TITLE

In-situ forming gels containing fluorometholone-loaded polymeric nanoparticles for ocular inflammatory conditions

Roberto Gonzalez-Pizarro[a], Paulina Carvajal-Vidal[a], Lyda Halbault Bellowa[a], Ana Cristina Calpena[a,b], Marta Espina[a,b], María Luisa García[a,b].

[a]Department of Pharmacy, Pharmaceutical Technology and Physical Chemistry, Faculty of Pharmacy and Food Sciences, University of Barcelona, 08028 Barcelona, Spain.

[b]Institute of Nanoscience and Nanotechnology (IN2UB), University of Barcelona, Barcelona, Spain.

ABSTRACT

Thermosensitive gels have been developed and optimized in such a way that they become gels at corneal temperature and with a viscosity that allows the adequate release of the Fluorometholone (FMT)-loaded PLGA nanoparticles (NPs) in order to improve ocular anti-inflammatory efficacy against a commercial formulation. It has been shown that gels avoid burst of drug release in the first hours with a slow and increasing profile after administration. NPs have maintained their average size and spherical shape within the gels as confirmed by transmission electron microscopy (TEM). In turn, the *in-situ* gelling of the formulations allows the administration in eye drop dosage form due to its state of sol at temperatures below 25 °C. Ocular tolerance studies have shown that no formulation causes eye irritation. The administration of the developed formulations has improved the precorneal residence time reflected in the ocular bioavailability, where deep tissues as aqueous humour and crystalline were reached. In conclusion, the use of thermosensitive gels for the topical application of NPs has demonstrated their effectiveness in the acute and preventive treatment of ocular inflammatory conditions.

KEY WORDS

Fluorometholone, PLGA nanoparticles, gel, ocular anti-inflammatory, poloxamer 407, thermosensitive, *in-situ* gelling.

1. Introduction

Fluorometholone (FMT) is one of the many corticosteroids used in ophthalmology for the treatment of allergic and inflammatory conditions of the anterior segment of the eye [1]. Since its incorporation to ocular treatment, this drug has shown certain advantages over other corticosteroids commonly used, such as high anti-inflammatory potency and low risk of increasing intraocular pressure that leads to a lower prevalence of corticoid-induced cataracts and glaucoma [2]. On the other hand, FMT has the disadvantage of having a low corneal

penetration that results in ineffective therapeutic levels for treatment in the posterior area of the eye [3]. Furthermore, in the eye drop dosage form, the formulation to be rapidly eliminated from the precorneal area as a consequence of tear turnover, having only a half-life of ocular residence around 1 – 2 minutes. To overcome these difficulties, various strategies have been developed to increase the half-life and therapeutic efficacy of ocular drugs ranging from increased viscosity with the use of ointments or gels to intravitreal injections for the treatment of diseases committed in the posterior area of the eye, as posterior uveitis [4].

One of the strategies currently used to increase the bioavailability of hydrophobic drugs such as corticosteroids is the development of nanoparticles (NPs) of poly (D, L-lactic-co-glycolic acid) (PLGA) [5]. Particularly, the incorporation of the drug into the polymer matrix creates a system that protects the drug from the enzymatic metabolism present in the tear film and allows a controlled and prolonged release of the drug [6–8]. The PLGA NPs, in turn, possess the ability to carry the drug to deep tissues, where conventional commercial formulations fail to reach [9].

The latest research to solve the problem of residence time in the precorneal area of eye drops focuses on the development of formulations that gelling *in-situ* when they come into contact with the eye due to the effect of pH, salts or temperature [10–12]. Poloxamer 407 (P407) is a triblock copolymer surfactant formed by polypropyleneglycol and two blocks of polyethyleneglycol at the ends, which has the characteristic of behaving as a Newtonian or viscoelastic solution (gel) depending on the temperature and concentration used [13]. The development of PLGA NPs incorporated in a thermosensitive gel would be able to solve the disadvantages of commercialized eye drops, which would allow gelling at corneal temperature [14], avoiding the burst of release of the drug from the NPs in the first hours administered, resulting in increased drug concentration in deep eye tissues such as aqueous humour and crystalline [15,16].

In the present study, a formulation of P407 with FMT-loaded PLGA NPs (FMT-PLGA-NPs), which gelling at corneal temperature, has been developed and optimized, with the aim of increasing its bioavailability in deep ocular tissues and treating inflammatory conditions of the anterior and posterior area of the eye. For this, morphometry and morphology of the NPs, rheological analysis, *in vitro* release profiles and short-term stability of the gels were also carried out. Eye tolerance, ocular bioavailability and anti-inflammatory efficacy studies were conducted with the aim to demonstrate the suitability of this system in the treatment of ocular inflammatory diseases.

2. Material and methods

2.1. Materials

FMT and PLGA RG 503H were purchased from Capot Chemical (Hangzhou, China) and Evonik Corporation (Birmingham, USA), respectively. Poloxamer 188 (P188) and P407 were given from BASF (Barcelona, Spain). Methylcellulose A4M (MC) and Benzalkonium chloride were purchased from Sigma-Aldrich (Madrid, Spain). Sodium alginate (65 – 75% guluronic and 25 – 35% mannuronic acid, ≈ 220 kDa) was provided by Fagron Iberica (Terrassa, Spain). Water through Millipore® MilliQ system was used for all the experiments and all the other reagents were of analytical grade.

2.2. Preparation and characterization of NPs

FMT-loaded NPs (FMT-PLGA-NPs) previously optimized in our group were prepared by the solvent displacement method [7]. Briefly, the PLGA (9.0 $mg \cdot mL^{-1}$) and FMT (1.5 $mg \cdot mL^{-1}$) were dissolved in 5 mL of acetone. The organic phase was added slowly dropwise, under stirring, into 10 mL of an aqueous solution of P188 (15 $mg \cdot mL^{-1}$) adjusted to pH 7.4. After that, the organic solvent was evaporated under reduced pressure.

Morphometry (average particle size (Zav), polydispersity index (PI)) and zeta potential (ZP) of NPs were determined in a Zetasizer NanoZS (Malvern Instruments, Malvern, UK) by dynamic light scattering (DLS) and electrophoretic mobility, respectively. Samples were diluted in MilliQ

water (1:10) and experiments were performed with disposable capillary cells DTS1070 (Malvern Instruments) at 25 °C. The reported values correspond to the mean ± SD.

The entrapment efficiency (EE) of FMT in the NPs was quantified indirectly by measuring the non-entrapped drug in the dispersion medium. The free FMT was separated by a filtration/centrifugation technique (1:10 dilution) at 25 °C and 5000 rpm for 10 min. The EE was calculated according to the following equation:

$$\mathrm{EE}\ (\%) = \frac{cFMT_0 - cFMT_1}{cFMT_0} \cdot 100 \qquad (1)$$

where $cFMT_0$ and $cFMT_1$ are the total amount of FMT and free FMT in the filtrated, respectively. The samples were evaluated by HPLC, according to the method described previously [7]. Data were processed using Empower 3® Software.

2.3. Elaboration and optimization of gels

An amount of 0.025 g of sodium alginate were dissolved in 10 mL of FMT-PLGA-NPs under agitation (800 rpm for 3 min) using the Unguator®. After one day of rest, MC (0 – 1% w/v) and benzalkonium chloride (0.01% w/v) were added and stirred at 1000 rpm for 5 min. The previous preparation was left to stand overnight to then add the P407 (15 – 25% w/v) under agitation of 1000 rpm for 5 min. The pH was adjusted to 7.4 with NaOH.

Design of Experiments (DoE) was employed using a multi-level factorial design 3^2 generated by StatGraphics Centurion XV. The matrix was developed to analyze the effects the two independent variables (concentrations of P407 [cP407] and MC [cMC]) on the three dependents variables (gelling capacity (Gt), sol-gel temperature transition ($T_{sol\text{-}gel}$) and viscosity (Vc)) [17]. Each independent variable was studied at three different levels coded (-1), a (0) and (+1) sign (see Table S.1 Supplementary Material).

2.4. Analysis of gels

2.4.1. Gelling capacity test

The determination of the Gt was carried out by placing 100 µL of gel in 2 mL of simulated tear fluid (SFL) at 34 ± 0.5 °C (average temperature of the ocular surface) [14,18]. The SFL was composed of 6.7% w/v sodium chloride, 2.0% w/v sodium bicarbonate, 0.08% w/v calcium chloride dehydrate in purified water [19]. The gelation was carried out by visual examination according to the Table 1.

2.4.3. Rheological properties of the gels

Rheological properties of gels were carried out using a rheometer HAAKE Rheostress 1 (Thermo Fisher Scientific, Karlsruhe, Germany) equipped with a cone-plate geometry set-up with a fixed lower plate and an upper cone (Haake C60/2Ti, 6 cm diameter). The temperature sweep test was performed to determine the $T_{sol\text{-}gel}$ *in-situ* of gels. For oscillatory testing, storage modulus (G') and loss modulus (G'') values were measured from 5 to 50 °C with a constant frequency of 1 Hz and a constant stress (0.5 Pa). The transition temperature was set when G' equals G'' (cross-over point). For rotational testing, viscosity curves and flow curves were recorded for 3 min during the ramp-up period from 0 to 100 s^{-1}, 1 min at 100 s^{-1} (constant share rate period) and finally 3 min during the ramp-down period from 100 to 0 s^{-1}. All determinations were performed in triplicate.

2.4.4. Flow ability test

The flow ability was tested using the inverted tube method. 3 mL of each formulation were added in a tube of 10 mL, to be incubated in a thermoregulated bath. Three test temperatures were used, storage temperature (5 ± 0.5 °C), room temperature (25 ± 0.5 °C) and precorneal temperature (34 ± 0.5 °C). The evaluation was carried out investing the tube after 30 s. The thermosensitive gel would be suitable when it does not flow after an incubation of 30 s at 34 °C [20].

