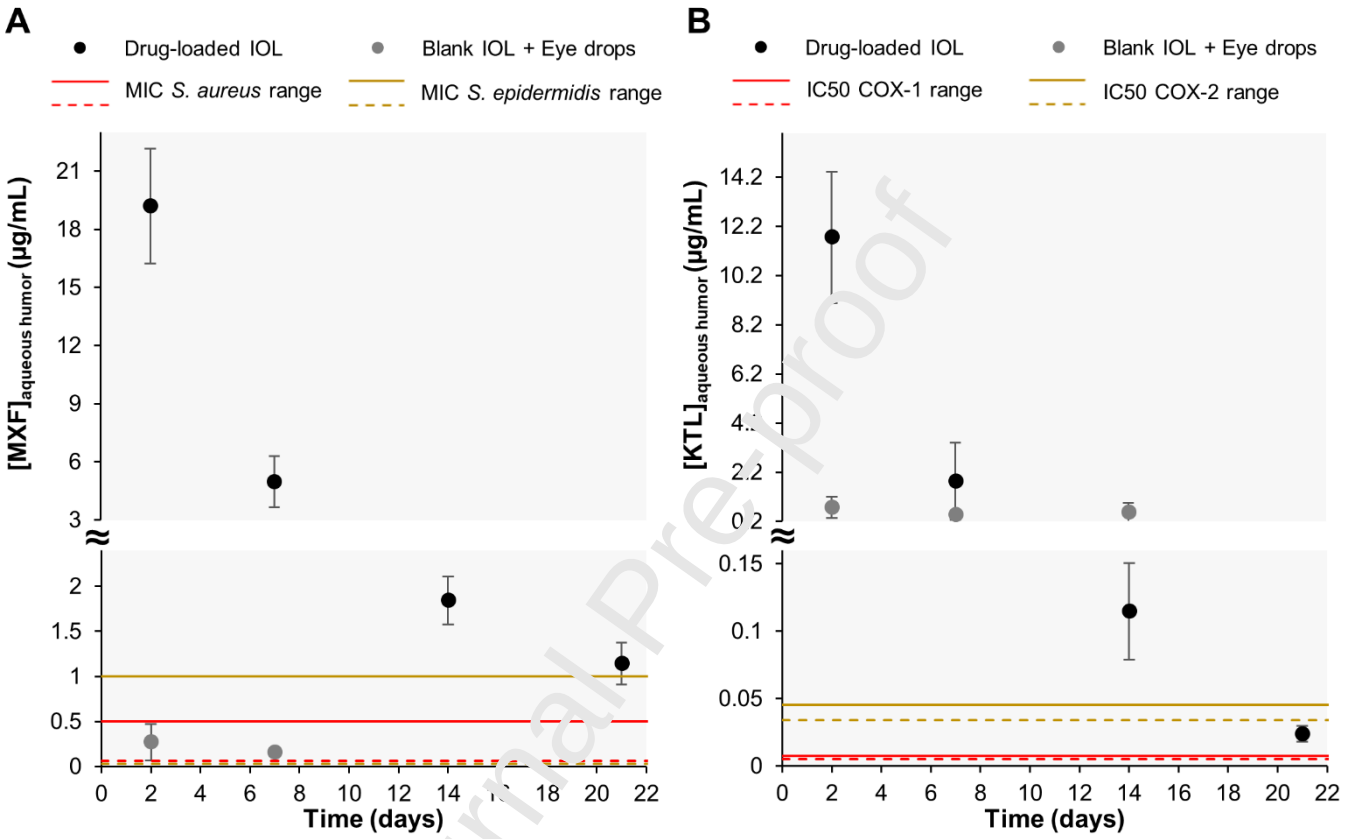

Figure 7. Concentration of MXF (**A**) and KTL (**B**) in the aqueous humor after the implantation of a drug-loaded CI26Y IOL (●), and a blank CI26Y IOL followed by eye drops therapy (●). The minimum (dashed line) and maximum (full line) of the MICs for *S. aureus* (red) and *S. epidermidis* (yellow) (**A**), and of the IC50 for COX-1 (red) and COX-2 (yellow) (**B**) are shown.

