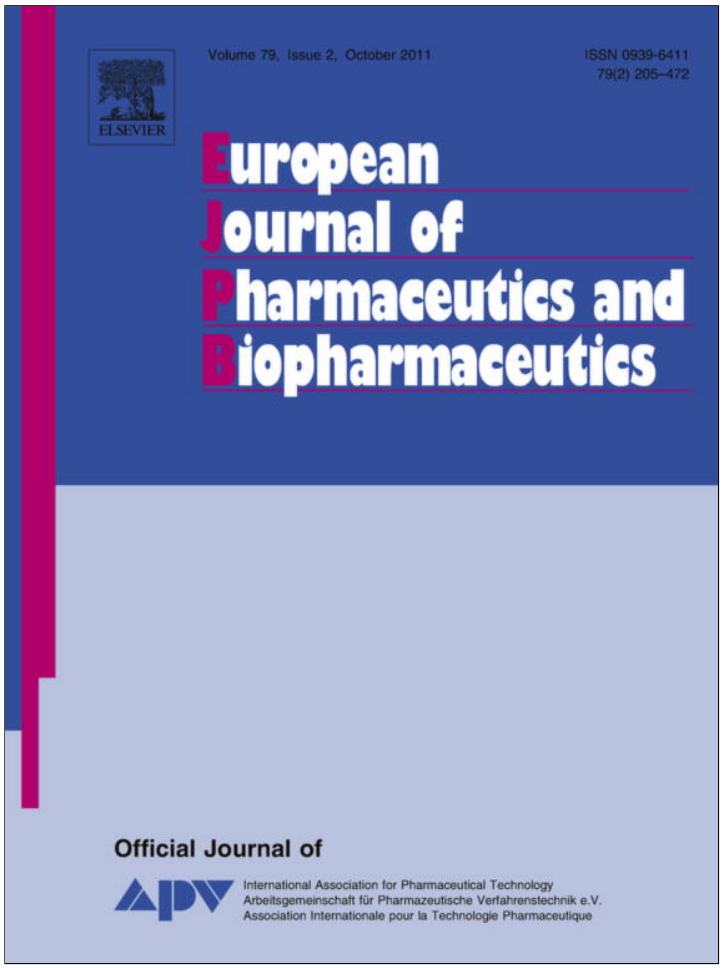

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Research paper

Enhancing and sustaining the topical ocular delivery of fluconazole using chitosan solution and poloxamer/chitosan *in situ* forming gel

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ABSTRACT

Fungal keratitis is a serious disease that can lead to loss of vision. Unfortunately, current therapeutic options often result in poor bioavailability of antifungal agents due to protective mechanisms of the eye. The aim of this work was to evaluate the potential of a chitosan solution as well as an *in situ* gel-forming system comprised of poloxamer/chitosan as vehicles for enhanced corneal permeation and sustained release of fluconazole (FLU). For this, *in vitro* release and *ex vivo* corneal permeation experiments were carried out as a function of chitosan concentration from formulation containing the chitosan alone and combined with the thermosensitive polymer, poloxamer. Microdialysis was employed in a rabbit model to evaluate the *in vivo* performance of the formulations. The *in vitro* release studies showed the sustained release of FLU from the poloxamer/chitosan formulation. *Ex vivo* permeation studies across porcine cornea demonstrated that the formulations studied have a permeation-enhancing effect that is independent of chitosan concentration in the range from 0.5 to 1.5% w/w. The chitosan solutions alone showed the greatest *ex vivo* drug permeation; however, the poloxamer/chitosan formulation presented similar *in vivo* performance than the chitosan solution at 1.0%; both formulations showed sustained release and about 3.5-fold greater total amount of FLU permeated when compared to simple aqueous solutions of the drug. In conclusion, it was demonstrated that both the *in situ* gelling formulation evaluated and the chitosan solution are viable alternatives to enhance ocular bioavailability in the treatment of fungal keratitis.

1. Introduction

Fungal keratitis is a serious disease that can lead to loss of vision if not diagnosed and treated promptly and effectively [1]. Previous ocular surface disease and trauma are the leading causes of fungal infection in the cornea [2], but prolonged chemo- or immunosuppressive therapy [3] and contact lens use [4] have also been reported as predisposing factors.

Unfortunately, current therapeutic options are limited [5]. Oral therapy requires high doses of an antifungal agent to reach therapeutic concentrations at the site of action, which may cause unwanted side effects. Antifungal subconjunctival and intracameral injections are customarily used but are very uncomfortable and may cause complications such as cataracts [6]. In most cases, surgery is recommended [7] even though surgical options, such as therapeutic keratoplasty, have a high incidence of infection recurrence [8]. In extremely severe cases, enucleation or evisceration is needed [9,10]. Topical treatment, which would be the ideal choice, is not efficient. Eye drops often result in poor bioavailability due to normal protective mechanisms of the eye such as rapid precorneal drainage allied with the poor permeability of the cornea to drugs [11,12].