2.4.5. Morphological characterization

The morphological examination of the gels was carried out by transmission electron microscopy (TEM) on a Jeol 1010 instrument. This study was used to evaluate if the

morphology of the NPs remained in the gel. The NPs were visualized with copper grids carbon-coated (carbon only). Samples (10 µL) were placed on grids and negative staining performed with 2% uranyl acetate.

2.4.6. *In vitro* release profile

To identify the cumulative release profile of the FMT from the optimized gels and FMT-PLGA-NPs compared with a commercial eye drop, a study was carried out in amber Franz cells using a dialysis membrane MW 12000–14000 Da, under sink conditions [21]. Isoptoflucon® is a commercial ophthalmic suspension of FMT that has been used as a reference for *in vitro* and *in vivo* studies. A volume of 300 µL of the samples was placed in the donor compartment and the receptor compartment was filled with receptor medium thermoregulated at 34 ± 0.5 °C in continuous agitation. Samples (300 µL) were withdrawn from the receptor compartment at fixed times and replaced by an equal volume of fresh receptor medium at the same temperature. The samples were quantified by HPLC using the same method as in EE. Data were processed using Empower 3® Software. Values were reported as the mean ± SD of the triplicates. Akaike's information criterion (AIC) and coefficient correlation (r^2) were determined for each model as an indicator of the model's suitability [22].

2.5. Stability analysis of gels

The physical stability of the optimized gels at 4 °C was evaluated by static multiple light scattering technology (S-MLS) using Turbiscan® Lab. Gels were placed in a cylindrical glass measuring cell that was scanned by a pulsed near-infrared light source (λ = 880 nm). Due to the opacity of the gels, only the backscattering profiles were used to evaluate the physical stability. The backscattering data were recorded every for 24 h at different times after preparation (1, 20 and 60 days).

2.6. Ocular tolerance

2.6.1. *In vitro* ocular tolerance

In vitro ocular tolerance was assessed using the HET-CAM® test to ensure that the gels are not irritating to ocular level. Hemorrhage, vasoconstriction and coagulation phenomena were measured by applying 300 µL of the formulation studied on chorioallantoic membrane of a fertilized chicken egg, monitoring it during the first 5 min after the application. The development of the test was carried out with 6 eggs for each optimized formulation, 3 for controls positive (NaOH 0.1 M) and negative (0.9% NaCl) [23]. The ocular irritation index (OII) was calculated by the sum of the scores of each injury according to the following expression:

$$\mathrm{OII} = \frac{(301-\mathrm{h})\cdot 5}{300} + \frac{(301-\mathrm{v})\cdot 7}{300} + \frac{(301-\mathrm{c})\cdot 9}{300} \qquad (2)$$

where h, v and c are times (s) until the start of hemorrhage, vasoconstriction and coagulation, respectively. The formulations were classified according to the following: OII ≤ 0.9 nonirritating; 0.9 < OII ≤ 4.9 weakly irritating; 4.9 < OII ≤ 8.9 moderately irritating; 8.9 < OII ≤ 21 irritating [24,25].

2.6.2. *In vivo* ocular tolerance

To corroborate the results obtained from the HEM-CAM® test, the formulations (optimized gels, FMT-PLGA-NPs and Isoptoflucon®) were evaluated using primary eye irritation test of Draize [26]. For this case, New Zealand albino male rabbits of 2.5 kg average weight were used, where 50 µL of each sample were instilled in the eye conjunctival sac (n = 6/group) and a gentle massage was applied to ensure circulation of the sample through the eyeball. Possible signs of irritation were observed at the time of instillation and after 1 h of exposure using the untreated contralateral eye as a negative control. The score Draize was determined by direct observation of the anterior segment of the eye and changes in ocular structures involving the cornea, iris and conjunctiva [7].

2.7. Therapeutic efficacy

The induction of inflammation with the objective of evaluating the anti-inflammatory effect of optimized gels and FMT-PLGA-NPs compared to the commercial drug (Isopotoflucon®) and

0.9% control group (NaCl), was carried out using New Zealand albino male rabbits (n= 6/group). The study was conducted with the application of 50 µL of 0.5% sodium arachidonate (SA) dissolved in PBS in the right eye, using the left eye as control. After 30 minutes of exposure, 50 µL of each formulation were instilled. In order to evaluate the prevention of inflammation, the formulations were administered 30 min before the induction of ocular inflammation. The evaluation of inflammation was performed from the application of formulations up to 150 min according to Draize modified scoring system [26].

2.8. Ocular bioavailability

The amounts of drug that permeated from optimized gels, FMT-PLGA-NPs and Isopotoflucon® were evaluated 4 h after its application. To this end, 50 µL of each formulation were administered to the rabbit's left eye. The experiment was carried out according to the Ethics Committee of Animals Experimentation from the University of Barcelona (CEEA-UB). The rabbits were anesthetized with intramuscular administration of ketamine (35 mg/kg) and xylazine (5 mg/kg) and euthanized by an overdose of sodium pentobarbital (100 mg/kg) administered for injection cardiac under deep anesthesia. The amount of FMT retained from the different parts of the eye (sclera, cornea, crystalline, aqueous humour and vitreous humour) was quantified by RP-HPLC.

2.9. Statistical analysis

The multiple comparisons were developed using two-way ANOVA with Tukey post hoc test with a significance of $\alpha < 0.05$ after having confirmed the normality and equality of variances by Bartlett in the groups. All analyzed data were presented as mean ± SD. GraphPad Prism® 6.01 software for windows was used to analyze the data.

3. Results and discussions

3.1. Characterization of NPs

FMT-PLGA-NPs, optimized by our research group previously [7], showed values Zav, PI, ZP and EE of 150.8 ± 0.7 nm, 0.082 ± 0.014, -27.9 ± 0.3 mV and 98.4 ± 1.4%, respectively. These results

ensure that the formulation FMT-PLGA-NPs meets the requirements for ocular administration as smaller populations of size 10 μm to avoid eye irritation and a high ZP, to increase colloidal stability between NPs [27,28].

3.2. Optimization of the gels

In the Fig. 1a, it is possible to visualize the significance and the type of effect in the Gt response. The Gt depends on several factors in its assessment, such as the ability to form a gel and the time that the gelled gel can remain in a SFL solution. In particular, cP407 and cMC have a significantly positive effect ($p < 0.01$) on the response of Gt. Analyzed each of the factors and their influence on the response of Gt, the cP407 has the ability to form the gel and cMC provides the system with longer gelled time, both proportional to the concentration used.

For the response of Vs, the same relation is fulfilled as in Gt, where both factors are statistically significant (Fig. 1b). The relationship is proportional to the concentration, being the cP407 has a greatear influence on Vs ($p < 0.0001$) while the cMC has a smaller influence ($p < 0.01$), but necessary to increase strength or keep the gel constituted longer.

From the Fig. 1c, it is possible to show that cP407 has a significant negative effect ($p < 0.001$) in the response of $T_{sol\text{-}gel}$, which means that as the concentration of cP407 increases the $T_{sol\text{-}gel}$ decreases [29]. In the Fig. S.1 Supplementary Material is possible to observe the surface responses of each independent variable.

According to the analysis of the factorial design used (Table 2), three formulations were selected (PG2, PG5 and PG8), due to the *in-situ* gelation property with the ability to remain constituted at the corneal temperature. Therefore, morphological characterization, stability studies, release, ocular tolerance, cytotoxicity, ocular bioavailability, anti-inflammatory and prophylaxis efficacy in the optimized formulations were carried out.

3.3. Analysis of gels

3.3.1. Gelling capacity test

The gelling capacity of the formulations PG2, PG5 and PG8 are directly related to the increasing amount of MC (0 – 1%) used. In the case of PG2 and PG5 (Fig. S.2 Supplementary Material), a score of "slow gelling and dissolves quickly" was obtained (Table 2), in which the formulation gelled in SFL keeping for at least 30 min. PG8 (Fig. S.2c Supplementary Material) gelled when contacting SFL, being able to keep in this state for 2 hours on average. These formulations, at a concentration of 20% of P407 have the characteristic of gelling *in-situ* at a temperature close to 34 °C and when the MC is added, the system acquires greater strength so that the gel stays longer.

3.3.2. Rheological properties

The gels PG2, PG5 and PG8 presented a non-Newtonian behaviour (Table 3), adjusting in the ascending section to a plastic flow (Herschel-Bulkley equation) and in the descending section, to a pseudoplastic profile (Cross equation) [30]. This bimodal behaviour at corneal temperature allows characterizing the gels when they are administered. Particularly, the optimized gels will need an increasing force of the blinking to flow in the ocular cavity, so that later the interactions between the polymeric chains of the P407 will be broken making it flow at an almost constant viscosity without reaching the value 0 of shear stress [31]. In the case of viscosity, these were proportional to the aggregate amount of MC in each formulation (Table 2). Overall, the rheological behaviour at a constant temperature makes the gel possess the slow release of the particles and a viscosity that allows it to be longer in contact with the eye [32]. The flow and viscosity curves of the gels can be observed in Fig. S.3 Supplementary Material.

3.3.3. $T_{sol\text{-}gel}$ and flow ability

The $T_{sol\text{-}gel}$ determination through the crossover between G' and G" of the optimized formulations are visualized in the Fig. 2. The selected gels presented similar $T_{sol\text{-}gel}$ among them, according to the graphs it is possible to show that gelation starts around 21 °C, increasing its viscosity (G") gradually as the temperature increases until it becomes equal with

the elastic behaviour (G'). At the corneal temperature, the gels reach the second crossover, where the viscosity remains constant [33]. The above is confirmed with the flow ability test, where the formulations do not flow at a temperature of 34 °C.