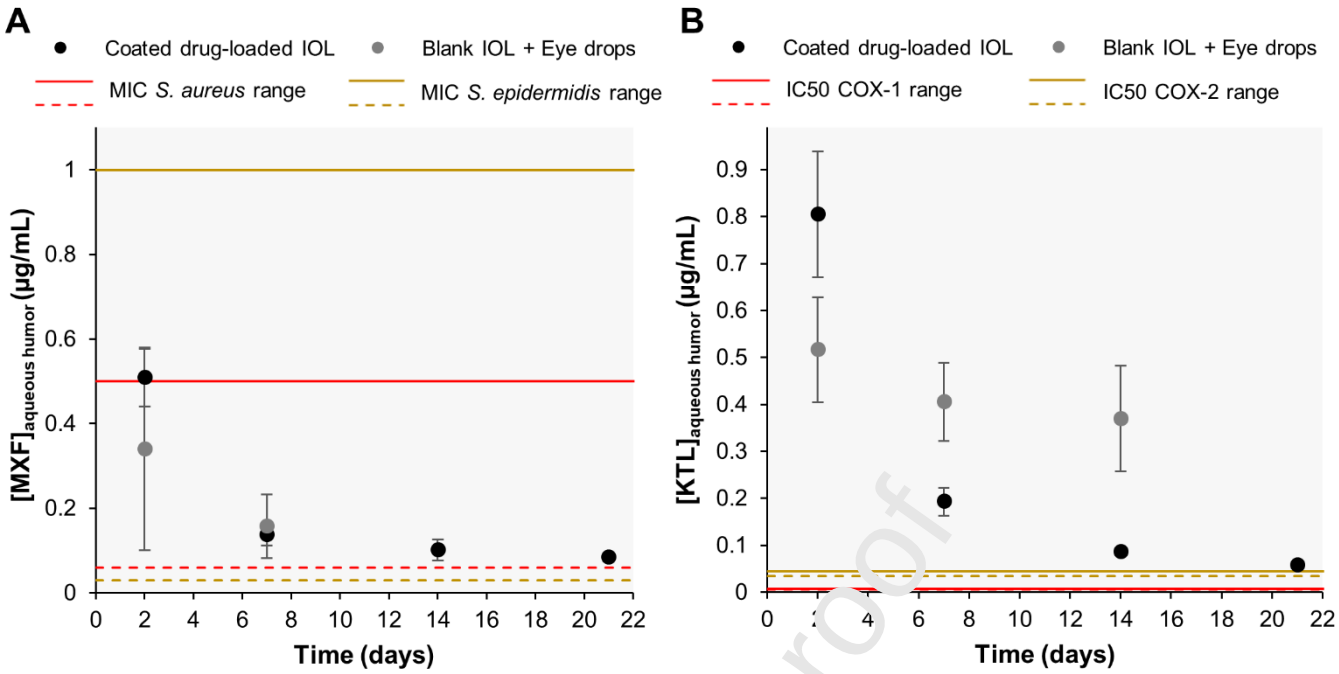

Figure 8. Concentration of MXF (**A**) and KTL (**B**) in the aqueous humor after the implantation of a coated drug-loaded GF IOL (●), and a blank GF IOL followed by eye drops therapy (●). The minimum (dashed line) and maximum (full line) of the MIC for *S. aureus* (red) and *S. epidermidis* (yellow) (**A**), and of the IC-50 for COX-1 (red) and COX-2 (yellow) (**B**) are shown.