The problem of short residence time of formulations on the eye surface may be overcome by the use of *in situ* forming gels. These systems are applied as solutions or suspensions and undergo gelation after instillation due to physico-chemical changes they undergo in the eye [13]. This allows for an easily reproducible administration of the formulation into the eye as a drop and an *in situ* phase transition to a gel on the surface of the cornea. This may improve the retention time of the formulation and, consequently, of the drug. Furthermore, the administration of solutions is well tolerated by patients, which could contribute to compliance with the regime.

Thermosensitive amphiphilic block copolymers, namely poly (ethylene oxide)–poly (propylene oxide)–poly (ethylene oxide) (PEO–PPO–PEO, poloxamers), have been extensively investigated as *in situ* forming gels [14–18]. They form micelles in solution that can self-organize and form a viscous gel depending on polymer

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concentration and temperature [19]. Though thermosensitive copolymers are widely employed, they suffer from a major drawback of having weak mechanical strength, which leads to rapid erosion [20]. One interesting approach to solve this problem focuses on blends of poloxamers with chitosan [21].

Chitosan is a biodegradable polymer that has demonstrated excellent ocular compatibility [22–24]. It presents positively charged amine groups in its chemical structure that could interact with the negatively charged mucous layer, conferring a mucoadhesive characteristic [25,26]. Due likely to this characteristic, chitosan solutions have been successfully used in prolonging contact time with rabbit's ocular surface [27].

Experimental evidence that poloxamer and chitosan can be used in combination for the preparation of *in situ* forming gels with improved mechanical and mucoadhesive characteristics for prolonged precorneal residence time *in vivo* was showed in our previous work [21]. At determined polymer concentration, a free-flowing solution at an environmental temperature, which could undergo phase transition upon instillation, was obtained. The adequate mechanical and mucoadhesive properties lengthened the residence time, and the problem of rapid precorneal elimination was overcome [21]. However, the potential of the poloxamer/chitosan gel for sustaining drug release and for overcoming cornea impermeability still needed to be evaluated.

Therefore, the aim of the present work was to evaluate the potential of an *in situ* gel-forming delivery system comprised of poloxamer/chitosan as well as a chitosan solution as vehicles for enhanced permeation and sustained release of drugs. To explore the feasibility of such strategies, fluconazole, which is a well-known and well-tolerated antifungal agent used in the treatment of fungal keratitis [28–30], was chosen. *In vitro* release and *ex vivo* permeation experiments were carried out as a function of chitosan concentration, and microdialysis was employed in a rabbit model to evaluate the *in vivo* performance of the formulations.

2. Materials and methods

2.1. Chemicals

Fluconazole (FLU) was purchased from Galena (Campinas, Brazil). Chitosan MMW (190,000–310,000 Da; 75–85% deacetylated – information provided by the manufacturer) was purchased from Sigma Aldrich (Steinheim, Germany). Poloxamer 407 was purchased from Embrafarma (São Paulo, Brazil), and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Ketamine hydrochloride (Dopalen) was purchased from Vetbrands (Jacareí, Brazil) and xylazine (Dopaser) from Hertape Calier (Juatuba, Brazil). For the release experiments, cellulose acetate dialysis membranes were used, 16,000-molecular weight cutoff (Fisher, Pittsburg, USA). All other reagents were analytical or HPLC grade. Deionized water (Milli-Q Millipore Simplicity 185, Bedford, MA, USA) was used to prepare all solutions. The linear probes used in microdialysis consisted of 0.3 × 10.0 mm, Gambro, Cuprophane, 3000-molecular weight cutoff (CMA Microdialysis, Stockholm, Sweden) glued to nylon tubing (standardized length of 50.0 mm).

2.2. Preparation of gels

Chitosan solutions were prepared dissolving the polymer in 0.5% v/w acetic acid solution. The *in situ* forming gels used in this study were prepared as previously described [21]. Briefly, poloxamer was dissolved into cooled FLU solution and, in cases where chitosan was added, it was first dissolved in a solution of acetic acid, 0.5% v/w. The final solution was kept in the refrigerator for at least 24 h to ensure complete dissolution. The final pH of the formulations ranged from 6.0 to 6.5. All formulations were isotonic with osmolalities ranging from 290 to 310 mOsm kg^{-1}. The amount of FLU added to the formulations was quantified by HPLC before the experiments, by appropriate dilution, to guarantee that the theoretical concentration dispersed in the day before was corrected.

2.3. In vitro drug release

FLU release rates from chitosan solutions (0.5, 1.0 or 1.5% w/w) and from *in situ* forming gels comprised of poloxamer (16% w/w) and chitosan (0.5, 1.0 or 1.5% w/w) were measured through a cellulose membrane in a Franz-type diffusion cell [21], with a diffusion area of 0.64 cm^2. Comparisons were made with the poloxamer (16% w/w) gel alone, and with aqueous solution, all of them containing 2 mg/ml FLU. Aqueous solution containing FLU 2 mg/ml was used as a control.