At the molecular level, at temperatures below 21 °C, the gel behaves like a Newtonian fluid where the P407 molecules are in a disordered state. From the temperature of 21 °C approximately, the molecules begin to be ordered in micelles, initiating the sol-gel transition, completely gelling at the corneal temperature. The gels PG2 and PG5 have a regular flow between a temperature of 5 and 25 °C. In the case of PG8, it flows with difficulty, mainly caused by the amount of MC (1%) present in the formulation. This gelling behaviour of the optimized formulations allow its administration as in eye drop dosage form and enabling the gelation when it comes in contact with the eyeball, which results in an increase in the residence time, avoiding the rapid elimination by the lacrimal stimulation. [32]. In Table S.2 Supplementary Material, the flow ability data of the rest of the gels of the design are visualized, which did not fulfill the condition of low flow at the corneal temperature and be gelled at 34 °C.

3.3.4. Morphological characterization

Through the analysis of TEM images (Fig. S.4 Supplementary Material), it was possible to visualize the NPs contained in the gels. According to these images, it was confirmed that the NPs maintained their spherical shape, average size and absence of aggregation, concordant with the data coming from the DLS and the TEM images of the optimized NPs made in the preliminary study [7].

3.3.5. *In vitro* release profile

In Fig. 3 it is possible to demonstrate the controlled release of FMT from the gels and FMT-PLGA-NPs. In the first instance, Isoptoflucon® presents a rapid release of FMT reaching 100% at 24 h, adjusting to a profile of order one (r^2 = 0.999, AIC = 69.523). The NPs presented a release close to 80%, characterized by a initial burst of release in the first 4 h reaching 45% of drug

released due to the drug weakly bound on the surface of the NPs [34]. The best model that adjusted the formulation FMT-PLGA-NPs was the hyperbola (r^2 = 0.982, AIC = 68.151), where the release in the last section of the profile (4 – 10 h), is slow and growing without reaching a plateau, by the affinity of the drug to the polymer matrix [35]. Finally, the formulations PG2, PG5 and PG8 presented a slow and increasing release of FMT of approximately 70%, 50% and 40%, respectively, at 24 h. As it is possible to observe in Fig. 3, at the time of 24 h PG2 reaches the released amount of FMT-PLGA-NPs, progressively increasing until the end of the experiment (48 h). PG2 does not present a burst release effect in the first 4 hours, allowing the drug to be released slowly and sustainably, due to the increase in viscosity and the constant erosion rate attributed to the *in-situ* formation of the gel [36]. It should be noted that the formation of the gel containing the nanoparticles prevents burst of release creating a repository of the drug within the gelling matrix, allowing a greater release of drug than FMT-PLGA-NPs [37,38]. In turn, although the three formulations of gels have similar $T_{sol\text{-}gel}$, they present a release at 24 h different from each other, probability due to the viscosity given by MC (0 - 1%). Finally, PG2 (r^2 = 0.984, AIC = 70.745), PG5 (r^2 = 0.925, AIC = 80.943) and PG8 (r^2 = 0.992, AIC = 42.868) adjusted a profile of order one, without reaching a plateau at the end of the study [39].

3.3.6. Stability analysis of gels

The gel formulations were evaluated for stability within a period of 60 days at storage conditions of 4 °C. According to Fig. 4 (a-c), it is evident that the PG5 and PG8 formulations have greater stability than PG2. This difference is mainly due to the viscosity that prevents the precipitation of the NPs inside the gelling system, at a temperature of 4 °C, since at this temperature the gels are in liquid state (suspension), increasing the instability that being in of gel [40,41]. Despite the above, the instability of PG2 is within the allowed margins (Backscattering ≤ 20%). Due to this behaviour of the gels at a temperature of 4 °C, they must be resuspended with gentle agitation before being administered.

3.3.7. Ocular tolerance

The *in vitro* eye tolerance study (HET-CAM® test) of the optimized gels, showed OII values of lower than 0.9, categorizing the formulations as nonirritating (Table S.3 Supplementary Material). In the case of NPs and the commercial drug (Isoptoflucon®) these have been evaluated in a previous study in our group, concluding that they are nonirritating [7]. In order to corroborate these data, the *in vivo* evaluation was carried out through the Draize test of the formulations of gels, NPs and Isoptoflucon® in albino New Zeeland rabbits. In this study, it was concluded that the evaluated formulations are not irritating (OII = 0) [41,42] (Table S.4 Supplementary Material).

3.3.8. Therapeutic efficacy

Through the realization of two *in vivo* studies, it was possible to confirm the anti-inflammatory capacity of the optimized gels, both in an acute treatment of ocular inflammation and in the prevention of it.

The optimized gels (PG2, PG5 and PG8) present evident significant differences regarding positive control ($p < 0.01$) and the commercial drug ($p < 0.05$) at 60 min of the anti-inflammatory efficacy test (Fig. 5a). The formulations PG2 and PG5 maintained significant differences regarding Isoptoflucon® until the end of the study. FMT-PLGA-NPs also had a significant anti-inflammatory effect ($p < 0.05$) up to 150 min. This proves that optimized thermosensitive gels increase the effect of the drug more than when it is loaded in the NPs or commercial eye drops. This is mainly due to the *in-situ* formation of the gel at corneal temperature, which leads to an increase in viscosity (PG2 < PG5 < PG8), resulting in prolongation of the residence time of the formulation in the precorneal area, increasing penetration ocular and in turn, avoiding the rapid elimination of the drug by tearing [16].

In order to observe the usefulness of the formulations in a prophylactic treatment of inflammation, these were applied at the beginning of the study and after 30 min inflammation was induced with SA (Fig. 5b). Gels and NPs reduced inflammation significantly ($p < 0.05$)

regarding positive control at 60 min. Particularly, PG2 and PG5 were the formulations that obtained significant differences evident when compared with the commercial drug ($p < 0.05$) from 60 min until the end of the study. In addition, Isoptoflucon® from 180 min up to 210 min, obtained a significant efficiency ($p < 0.05$) in decreasing inflammation regarding SA. At the end of the study, it was concluded that PG2 and PG5 decrease inflammation more effectively than other formulations when compared with SA ($p < 0.0001$) and Isoptoflucon® ($p < 0.01$). It is also possible to visualize that the anti-inflammatory degree of the prophylaxis is greater than in the acute treatment, because the drug is longer in contact with the eyeball, in turn, the residence time is increased when the formulation is in a state of gel. PG2 and PG5 would be suitable for the prevention of inflammation without the need for repetitive administrations of the formulation to obtain a significant therapeutic effect.

3.3.9. Ocular bioavailability

The remaining amount of FMT (determined by RP-HPLC) in the different ocular tissues of PG2, PG5, PG8, FMT-PLGA-NPs and Isoptoflucon® are visualized in Fig. S.5 Supplementary Material. At the corneal level, the formulation FMT-PLGA-NPs and Isoptoflucon® presented similar values without significant differences and other formulations were not detectable at this level. In Fig. S.5 Supplementary Material it is possible to visualize that the FMT-PLGA-NPs ($p < 0.0001$) and PG8 ($p < 0.01$) showed a concentration obviously higher than the commercial drug in the sclera. PG5 and PG were those formulations that presented an accumulation at the aqueous humour level of 2 and 3 times more than Isoptoflucon®, respectively. At the level of the crystalline, it is possible to show that only the FMT-PLGA-NPs and PG5 obtained a significant difference against to the commercial drug ($p < 0.0001$), this because the gel has an intermediate viscosity that allows it to release the drug slowly and remain a longer residence time in the precorneal area. In turn, NPs due to their ability to protect the drug from ocular metabolism and increase the solubility of the drug to be encapsulated in the lipophilic matrix of PLGA are the potential reasons for the accumulation of these two formulations in the

crystalline [6,43]. Finally, at the vitreous humour level, it is not possible to show significant differences in the accumulation of the drug among all the formulations analyzed. According to the release information, the anti-inflammatory efficacy and the bioavailability, PG5 is the one of the developed gels that presents the characteristics that best suit a formulation with anti-inflammatory activity and with the slow and prolonged release of the drug, which allows it to reach deep ocular tissues.

4. Conclusions

Using a multifactorial design, it was possible to select three formulations of gels loaded with FMT-PLGA-NPs (PG2, PG5 and PG8), which have in common the same amount of P407 (20% w/v) and with a variable MC concentration (0 - 1% w/v). The optimized gels presented suitable rheometric characteristics for use as an ophthalmological suspension of *in-situ* gelation, such as fluidity at room temperature, a gelling capacity in SFL, a viscosity and $T_{sol-gel}$ that allow it to be in a gel state at corneal temperature and with an optimal viscosity to increase the residence time in the cornea than other commercial eye drops. In turn, with the analysis of TEM images corroborated that the NPs in the gels maintained their spherical shape and their Zav, without evidence of agglomerates. The gelling system gave the formulations a proven stability of 60 days and with the recommendation of a gentle agitation before its administration, due to the behaviour of suspension at room temperature. In the *in vivo* studies of efficacy and inflammatory prophylaxis, the optimized gels obtained a greater capacity in decreasing the OII in contrast to the Isoptoflucon® and without effects of ocular irritation (studies in HET-CAM® and Draize test). According to the release information, the anti-inflammatory efficacy and the bioavailability, PG5 is the one that presents the characteristics that best suit a formulation with anti-inflammatory activity and with the slow and prolonged release of the drug, which allows it to reach deep ocular tissues such as aqueous humour and crystalline. Finally, the formulation

of PG5 would be useful for the treatment of inflammatory eye diseases, avoiding the repetitive administration of eye drops, which would improve the patient's adherence to treatment.