3.6.2. Slit-lamp examinations

The eyes with implanted IOLs were inspected by slit-lamp microscopy, at pre-defined times between days 1 and 21. The slit-lamp photographs obtained with both CI26Y and GF IOLs are presented in Figure 9A and B, respectively. Mild acute inflammation response and anterior chamber exudation were detected on days 1 and 3 after the surgery for both CI26Y and GF IOLs, however the eyes were clear by day 7. Neither postoperative infection, nor PCO signs were detected during the whole period of observation.

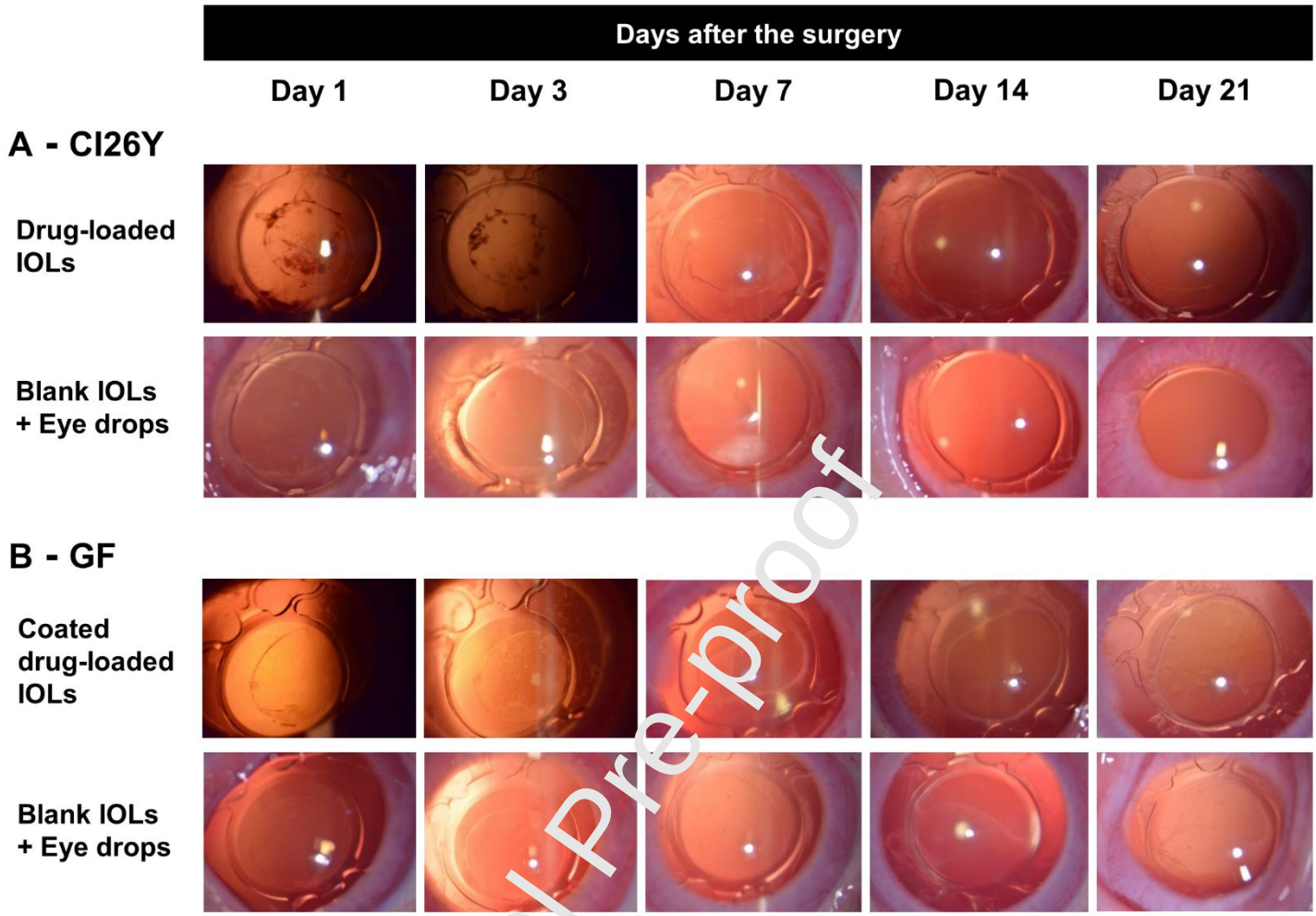

Figure 9. Slit-lamp photographs of the eyes from day 1 until day 21 after surgery for: **A**) test groups with CI26Y IOLs drug-loaded (up) and control groups (down), and **B**) test groups with GF IOLs coated and drug-loaded (up) and control groups (down).

3.6.3. Histological analysis

A more detailed investigation was done to confirm the *in vivo* biocompatibility of the IOLs by observation of histopathological sections of the iris and the cornea. At day 21, the rabbits were