The donor compartment was filled with 1 ml of studied formulation while the receptor compartment contained 35 ml of pH 7.4 HEPES buffer solution. The system was maintained under magnetic stirring (600 rpm) and at 35 °C with an outer bath. Samples (1 ml) were withdrawn from the receiving solution each hour for 6 h and replaced with fresh receiving fluid. The amount of FLU that permeated across the membrane, i.e., the amount of the drug in the receiving solution, was analyzed by HPLC as described in Section 2.5. The diffusion coefficients (D) of FLU from each vehicle were calculated using the following equation [31,32].

$$Q = 2C_o(Dt/\pi)^{1/2} \quad (1)$$

where Q is the cumulative amount of drug released per unit area, C_o is the initial drug concentration in the vehicle, and D is the diffusion coefficient and t is time.

The drug release rate (K) was also determined by the slope of the linear portion of the plots of the FLU cumulative amount versus the square root of time.

2.4. FLU ex vivo corneal permeation

Corneas used in the *ex vivo* experiments were obtained from porcine eyes, which were collected immediately after the animals were slaughtered (Frigorífico Pontal Ltda, Pontal, SP, Brazil). The eyes had not been heat treated in the abattoir in any way. They were then kept at 4 °C while transported to the laboratory and used within 1.5 h of enucleation. Any eye with a collapsed anterior chamber was discarded. Corneoscleral buttons were dissected using standard eye bank techniques, and care was taken to minimize tissue distortion.

Immediately after corneal preparation, the tissue was placed between the donor and the receiving compartments of the vertical modified Franz-type diffusion cell [33]. The receiving compartment was filled with HEPES buffer solution (25 mM, pH 7.4), and the donor compartment was filled with 1.0 ml of the formulations described in Section 2.3 (the *in situ* chitosan/poloxamer forming gels, the chitosan solutions, the poloxamer gel, and the water) containing 2 mg/ml of FLU. The diffusion-cell system was maintained under identical magnetic stirring and outer bath temperature as described for the *in vitro* release studies (see Section 2.3). Corneal integrity and barrier properties when mounted on the described system are assured for 6 h [33]. For each formulation, six corneas were used ($n = 6$).

During the experiment, samples (1 ml) were withdrawn from the receiving solution each hour for 6 h and replaced with fresh receiving fluid. The amount of FLU that permeated across the cornea, i.e., the amount of the drug in the receiving solution, was analyzed by

HPLC (see Section 2.5). The permeation flux (J) of FLU through the cornea from each formulation was then determined by the slope of the linear portion of the graphic obtained when the amount of drug that permeated across the cornea was plotted against time.

2.5. Quantification of FLU by HPLC

The amounts of FLU, which had diffused through the cellulose membrane or the cornea, were determined by a spectrophotometric high-performance liquid chromatography (HPLC) method. For these analysis, C_{18} reversed-phase columns (125 × 4 mm, 5 μm) were used, with water/acetonitrile/methanol (80:15:5 v/v) as the mobile phase at a flow of 1.0 ml/min, detection at 210 nm, and an injection volume of 50 μl. The calibration curve was linear (r = 0.999) for FLU over a concentration range of 0.1–200.0 μg/ml. Intra- and inter-day precision and accuracy of the method showed a% CV and a relative error (% E) not greater than 5% and 3%, respectively.

2.6. In vivo absorption experiments – microdialysis

2.6.1. Animals

New Zealand albino rabbits weighing between 2.0 and 2.5 kg were used. This research followed the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the ethical committee of the University of São Paulo (CEUA *Comissão de Ética do uso de animais* – Protocol no. 06.1.90.53.8). This work was strictly conducted according to the committee guidelines on the use of animals. The rabbits were kept under anesthesia throughout the experiment by intramuscularly injecting ketamine hydrochloride (35 mg/kg) and xylazine (3.5 mg/kg), every 40 min. These animals did not recover from the anesthesia during the entire length of the experiment. They were sacrificed at the end of the experiment by asphyxiation in a saturated CO_2 chamber.

2.6.2. Probe implantation

The linear probe was implanted in the aqueous humor using a 22-gauge needle. Previously, the animal's eye was luxated and fixed using a chirurgic glove. Then, a small incision of approximately 2 mm was made just above the corneal scleral limbus so that the needle containing the probe attached to its bevel edge could traverse through the center of the anterior chamber to the other end of the cornea leaving the probe with the membrane in the middle of the anterior chamber. The needle was then removed, and the extremities of the probe were connected to polyethylene microtubes leading to the microinjection system and the collection tube. A diagrammatic representation of the microdialysis probes implanted in the eye is depicted in Fig. 1. The probes were perfused with pH 7.4 isotonic phosphate-buffered saline (IPBS) with a flow rate of 2 μl/min, using a microinjection pump (Bioanalytical Systems, West Lafayette, IN, US). After probe implantation, the eyes were returned to its normal position and the animals were allowed to stabilize for at least 2 h for the restoration of intraocular pressure and replenishment of the aqueous humor that can be partially lost during the probe implantation [34].