Conflict of interest

No conflicting relationship exists for any author.

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Appendix A. Supplementary Material

Supplementary data associated with this article can be found, in the online version, at ees.elsevier.com/colsub/download.aspx?id=905732&guid=ffd623a1-0fd8-449a-aafc-c46e0783a017&scheme=1

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Table captions

Table 1
Coding for the gelling capacity test.

Coding	Observation
0	No gelling occurs.
1	Slow gelling and dissolves quickly.
2	Immediate gelling and remains for a few hours.
3	Immediate gelling and remains for an extended period.

Table 2
Values of the two experimental factors according to the matrix designed by 3^2 multifactorial and the measured response.

Factorial points	cP407 (w/v)	cMC (w/v)	Gt	Vs (mPa·s) at 100 s^{-1}	$T_{sol-gel}$ (°C)
PG1	15	0.0	0	129.6 ± 5.2	33.11 ± 0.41
PG2	20	0.0	1	3133.0 ± 26.5	21.55 ± 0.36
PG3	25	0.0	1	4879.5 ± 110.5	12.17 ± 0.07
PG4	15	0.5	0	226.6 ± 10.2	31.06 ± 0.22
PG5	20	0.5	1	3347.0 ± 76.6	20.85 ± 0.01
PG6	25	0.5	2	5521.5 ± 108.2	13.77 ± 0.19
PG7	15	1.0	1	262.1 ± 5.7	30.19 ± 0.31
PG8	20	1.0	2	3611.0 ± 109.6	22.20 ± 0.38
PG9	25	1.0	3	6486.5 ± 117.3	14.84 ± 0.40

Table 3
Best adjustment model of optimized gels.

Parameter	PG2	PG5	PG8
Rheological model	Hershel-Bulkley[a] (r = 0.999)	Hershel-Bulkley[a] (r = 0.994)	Hershel-Bulkley[a] (r = 0.955)
	Cross[d] (r = 0.987)	Cross[d] (r = 0.997)	Cross[d] (r = 0.994)

[a] ascending section; [d] descending section.

Figure 1

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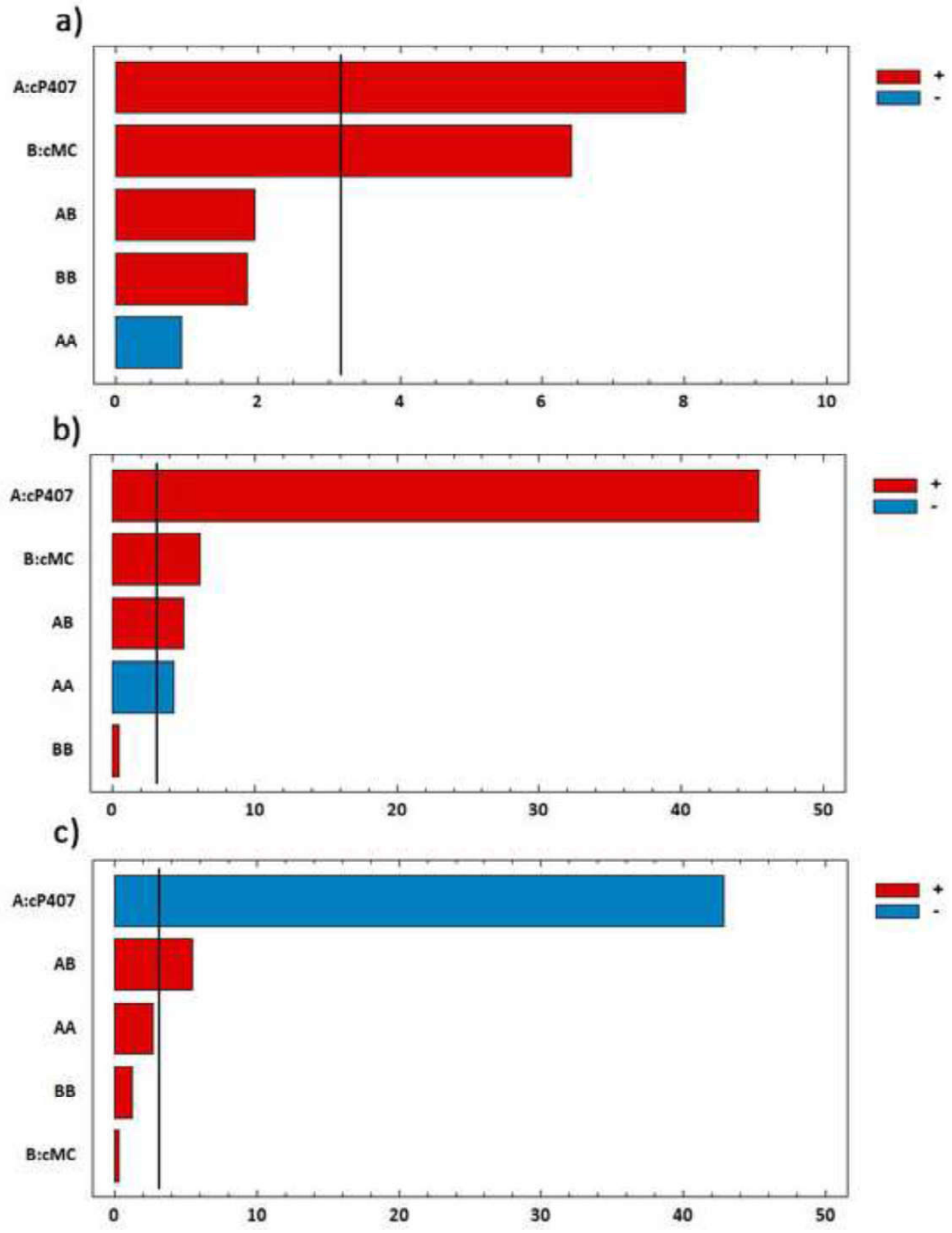

Figure 2

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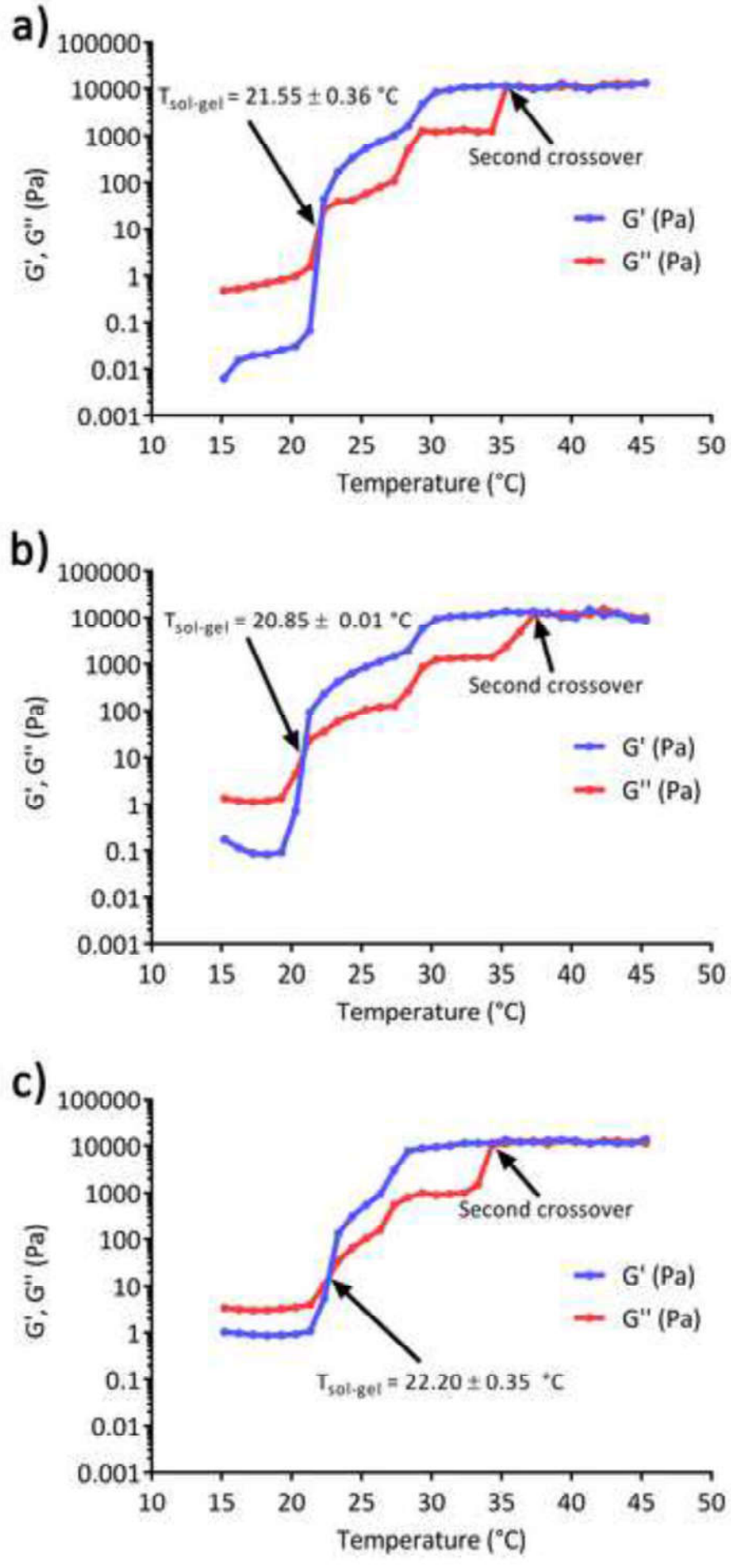