sacrificed, the eye balls were enucleated and the ocular tissues were cross-cut, stained by the HE protocol and examined by microscopy. Cross-sections of the cornea and of the iris, presented in Figure 10A for CI26Y and 10B for coated GF IOLs, demonstrate that all ocular tissues present a normal morphology. It is possible to identify the normal layers of the cornea: the epithelium with a basement membrane, the Bowman's layer, the stroma, the Decement's membrane and the endothelium [48,49]. The iris exhibits the typical blood vessels and folds [49,50]. Furthermore, no significant differences were detected between the group with the drug-loaded IOLs and the group with the blank IOLs followed by eye drops therapy, for both types of IOLs.

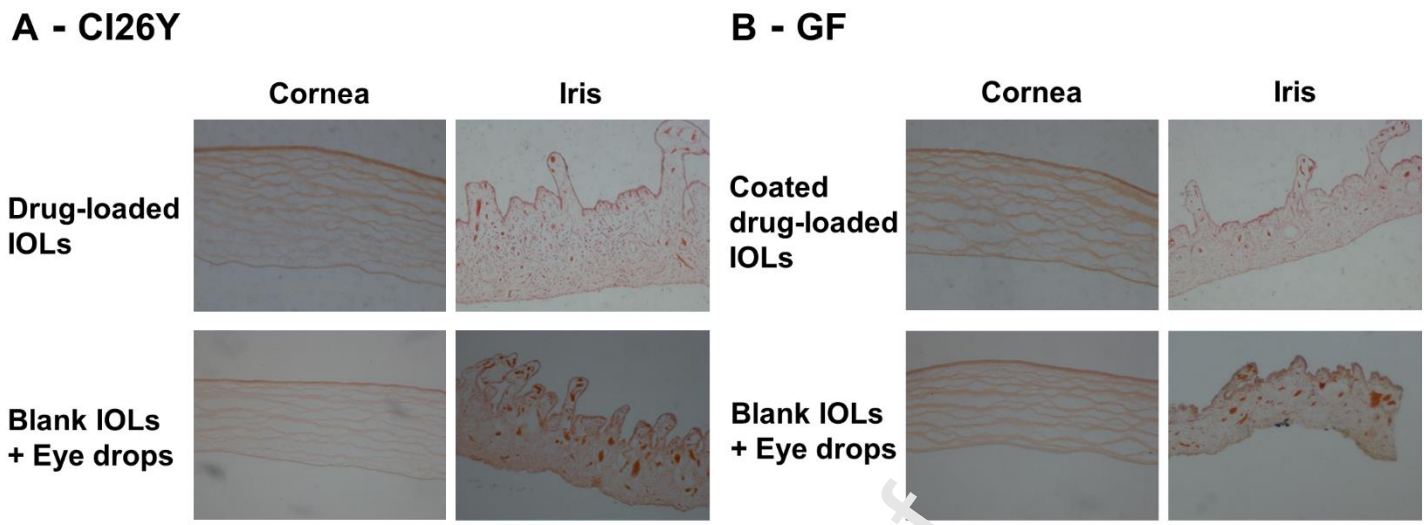

Figure 10. Histological examinations of the cross sections of the cornea (left) and the iris (right) of the eyes where implantation of drug-loaded IOLs (up) and blank IOLs followed by eye drops therapy (down) was performed, for CI26Y (**A**) and coated GF (**B**) IOLs.