2.6.3. Microdialysis

After probe implantation, 50 μl of isotonic formulations containing 0.4% w/w FLU was topically administered. The corneal permeation of FLU from a poloxamer/chitosan *in situ* forming gel containing 16 and 1.0% w/w of each component, respectively (Chi1.0P16), was compared with the drug permeation from a 1.0% w/w chitosan solution (Chi1.0). An aqueous solution containing 0.4% FLU was used as a control. Samples were collected at predetermined time points by microdialysis and analyzed by HPLC as described in Section 2.5. Samples from the experiments carried out

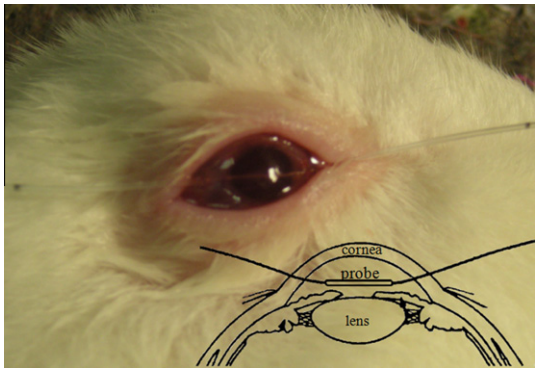

Fig. 1. Rabbit's eye after linear probe implantation according to the schematic diagram (in detail). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with Chi1.0P16, and control formulations were collected every 30 min during 6 h. From those performed with Chi1.0, samples were collected every hour, also for 6 h. All experiments were carried out in four replicates. The pharmacokinetic parameters, as the peak aqueous humor concentration (C_{max}) and the areas under the concentration–time curve (AUC), were calculated based on the concentration of FLU in the aqueous humor over the 6 h. This concentration was calculated by dividing the dialysate concentration by the *in vivo* probe recovery concentration (see Section 2.6.4. for *in vivo* probe recovery determination).

2.6.4. Probe recovery

– *In vitro probe calibration:* to determine the *in vitro* relative recovery of FLU through the membrane, four probes connected to one microinjection pump were bathed in IPBS containing known concentrations of the drug (from 1 to 10 μg/ml) and perfused with IPBS at 2 μl/min. Dialysate was collected and measured using HPLC. The *in vitro* recovery (R) of FLU was calculated according to the following equation:

$$R = \frac{C_d}{C_i} \quad (2)$$

where C_d is the dialysate concentration and C_i is the known concentration of FLU in IPBS [34].

– *In vivo probe calibration* (*retrodialysis*): an *in vivo* retrodialysis method was employed for probe calibration in rabbits [35]. This method is based on the dynamic equilibrium principle, which states that the amount of drug lost to the surrounding medium should be equal to the amount of drug gained from the surrounding medium. The probe was implanted in the aqueous humor of the rabbit's eye, and the microdialysis experiment was carried out by probe's perfusion with a known concentration of the FLU (5 μg/ml). These experiments were carried out in duplicate. The dialysate was collected, and the *in vivo* recovery (R) was calculated using the following equation:

$$R = \frac{C_i - C_d}{C_i} \quad (3)$$

The concentration of FLU in the aqueous humor during the pharmacokinetic experiment was calculated by dividing the dialysate concentration by the *in vivo* recovery obtained above.

2.7. Statistical analysis

The data obtained from all experiments were subjected to an unpaired t-test. Values with $P < 0.05$ were considered statistically significant (Prisma, Graphpad Software, La Jolla, US).

3. Results

3.1. In vitro drug release

Cumulative amount of FLU released from all the formulations plotted against the square root of time revealed a linear relationship ($r > 0.98$), indicating that this drug released from the vehicles following the Higuchi diffusion model. Comparison of the release rates (K) shows the expected higher K from the aqueous solution (Fig. 2); consequently, the highest drug diffusion coefficient for FLU was also obtained from the aqueous solution ($D = 2.77 \times 10^{-5}$ cm^2/s). Diffusion coefficient for 16% w/w poloxamer and 1.0% w/w chitosan separately and in combination was, respectively, 1.27, 1.67, and 0.43×10^{-5} cm^2/s. All polymeric formulations presented significant lower K values, although there were no statistical differences between the samples when only one polymer was used. When poloxamer/chitosan was used in combination, the FLU release was retarded independently of chitosan concentration. Hence, the *in situ* forming gel containing chitosan showed a superior capacity for sustaining drug release.

3.2. Ex vivo drug permeation

Linear correlation coefficients were obtained when the cumulative amount of FLU in the receptor solution was plotted over time, indicating that the permeation of FLU from the different formulations through porcine corneas followed zero order kinetics, where the flux is independent of the drug concentration [36]. The total amount of FLU permeating from the poloxamer gel (88.46 μg/cm^2) was not statistically different from the aqueous solution (74.38 μg/cm^2). A twofold increase in the total amount of FLU that permeated from the gel was observed when 1.0% w/w chitosan was added (192.44 μg/cm^2). Higher drug permeation was observed from chitosan solutions in comparison with all other formulations (345.69 μg/cm^2 permeated from 1.0% w/w chitosan solution).

3.3. In vivo microdialysis

3.3.1. Probe recovery

In vitro probe recovery experiments showed a linear relationship ($r = 0.999$) between the drug concentration in IPBS and in the dialysate over the studied concentration range of FLU. In this way, the diffusion through the dialysis membrane was independent of FLU concentration, which is a prerequisite for the microdialysis experiments. The average of FLU recovered in the *in vitro* experiments was 22%, which was very similar to the *in vivo* recovery, 24%, used to estimate the real drug concentration in the aqueous humor after topical administration of the formulations.