Figure 3

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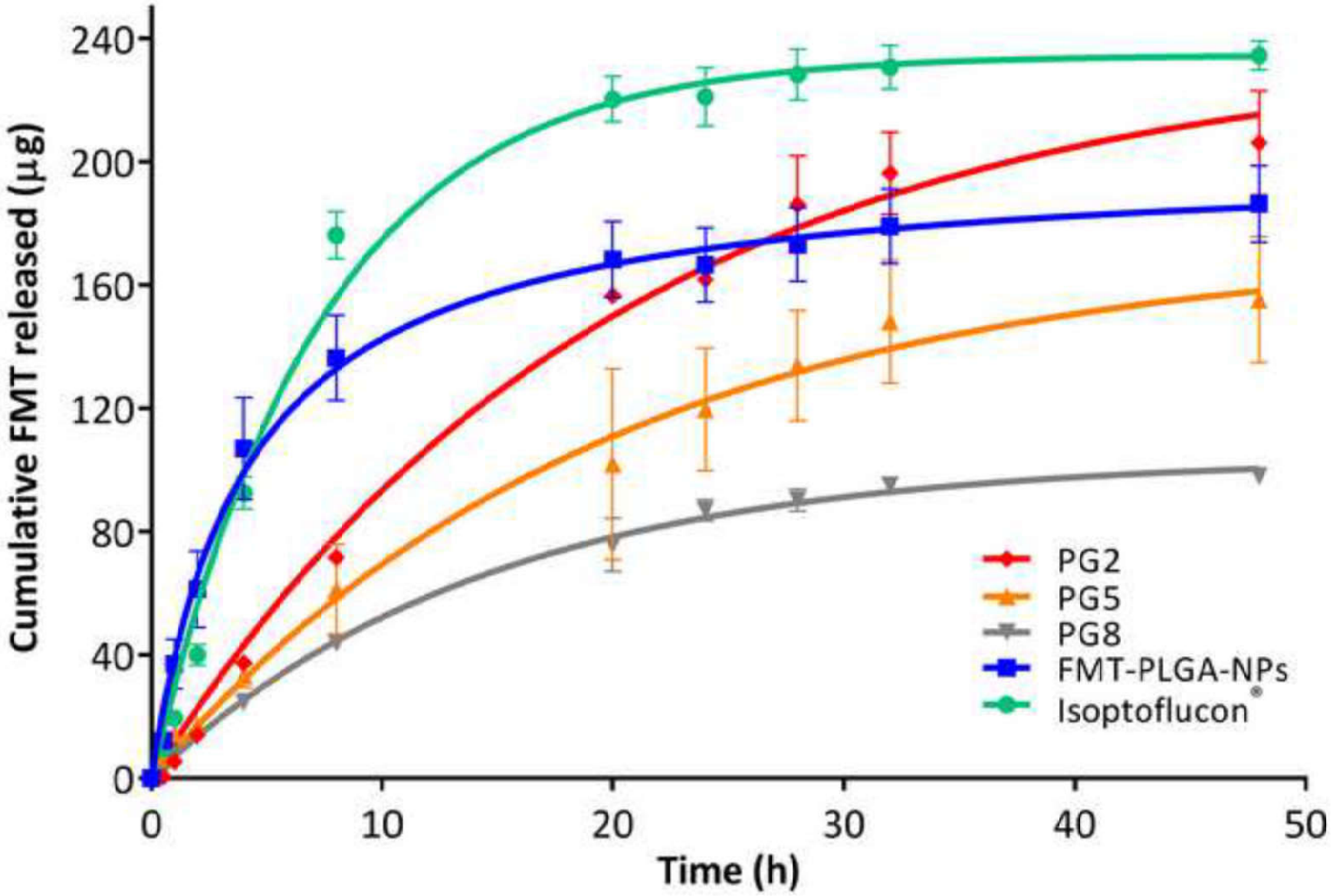

Figure 4

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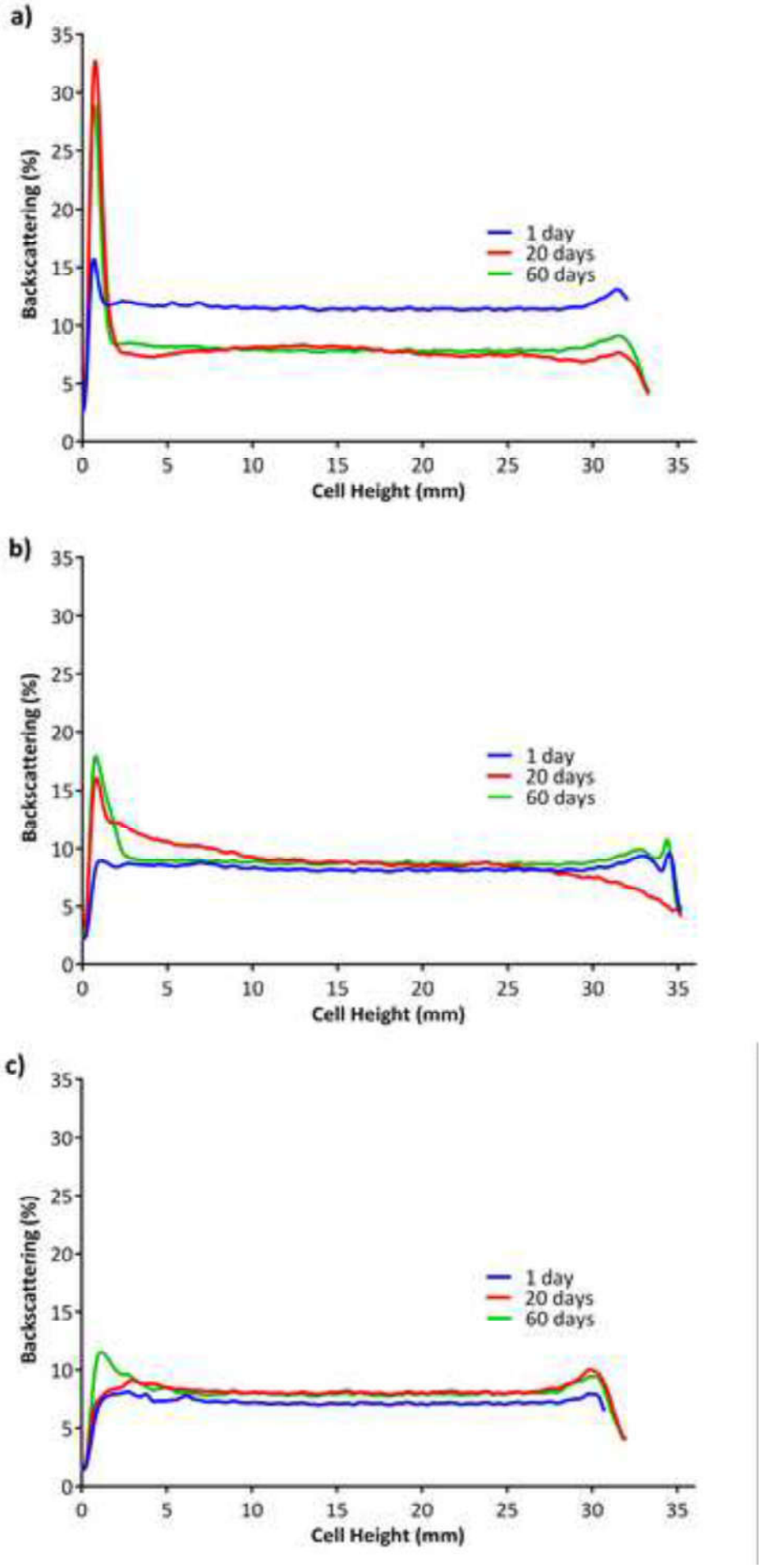

Figure 5

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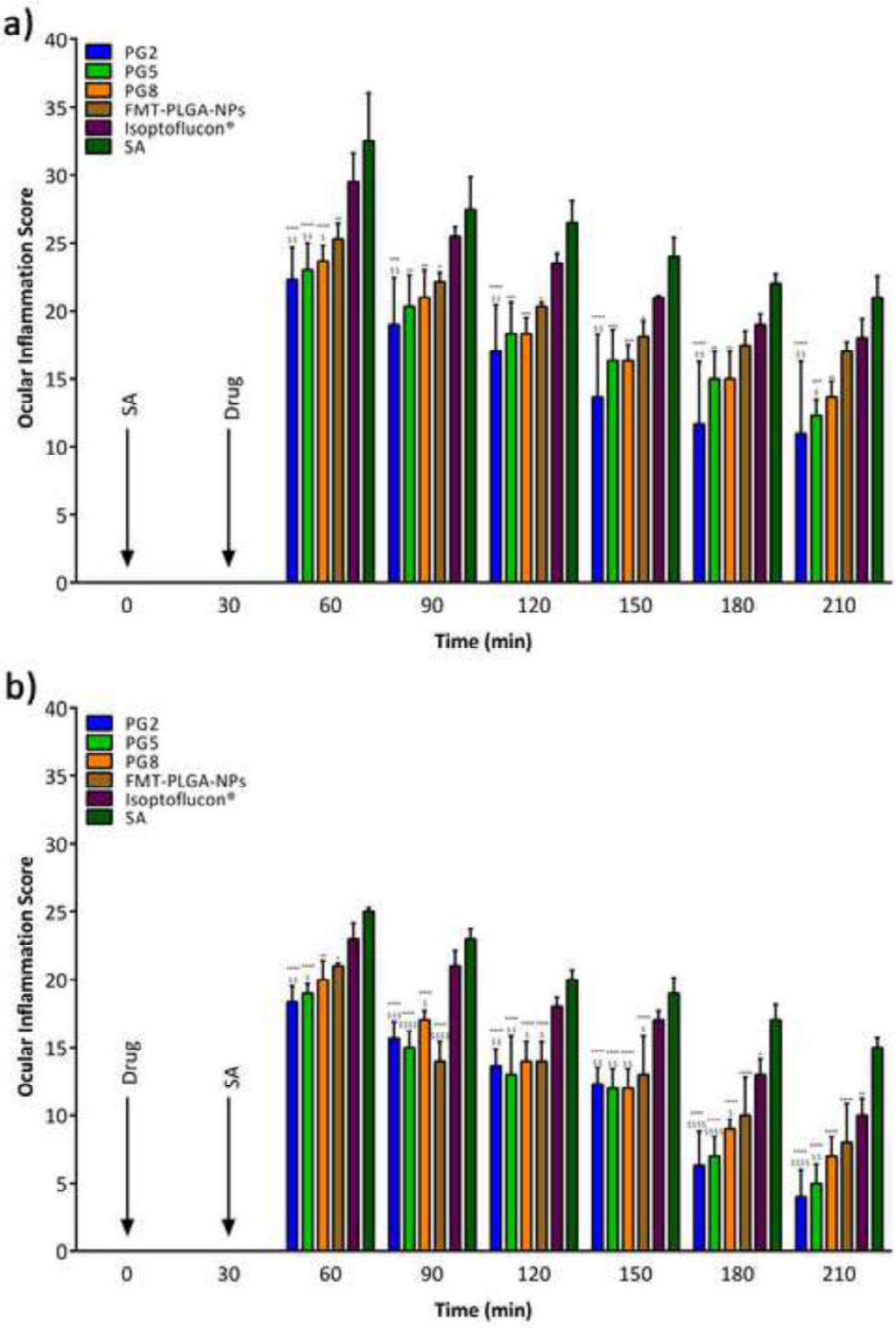