4. Discussion

The potential of hydrophilic CI26Y IOLs double loaded with MXF and KTL, as an alternative to eye drops in the prevention of endophthalmitis after cataract surgery, was positively assessed in a previous work [34]. However, the efficacy of these devices needed to be further investigated with *in vivo* studies. Moreover, if we consider the prevention of PCO, the choice of lens material is important and the possibility of using hydrophobic GF IOLs should be explored. Comparison of the release profiles of MXF and KTL from both CI26Y and GF materials confirmed our expectations (Figures 1 and 2). GF disks present a low liquid uptake, and therefore incorporate and release small amounts of MXF and KTL, in comparison with the CI26Ydisks. An attempt to increase the drug loading/release from GF was made through the deposition of a hydrophilic coating of CI26Y material on one side of the GF samples, which could serve as a drug reservoir. With this modification, a significant increase in the drug released was observed for both drugs (Figure 2), which renders the option of GF viable. Before the *in vivo* studies, sterilization of the materials has to be done. HHP was chosen as the sterilization method because it was recently applied with success to sterilize intraocular lens materials [40]. The effect of sterilization on the drug release profiles was investigated. Figures 1 and 2 show that HHP slightly increased the release of MXF and KTL from CI26Y disks but did not affect the drug release from coated GF disks.

The liquid uptake capacity of CI26Y and of coated GF disks increased after drug loading and sterilization. The transmittance below 425-430 nm was reduced to zero which is similar to the average value < 5% for the transmittance of healthy human crystalline in this range of wavelengths [33]. Thus, the drug loaded, sterilized IOLs mimic the natural protection against UV radiation. The transmittance in the range 520-650 nm was not affected by the presence of drugs, being > 90 % as usually reported in the literature for IOLs materials [51].

Microbiological tests showed that the antibacterial activity of the drug release solutions against *S. aureus* and *S. epidermidis* was maintained after 26 days of release, which confirmed that the preparation and sterilization of the drug-loaded materials did not induce activity loss of the antibiotic. The sterilization

tests performed with blank disks proved that HHP is a suitable sterilization method for both CI26Y and GF materials.

Adhesion tests of HLECs to the surface of CI26Y and GF disks showed that incorporation of MXF and KTL reduced significantly the number of adherent cells, helping in the prevention of PCO. These results are somehow unexpected since MXF and KTL are not reported in the literature as pharmacological agents against PCO. Yet, anti-inflammatories, in particularly NSAIDs, such as celecoxib, diclofenac sodium, indometacin and rofecoxib, were already investigated for this purpose and led to promising results [52]. The inhibition of HLECs adherence was particularly significant on CI26Y, which may be related to the higher drug content in these hydrophilic disks. We must stress here that the hydrophobic material, in spite of presenting some cell adhesion, is still a good option for the prevention of PCO because of its known improved capacity of adhesion to the capsular bag [14].

The actual performance of the studied IOL materials was finally assessed with *in vivo* tests. Sterilized, drug-loaded CI26Y and coated GF IOLs were implanted in the eyes of the rabbits of the test group. In the control group, blank CI26Y and GF IOLs were implanted and the rabbits followed a therapy with eye drops after the surgery. The concentrations of MXF in the aqueous humour of the rabbits of the test group implanted with CI26Y are always higher than the concentrations measured in the correspondent control group, and they stay above the MICs of *S. aureus* and *S. epidermidis* for 21 days. In contrast, the concentration of MXF measured in the control group is very close to the minimum values of the MICs. The concentration of KTL in the test group exceeds that found in the control group for the first seven days, but, in both groups, the concentration of KTL stays above the IC50 for COX-1 and COX-2 for 14 days that is the recommended period for the instillation of NSAIDs eye drops after the cataracts surgery. The results obtained with the coated GF IOLs are different due to the lower loading capacity of these devices, being the concentration of drugs released from these IOLs higher or similar to that measured in the control group, until day 7 for MXF, and for more than 2 days, but less than 7 days, for KTL. Even so, for the test group, the concentrations of MXF remain in the range of the MICs and of KTL above the IC50 of both COX for 21 days.