3.3.2. FLU corneal permeation after topical administration

Due to the low percentage of FLU recovered (around 24%), microdialysis studies were carried out with 0.4% of FLU incorporated in the formulations to obtain sufficient amounts of the drug in dialysate for direct quantification. Also to allow direct quantification, the perfusate was analyzed every 30 min because only after this period, one has sufficient volume (60 μl) of perfusate to be analyzed (recall that the volume of FLU sample required by the analytical method to be injected was 50 μl and the microdialysis

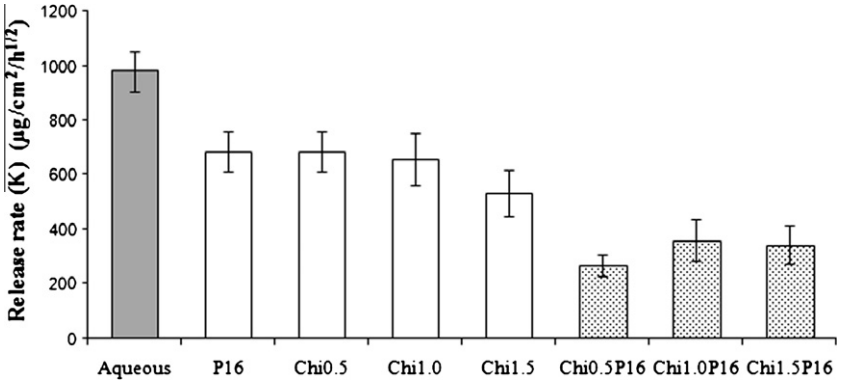

Fig. 2. Drug release rates, studied *in vitro*, through a cellulose acetate membrane from different polymeric formulations. The results are represented by the slope obtained from the linear portion of the release profile curve, cumulative drug amount versus time. Different bar fillings represent statistical difference ($P < 0.05$). Data represent mean ± SD ($n = 6$). Aqueous, *P*, and Chi are abbreviations for, respectively, aqueous solution, poloxamer, and chitosan. The numbers represent the polymer concentration (% w/w) in the formulation.

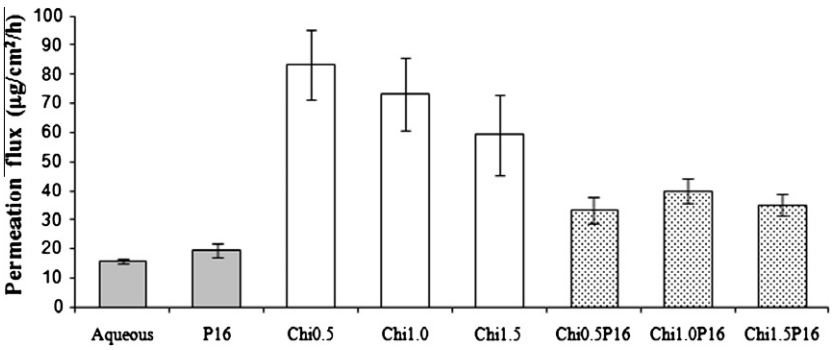

Fig. 3. *In vitro* drug release rates trough porcine corneas from different formulations. The results are represented by the slope obtained from the linear portion of the release profile curve, cumulative drug amount versus time. Different bar fillings represent statistical difference ($P < 0.05$). Data represent mean ± SD ($n = 6$). Aqueous, *P*, and Chi are abbreviations for, respectively, aqueous solution, poloxamer, and chitosan. The numbers represent the polymer concentration (% w/w) in the formulation.

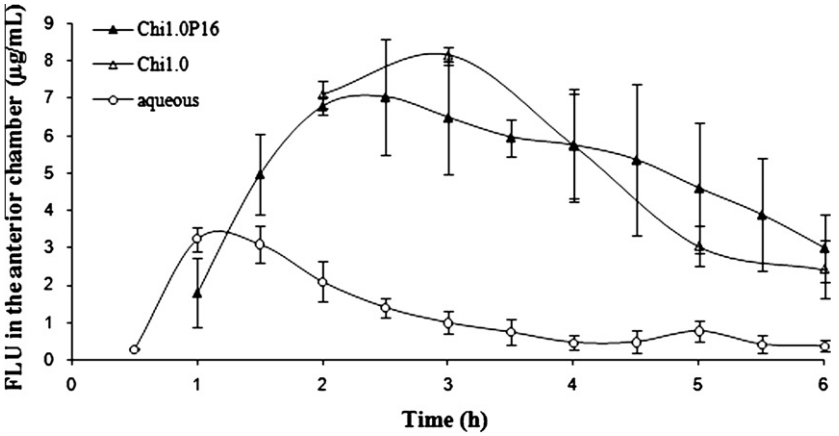

Fig. 4. FLU aqueous humor concentrations in rabbits after the topical administration of the following: (i) poloxamer/chitosan *in situ* forming gel (Chi1.0P16), (ii) chitosan solution (Chi1.0), and (iii) aqueous solution (aqueous). All formulations contained 0.4 mg/ml FLU. Data represent mean ± SD ($n = 4$).

probes were perfused with IPBS with a flow rate of 2 µl/min). The exception was when the chitosan solution was administered, to which a time point every hour was chosen. The reason for this was also analytical: with the analytical method that we developed, it was not possible to quantify FLU in the perfusate neither after 30 min nor after 1 h when Chi1.0 was administered. We decided then to sample at each hour, instead of every 30 min, to ensure drug amounts above the quantification limit of the method. With this experimental condition, it was possible to determine the C_{max} and T_{max} (see Fig. 4) for Chi1.0 with an acceptable reproducibility (coefficient of variation between the replicates was smaller than 31%). Therefore, to reduce the number of animals used to a minimum, we considered the FLU concentration profile obtained for Chi1.0 satisfactory for comparison purposes to the others formulations, even though the number of time points assessed was different from the others formulations.