Figure captions

Fig. 1. Optimization of the gels. Pareto' diagram of the analyzed effect on a) Gt, b) Vs, (c) $T_{sol\text{-}gel}$.

Fig. 2. Sol-gel temperature transition of gels. a) PG2, b) PG5, c) PG8.

Fig. 3. *In vitro* profile release of gels (adjust to first order equation), FMT-PLGA-NPs (adjust to hyperbola equation) and Isoptoflucon® (adjust to first order equation).

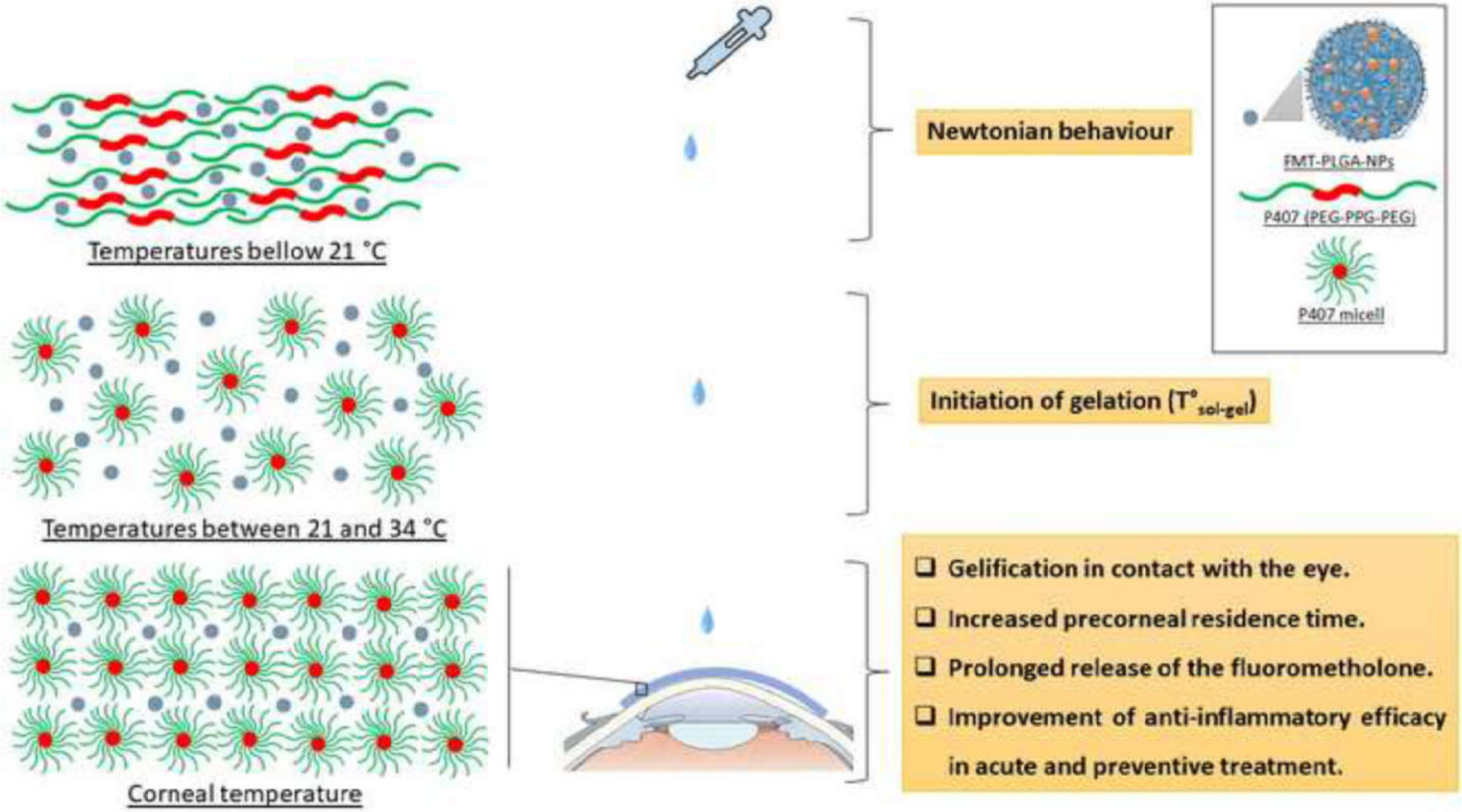
Fig. 4. Backscattering profile of optimized gels at 4 °C. a) PG2, b) PG5, c) PG8.

Fig. 5. Comparation of anti-inflammatory efficacy of PG2, PG5, PG8, FMT-PLGA-NPs and Isoptoflucon®. a) Inflammation treatment, b) inflammation prevention. Values are expressed as mean ± SD; ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ significantly lower than the inflammatory effect induced by SA; $$ $p < 0.01$, $$$ $p < 0.001$ and $$$$ $p < 0.0001$ significantly lower than the inflammatory effect induced by Isoptoflucon®.

***Highlights (for review)**

- DoE was useful for the for the optimization of *in-situ* forming gels.
- *In-situ* gelation allowing a slower and sustained release than commercial eye drops.
- Developed gels are non-irritating confirmed by ocular tolerance *in vitro/in vivo*.
- Gels are effective both in the treatment and in the prevention of inflammation.

Graphical Abstract



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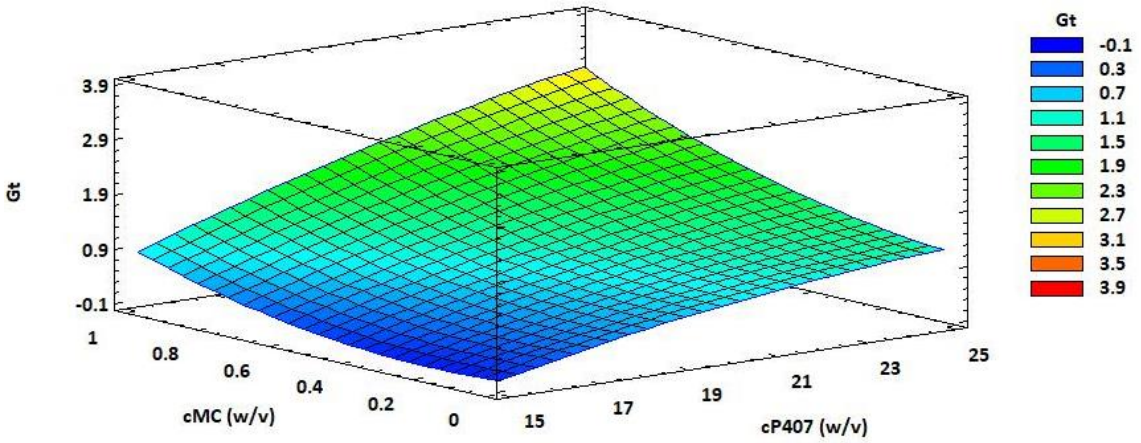

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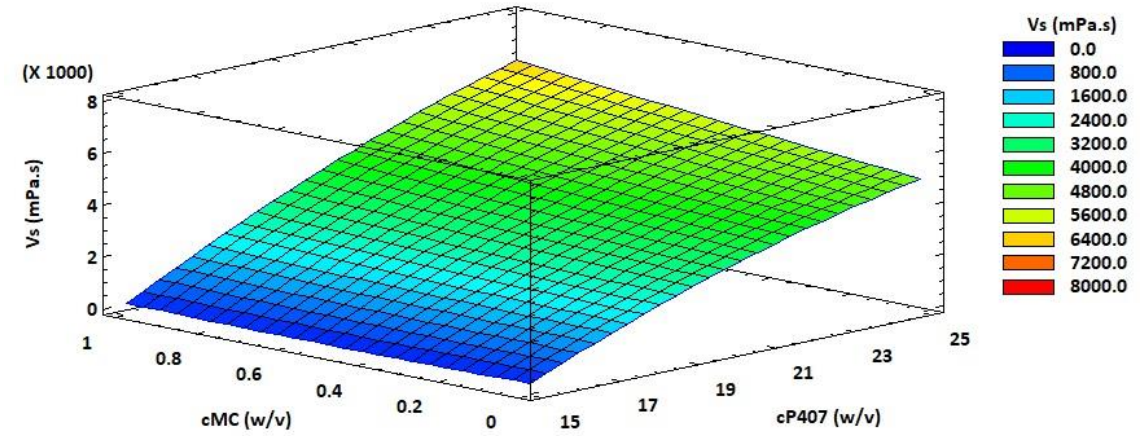