It is important to stress that the aqueous humour samples in the control group were collected 3.5 h after the second instillation of eye drops of the day. This means that the concentration of both drugs will be higher at the time of the instillation, and may drop below the minimum therapeutic levels at the time of the next instillation, assuming a 6 h period between consecutive instillations to ensure a therapy of four times a day. This suggests that both CI26Y and coated GF IOLs loaded with MXF and KTL, which ensure a sustained drug release, would be more effective than the eye drops therapy in the prevention of ocular inflammation and endophthalmitis.

The eyes of the rabbits of the test and the control groups were examined through slit-lamp examinations during the course of the experiment. The mild inflammation observed in the test group on days 1 and 3 disappeared after day 7, meaning that the released KTL was effective for the treatment of post-surgery inflammation. Some doctors recommend starting the eye drops therapy one day before the surgery [53,54]. This procedure was not adopted in the present work, to avoid interference with the concentration measurements in the test group. However, in the future, the treatment for post-operative inflammation using drug-loaded IOLs can be complemented with eye drops therapy before the surgery. Neither ocular infection/endophthalmitis nor PCO signs were detected in the eyes of the rabbits of both test and control groups. However, we cannot conclude from these results that the implanted IOLs are efficient in the prevention of PCO, since this complication may appear months or years after the surgery [55]. Finally,

histological analysis of the iris and the cornea of the eyes implanted with drug-loaded and blank IOLs reveal similar capsular and uveal biocompatibility

Overall, the experimental results obtained with the *in vitro* and *in vivo* tests indicate that the studied hydrophilic and hydrophobic IOLs, loaded with MXF and KTL, release the drugs in a controlled manner and advantageously substitute the eye drop therapy in the prevention of ocular infection/endophthalmitis. Furthermore, they may have a beneficial action against PCO, since the presence of the drugs decreased cell adhesion. Future clinical application of these IOLs will depend on their approval by FDA as a combination product, more specifically, a combination product type 4 because these devices have an additional function besides delivering the drug [56].

5. Conclusions

Hydrophilic IOLs and hydrophobic IOLs, coated with one hydrophilic layer, were successfully loaded with MXF and KTL to achieve controlled release platforms against ocular inflammation and endophthalmitis. Both types of devices seem to be more effective than the commonly used eye drops therapy, because they ensure sustained concentrations of MXF and KTL in the aqueous humour, respectively above the MICs and the IC50 of both COXs, during the period recommended for the prevention of those ocular diseases. The drug-loaded IOLs were sterilized with a new sterilization method (HHP), which did not affect the antibacterial properties of the released antibiotic, kept adequate liquid uptake and transmittance of the IOLs, and had no hazardous biological effects. Adhesion of HLECs on the surface of the drug loaded IOLs was reduced in comparison with the blank IOLs, which may contribute to reduce the PCO incidence.

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Highlights

- Hydrophilic and coated hydrophobic IOLs may be loaded simultaneously with MXF and KTL.
- Drug-loaded IOLs release both drugs in a sustained way.
- Drug-loaded IOLs decrease the adherence of HLECs and are biocompatible.
- Drug-loaded IOLs lead to therapeutic levels of MXF and KTL in the aqueous humor.
- Drug-loaded IOLs have better performance than eye drops in i*n vivo* tests.