Similar to what happened to the Chi1.0, Chi1.0P16 gel did not permeate the cornea in a quantifiable amount after 30 min, but did after 1 h. Because of the different initial time points obtained for each formulation, AUC values of chitosan solution and poloxamer/chitosan gel were calculated considering only the concentration values starting from 2 h, where the data for both formulations were available.

The concentration–time curves of fluconazole in aqueous humor after the topical administration of Chi1.0P16, Chi1.0 and aqueous solution, all of them containing 0.4% of the drug, are illustrated in Fig. 4. The peak aqueous humor concentration (C_{max}) of 3.23 ± 0.32 µg/ml was reached at 60 min for the aqueous solution, whereas the maximum concentration of 7.24 ± 2.81 and 8.14 ± 0.24 µg/ml was reached at around 170 and 180 min for, respectively, the thermosensitive gel formulation (Chi1.0P16) and the chitosan solution (Chi1.0) (Table 1). The areas under the concentration–time curve (AUC), calculated from the second hour of experiment until the sixth hour, obtained after the administration of FLU in aqueous solution, in Chi1.0 and in Chi1.0P16 were, respectively, 3.07, 21.67, and 21.94 µg h/ml (Table 1). For the aqueous solution, the AUC was also calculated from 1 h to 6 h of experiment, being 5.94 µg h/ml, i.e., around 3.5-fold smaller than those obtained for the formulations that contained chitosan.

Table 1
Peak aqueous humor concentration (C_{max}), time to achieve maximum concentration (t_{max}), and area under the concentration–time curve (AUC) calculated from the second hour of experiment until the sixth hour, obtained after the administration of FLU in aqueous solution, chitosan solution (Chi1.0), and chitosan/poloxamer *in situ* forming gel (Chi1.0P16), all of them containing 0.4% of the drug.

Formulation	C_{max} (µg/ml)	t_{max} (min)	AUC (µg h/ml)
Aqueous solution	3.23 ± 0.32	60	3.07
Chi1.0	8.14 ± 0.24	180	21.67
Chi1.0P16	7.24 ± 2.81	170	21.94

4. Discussion

In the present work, the potential of chitosan-based formulations for overcoming cornea impermeability was evaluated *ex vivo* as a function of chitosan concentration for a well-known antifungal drug, the FLU. The *in vivo* performance of the formulations chosen from the results obtained was also assessed using microdialysis.

In a previous work [21], we obtained, by the combination of poloxamer and chitosan, an *in situ* forming gel with improved mechanical and mucoadhesive properties, as well as improved retention time. We demonstrated that poloxamer/chitosan formulation in a concentration of 16% of poloxamer and 0.5–1.5% of chitosan showed optimal gelation temperatures ($T_{sol/gel} \sim 32$ °C) and were able to withstand low shearing forces at 35 °C. The mechanical properties of these formulations, especially the one containing 1% of chitosan, indicated that they have high hardness values and adhesiveness besides showing prolonged retention time when administered in humans *in vivo* [21]. Because chitosan solutions alone also showed a prolonged precorneal residence time in rabbits [27], it would be interesting if these chitosan-based formulations could sustain the release of drugs incorporated therein in order to guarantee a prolonged pharmacological effect. Therefore, to investigate the potential of the chitosan solutions and the poloxamer/chitosan gels in sustain drug release, the FLU release was measured *in vitro* to gain information about release kinetics and about how each formulation affects drug diffusion. Note that, despite the different mathematical models proposed to determine drugs diffusion coefficient from poloxamer gels [37,38], due to our experimental condition (the gel was physically separated from the receptor solution by a membrane), the semisolid characteristics of the gel and the proposed topical application, the Higuchi model [31] was applied to determine FLU diffusion coefficient from the formulations studied. FLU release from the systems developed followed the Higuchi square root law quite well ($r > 0.98$), like the release of other drugs from poloxamer gels (e.g., [37,39]).

Poloxamer and chitosan when used separately retarded drug release compared with a simple FLU aqueous solution, being this retard more pronounced when they were used in combination (see Fig. 2). The highest FLU sustained release from the poloxamer/chitosan gel can be explained by the increased mechanical strength

of this formulation [21], which can affect drug diffusion. More specifically, drug diffusion can be well described by Fick's equation, which correlates the drug's flux with its chemical potential gradient in the formulation [40]. Therefore, the diffusion coefficient of the drug is affected by the structure and pore size of the gel, the polymer composition, the water content, and the nature and size of the drug [40]. The addition of chitosan to the poloxamer dispersion must tighter poloxamer network, which resulted in a gel with improved mechanical properties and in a slower drug release system.