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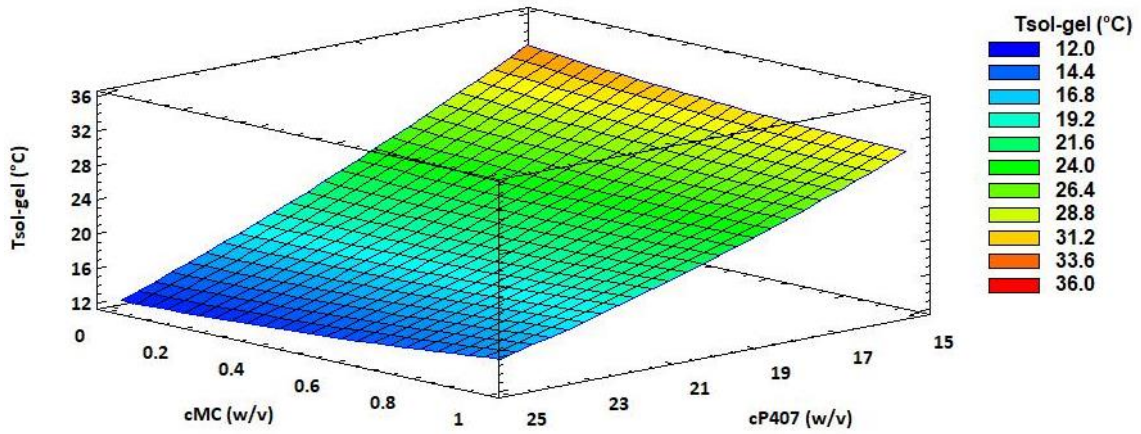
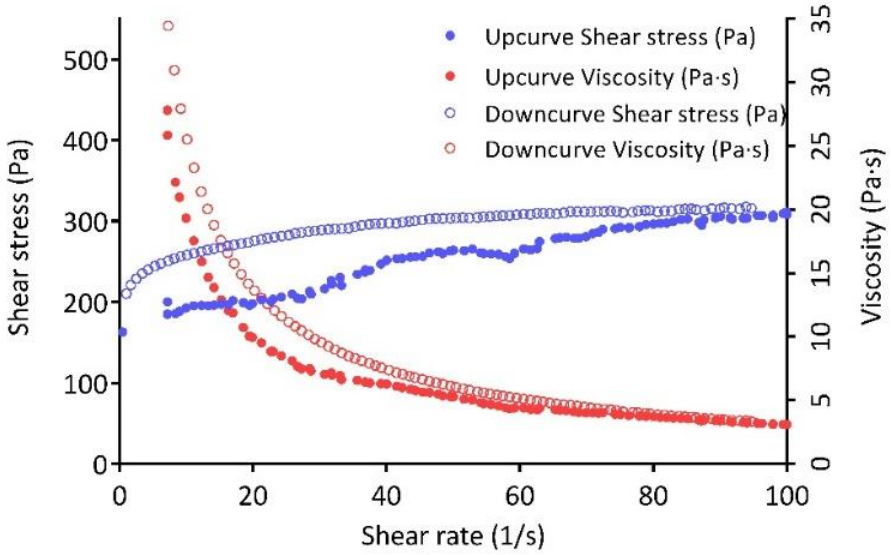


Fig. S.1. Surface response. a) Gt, b) Vs, c) $T_{sol\text{-}gel}$.

Fig. S.2. Photos of the formulations optimized in the gelling capacity test. a) PG2, b) PG5, c) PG8.

a)



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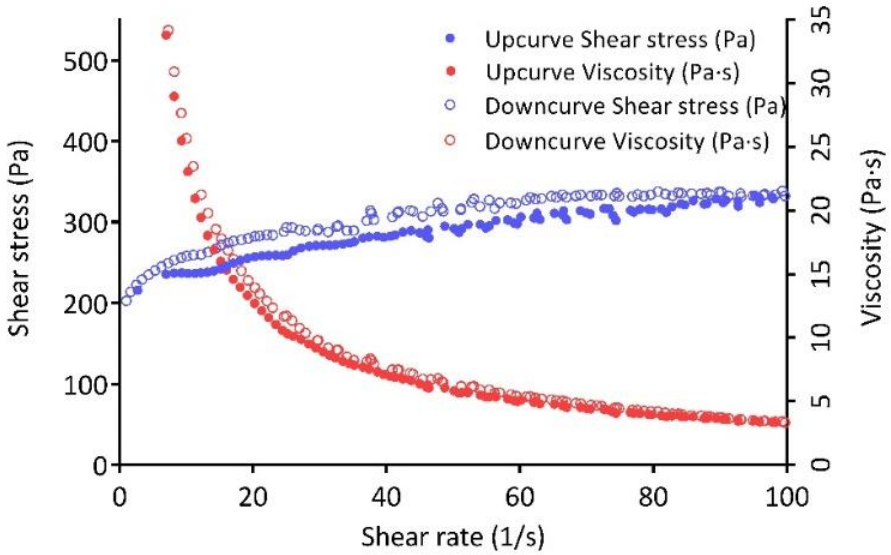


c)

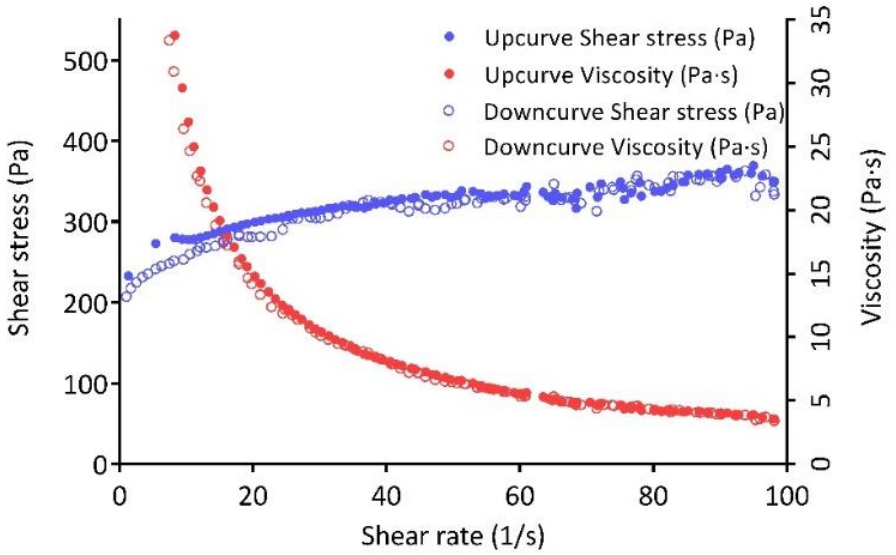


Fig. S.3. Flow and viscosity of the optimized gels. a) PG2, b) PG5, c) PG8.

Table S.1
Independents variables and codes used in the experimental design.

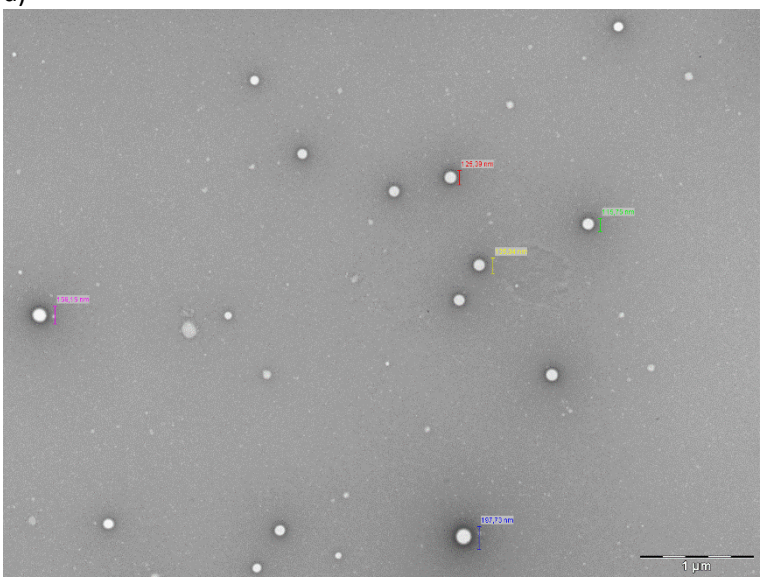
Independents variables (w/v)	Levels		
	-1	0	+1
cP407	15.0	20.0	25.0
cMC	0.0	0.5	1.0

Table S.2
Flow ability of optimized gels.