The FLU-sustained release given by chitosan/poloxamer combined formulations is an important characteristic of these systems, but should be aligned with others to guarantee the best drug therapeutic effect. Improving corneal FLU permeation together with a prolonged release is important, for example, for the treatment for resistant fungi that exhibit higher minimum inhibitory concentrations. To evaluate what extent the systems developed would influence the FLU permeation, *ex vivo* permeation experiments through porcine cornea were carried out. In these studies, corneal impermeability showed to be the rate-limiting step for FLU diffusion, as explained below: in spite of the fact that FLU release rate was higher from the aqueous solution than from the poloxamer gel (see Fig. 2), there was no significant difference between the FLU corneal permeation flux (see Fig. 3) of the aqueous solution and that of the poloxamer gel. If the cornea was not the rate-limiting step, formulation that released the drug faster should show higher drug permeation earlier, because in the *ex vivo* experiments carried out, the amount of drug released is not diluted nor drained (as happens *in vivo*) and is completely available for permeation. On the other hand, solutions composed by chitosan, a potential penetration enhancer for hydrophilic drugs [41], did increase FLU permeation. The mechanism of chitosan's penetration enhancement is mainly due to a transient widening of the tight junctions between cells [41]. Therefore, chitosan is acting in the membrane (cornea) to increase FLU permeation. Note in Fig. 3, however, that the same chitosan concentration increased less FLU permeation when it was dispersed in the poloxamer gel than when it was alone in solution. This apparent lower enhancing effect of chitosan when incorporated to the gel formulation can be explained by the lower diffusion of the drug from such formulation, as it was observed with the *in vitro* experiments (see Fig. 2), and not by a real decrease in chitosan penetration enhancer potential.

It is important to note as well that, in spite of the fact that higher FLU permeation was observed from chitosan solutions in comparison with all other formulations, its transport-enhancing effect had no significant correlation with chitosan concentration (see Fig. 3). The permeation-enhancing effect of this polysaccharide has been observed to be independent from its concentration in other works found in the literature. For instance, two different chitosan concentrations (0.4 and 1.0% w/v chitosan glutamate) did not change the transport of 9-desglycinamide, 8-L-arginine vasopressin (DGAVP) in cell culture [42] and across rat perfused intestine [43].

In summary, poloxamer/chitosan systems showed to be capable of (1) sustaining FLU release and (2) increasing drug corneal permeability. Chitosan solutions, in turn, presented higher permeation rates *ex vivo* than the *in situ* forming gel system.

Sustaining drug release *in vitro* and increasing drug penetration *ex vivo* are not the only characteristics that a formulation should have to be effective; optimizing other formulation properties is advisable to achieve greater bioavailability and consequently effectiveness. It is primarily important that the formulation possesses acceptable sensorial characteristics to allow for easy administration, leading to patient compliance. Once administered, it must remain in contact with the ocular surface for a prolonged time. In this way, the *in vivo* performance of the *in situ* forming gel containing poloxamer/chitosan (16.0 and 1.0% w/w, respectively), which in addition to sustain FLU release *in vitro* also exhibited a prolonged retention time *in vivo* in humans [21], was compared with that of the 1.0% w/w chitosan solution, which showed here to promote greater *ex vivo* drug permeation.

Note that, despite the non-significant difference on the amount of FLU permeated when 0.5–1.5% w/w of chitosan was used, formulations containing 1.0% of chitosan were chosen for the *in vivo* studies. This choice was based on our previously published study with the chitosan/poloxamer systems [21]. In this study, we showed that the formulation formed by 16% of poloxamer and 1% of chitosan had more desirable mechanical properties than the others. Specifically, we have shown that an increase from 0.5% to 1.5% on the content of chitosan in the formulations containing 16% poloxamer significantly increased formulation hardness and mucoadhesive force, which could make drainage more difficult and prolong its retention time in the eye. Although the formulation containing poloxamer and chitosan 1.5% w/w presented the greatest hardness values at 35 °C, before administration (at 25 °C), these values were also higher, which could hamper its administration. Formulations containing 1.0% w/w of chitosan presented an adequate solid–gel transition temperature, and it was able to withstand a low shearing force at 35 °C. It also presented higher hardness (especially at 35 °C), adhesiveness, and mucoadhesive force than did the formulation that contained 0.5% of chitosan. For those reasons, the formulation containing poloxamer/chitosan 16/1.0% w/w and the 1.0% chitosan solution were chosen to be evaluated *in vivo*.

In order to assess the FLU corneal absorption by microdialysis, FLU recovery by the probe was first evaluated. The recovery values encountered in the microdialysis experiments were in accordance with values found in the literature. Macha and Mitra [34], for example, while validating the anterior chamber microdialysis technique in rabbits using a probe with similar dimensions of ours (0.32×10.00 mm), obtained *in vitro and in vivo* recoveries of 15% and 18%, respectively, for fluorescein [34]. These percentages are even smaller than the ones that we obtained for FLU, but *in vitro* and *in vivo* recovery were very similar both in [34] and in the present work. Note that similar *in vitro* and *in vivo* recoveries can be indicative of probe implantation without lesions to adjacent tissues. Wei et al. [44], for instance, obtained for timolol *in vitro* and *in vivo* recoveries of around 58% and 17%, respectively. To explain such difference, the authors reported that the probe may have touched the iris when spanning the entire rabbit anterior chamber and elicited fibrin formation, which ultimately lowered recovery [44]. In accordance with our findings, other authors reported very similar *in vitro* and *in vivo* recoveries during anterior chamber microdialysis [35].

In vivo FLU corneal permeation from the aqueous solution showed a C_{max} after only 1 h of drug administration. In spite of this faster C_{max}, however, the amount of drug permeated from the aqueous solution, determined by the AUC in 6 h of experiment, was the smallest one (see Table 1). Moreover, after C_{max}, the FLU concentration in the aqueous humor dropped rapidly when the aqueous solution was applied, while the FLU concentration was sustained from the chitosan solution and the thermosensitive gel (see Fig. 4).

The *in vivo* sustained concentration profile showed by these both chitosan formulations might be related to: (1) a sustained drug release rate from the formulations and/or (2) a prolonged precorneal residence time of the formulations. The poloxamer/chitosan gel proved to sustain drug release as observed *in vitro* (see Fig. 2) and also to prolong retention time on an *in vivo* situation [21]. Chitosan solution sustained FLU release, even in less proportion than the binary formulation (Fig. 2), and also prolonged retention time *in vivo* when evaluated in rabbits [27].

Regarding the corneal impermeability, FLU permeation from the chitosan solution was 3.5-fold greater than that obtained with the

aqueous solution (see Fig. 3). This increase in the permeated drug amount caused by the chitosan 1% solution shows a good relationship with the *ex vivo* experiments, where a 4-fold increase was observed. On the other hand, the drug C_{max} was achieved practically at the same time from the chitosan solution and from the thermosensitive gel (Table 1), despite the different diffusion rates showed by these two formulations *in vitro* (recall that, *in vitro*, poloxamer/chitosan gel significantly sustained FLU release compared with the chitosan solution). This fact can be easily explained by the *in vivo* existence of the precorneal fluids drainage, which is obviously absent in an *in vitro* model. Though both formulations (chitosan solution and thermosensitive gel) are mucoadhesive, the chitosan solution releases FLU faster than the thermosensitive gel, allowing a greater drainage of the drug, and decreasing its ability to permeate the cornea properly. Therefore, despite the chitosan solution ability to permeabilize the corneal membrane in higher extension than the binary formulation (as shown in Fig. 2), FLU corneal permeation was similar from that of the thermosensitive gel in an *in vivo* situation. Moreover, when compared to the aqueous solution, the increase in the *in vivo* drug permeating from the thermosensitive gel (3.5-fold) was greater than that observed *ex vivo* (approximately 2-fold), emphasizing the importance of the precorneal drainage and the ability of the gel in sustaining FLU release and in remaining longer in the cornea.

It is important to note that there are remarked physiological differences between rabbit and human models, especially concerning the blinking rates that are slower in rabbits [34]. It is also important to have in mind that the corneal thickness is thinner in rabbits (0.35 mm versus 0.52 mm in humans) [45]. Therefore, besides the similar *in vivo* FLU permeation through rabbits' cornea for the two formulations tested, it is not possible to affirm that both the poloxamer/chitosan *in situ* forming gel and the chitosan solution will have the same performance when applied in humans, thus making further studies necessary.

Regarding the potential of the studied formulations for a topical treatment of fungal keratitis, although the amount of drug retained in the cornea was not evaluated, a greater permeation may also indicate a higher FLU amount in the cornea after topical administration, which may be especially advantageous for the treatment of keratitis. Furthermore, the topical application of chitosan-based formulations could be a useful tool to replace the aqueous solutions normally used and diminish the administration frequency of ophthalmic topical applied solution. The proposed formulations thereby can conquer better patient compliance in a multidose regime, which would be crucial for the therapy success. The *in situ* gel-forming system developed can be easily sterilized by autoclaving without changes on their properties, is easy to instill, and can be precisely administered, since it is a solution at room temperature and transition occurred only after contact with the ocular surface [21]. Both the gel and the chitosan solution exhibited an enhanced permeation of FLU, in comparison with aqueous solution. The easy administration and sustained drug release over a prolonged period may also lead to improved patient compliance and better clinical outcome. Further clinical studies are necessary to compare poloxamer/chitosan *in situ* forming gel with the chitosan solution and to establish the most adequate administration regime.

5. Conclusion

The present study demonstrated that the poloxamer/chitosan *in situ* forming gel retarded FLU release when compared to the chitosan solution; however, chitosan solutions alone were able to deliver greater FLU amounts through the porcine cornea *ex vivo* than the *in situ* forming gel. *In vivo*, both formulations exhibited similar performance with sustained release. Therefore, both the *in situ* gelling formulation evaluated and the chitosan solution has potential for ophthalmic use for the treatment of fungal keratitis. These formulations outstand for being simple to prepare and constituted of commercially available polymers at relative low costs.

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