Formulation	**Flow ability at different temperatures (°C)**		
	5 ± 0.5	25 ± 0.5	35 ± 0.5
PG1	+++	+++	+++
PG2	+++	++	-
PG3	+++	-	-
PG4	+++	+++	+++
PG5	++	++	-
PG6	++	-	-
PG7	++	++	++
PG8	+	+	-
PG9	+	-	-

+++ = freely flow, ++ = regular flows, + = flow with difficulty, - not flow.

a)



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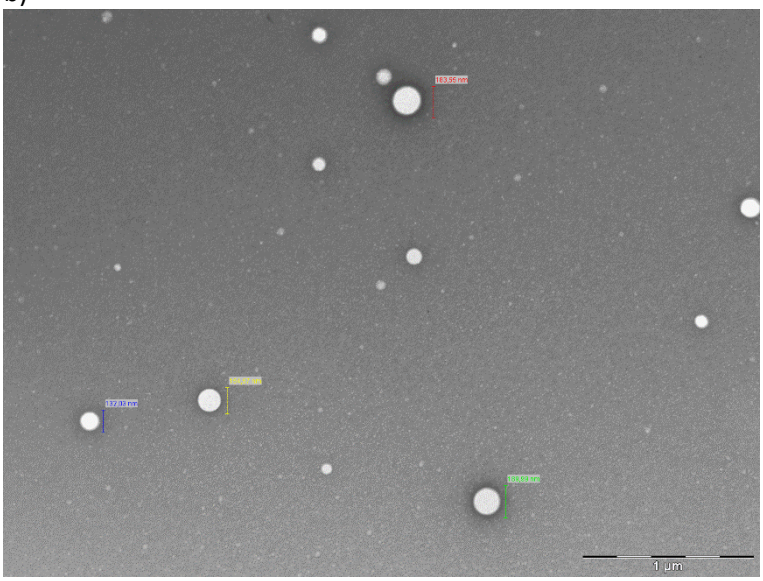

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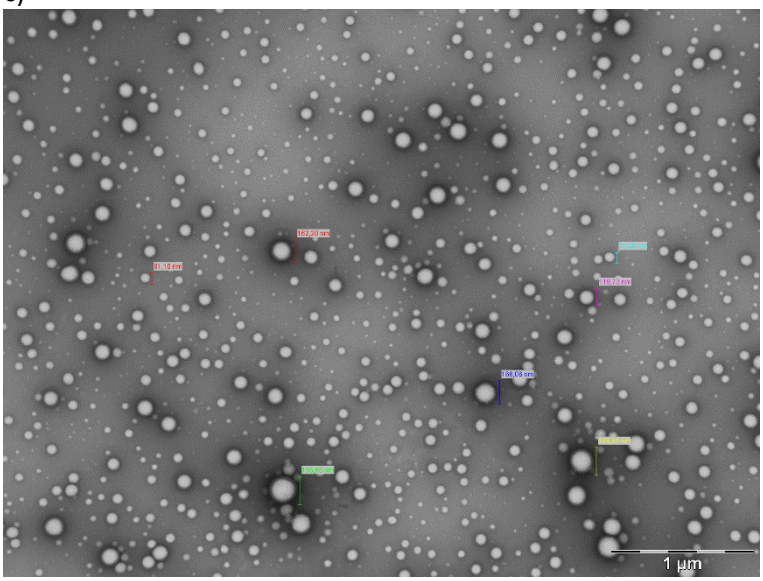


Fig. S.4. Transmission electron microscopy analysis of gels. a) PG2, b) PG5, c) PG8.

Table S.3
Ocular tolerance by HET-CAM® test.

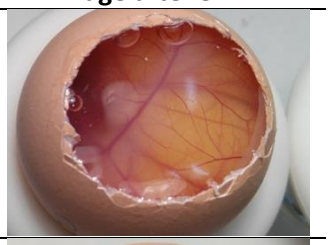
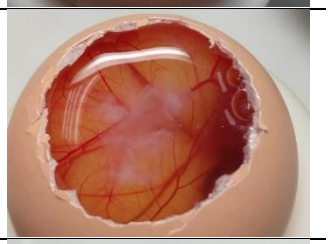
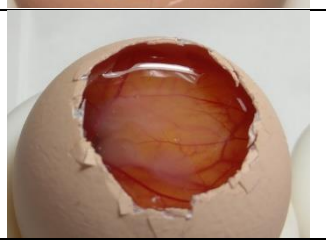
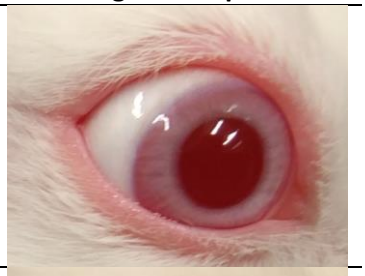
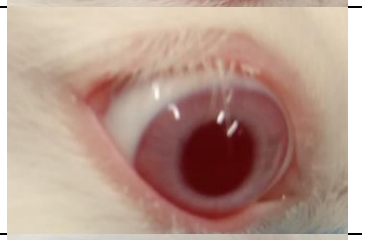
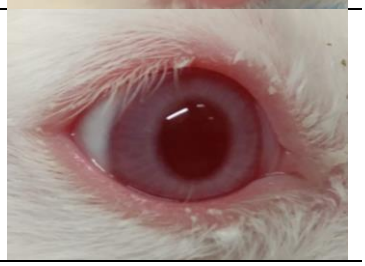
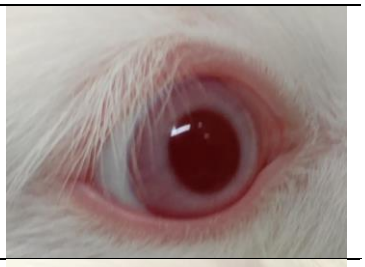
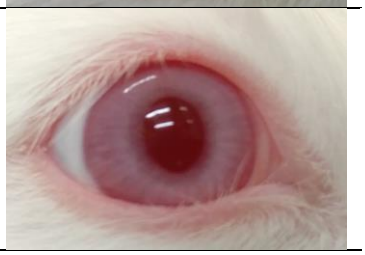
Formulation	Medium Score	Classification	Image after 5 min
PG2	0.61 ± 0.20	Nonirritating	
PG5	0.40 ± 0.20	Nonirritating	
PG8	0.41 ± 0.07	Nonirritating	

Table S.4
Ocular tolerance by Draize test.

Formulation	Medium Score	Classification	Image at endpoint
PG2	0.00 ± 0.00	Nonirritating	
PG5	0.00 ± 0.00	Nonirritating	
PG8	0.00 ± 0.00	Nonirritating	

FMT-PLGA-NPs	0.00 ± 0.00	Nonirritating	
Isoptoflucon®	0.00 ± 0.00	Nonirritating	

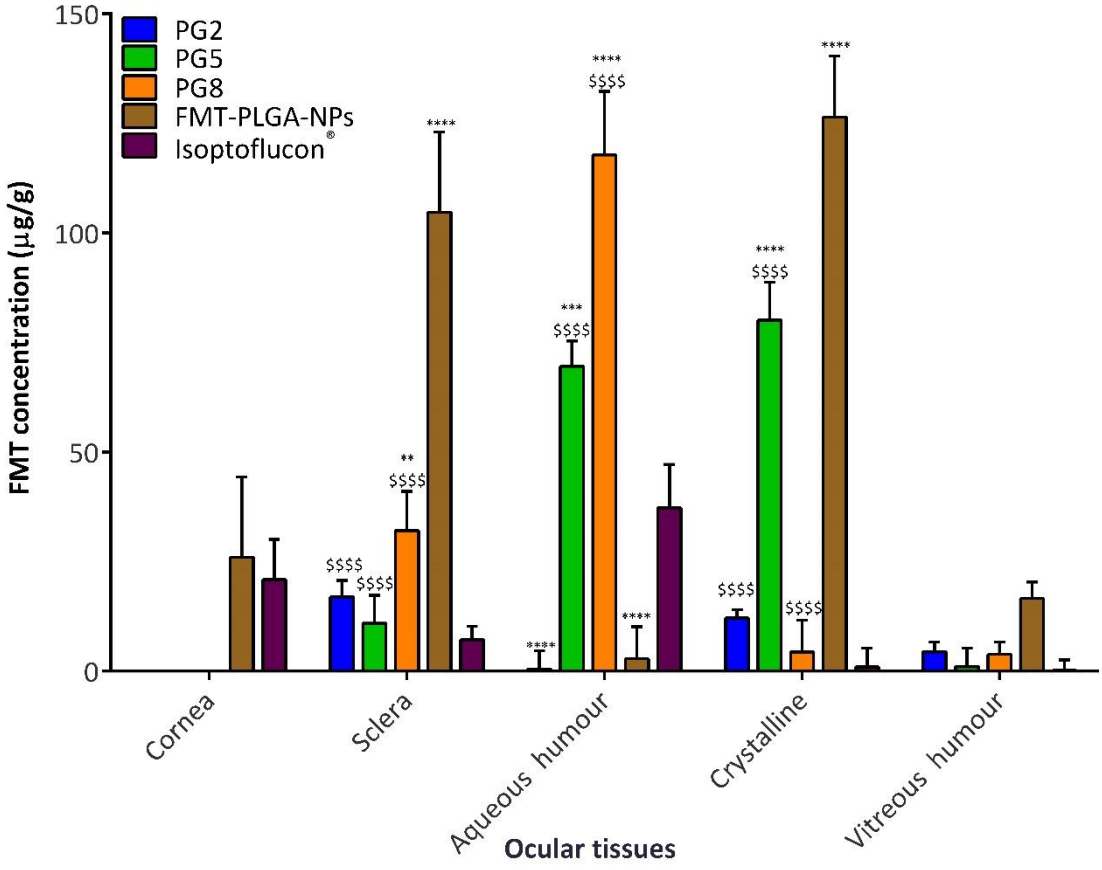


Fig. S.5. Bioavailability of PG2, PG5, PG8, FMT-PLGA-NPs and Isoptoflucon® in the different ocular structures. Values are expressed as mean ± SD; ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ significance with respect to the concentration of FMT from Isoptoflucon®; $$ $p < 0.01$, $$$ $p < 0.001$ and $$$$ $p < 0.0001$ significance with respect to the concentration of FMT from FMT-PLGA-NPs.