Liposomal voriconazole (VOR) formulation for improved ocular delivery

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ABSTRACT

Treating infectious eye diseases topically requires a drug delivery system capable of overcoming the eye's defense mechanisms, which efficiently reduce the drug residence time right after its administration, therefore reducing absorption. In order to try to surpass such administration issues and improve life quality for patients with fungal keratitis, liposomal voriconazol (VOR) formulations were prepared. Formulations were composed of soy phosphatidylcholine (PC) containing or not 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and cholesterol. Liposomes were characterized by their drug entrapment efficiency (EE), drug recovery (DR), average diameter (size) and polydispersivity index (PdI). *In vitro* mucosal interaction and irritancy levels, *ex vivo* permeation, as well as the short-term stability were also assessed. Liposomal VOR formulation produced with 7.2:40 mM VOR:PC showed to be the most promising formulation: mean size of 116.6 ± 5.9 nm, narrow PdI (0.17 ± 0.06), negative zeta potential (~ -7 mV) and over 80% of EE and yield, remaining stable for at least 30 days in solution and 90 days after lyophilization. This formulation was classified as 'non-irritant' after HET-CAM's test and was able to deliver about 47.85 ± 5.72 μg/cm^2 of VOR into porcine cornea after 30 min of permeation test. Such drug levels are higher than the minimal inhibitory concentrations (MIC) of several fungi species isolated from clinical cases of corneal keratitis. Overall results suggest VOR can be effectively incorporated in liposomes for potential topical treatment of fungal keratitis.

1. Introduction

The treatment of ocular infections remains among the most challenging subjects of ophthalmology [1]. The eye is a small organ

protected from the external environment and separated from the body by a complex structure of biological barriers and defense mechanisms (i.e. tight junctions of the corneal epithelium, blinking, tear secretion and even tear film composition), which efficiently reduce the drug residence time right after its administration, therefore reducing absorption [2,3].

The treatment of mycotic ocular infections is specially complicated due to epidemiological characteristics of this disease combined with sociodemographic indicators, i.e. more resistant fungi tend to prevail in hot and humid environments, affecting mainly rural workers who live in poor insanitary environment [4,5]. In addition, patients with mycotic keratitis generally delay seeking medical care from onset of complaints compared to patients with nonmycotic infections [6]. Hence, current therapies are mostly invasive or require the use of high doses of antimycotics, reducing the adhesion to the treatment and, so, increasing the chances of a bad prognosis [7,8]. In this scenario, an efficient biocompatible formulation capable of delivering a potent antifungal agent is essential for treatment effectiveness.

Voriconazole (VOR) is a second-generation antifungal agent from the 'azole' family, which possesses broad-spectrum activity even against resistant fungal species [9–11].

Although several adverse effects have been reported following VOR systemic exposure [12–15], animal and clinical studies have suggested excellent outcome on treating fungal keratitis by its topical, intrastromal and/or systemic applications [16–19]. In all these studies, researchers have improvised with off-label usage of commercially available product for injection in the form of lyophilized powder of cyclodextrin-voriconazole complex. So far, no topical ocular formulation of VOR has been available in the market, probably due to its poor aqueous solubility [20].

Nanoencapsulation techniques could be applied to overcome such drug physicochemical challenges and improve ophthalmic formulation performance, prolonging drug residence time, enhancing drug penetration into the cornea and improving sensorial felling, hence, patient compliance. Recently, a microemulsion of VOR was formulated to this end and indeed exhibited about 3-fold higher drug permeation trough excised cornea in comparison to the VOR suspension [21]. Such results encourage the development of lipid nanoparticles entrapping VOR for ocular delivery. Liposomes can be fairly ideal drug delivery systems for topical treatment of ocular infections, as they are considered to be non-toxic, biodegradable, can be easily produced and lyophilized and present good interaction with mucosal structures.

The dual nature of the cornea provided by its superficial layers, the lipophilic epithelium and the hydrophilic stroma, is a limiting factor which enables only a few molecules to suit as candidates for ocular drug delivery. In this way, liposomes represent an interesting approach, since they are able to carry different drug molecules across these main layers, enhancing drug bioavailability [22,23]. Accordingly, a recent study has shown topical liposomal fluconazole (2 mg/mL) as superior to fluconazole solution in eliminating experimental *Candida albicans* infection of the rabbit cornea [24].

In this way, this article proposes a liposomal VOR formulation for the treatment of mycotic ocular infections.

2. Materials and methods

2.1. Materials

Cholesterol (Chol), soybean phosphatidylcholine (PC) and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Voriconazole (VOR) was purchased from Hangzhou Dayangchem Co., Limited (Hangzhou, China). Acetonitrile was purchased from J. T. Baker (Phillipsburg, USA). Water was purified using a Milli-Q system (Millipore, Billerica, USA) with a 0.22 μm pore end filter. All other chemicals and reagents were of analytical grade or superior.

2.2. Preparation of liposomal VOR formulation

The VOR liposomes were prepared by the thin-film hydration method. VOR and PC were dissolved in chloroform/methanol (4:1, v/v) in a round-bottom flask. The organic solvent was removed by evaporation promoting the formation of a thin lipid film on the glass wall. The dried lipid film was maintained overnight under reduced pressure to remove traces of solvent. The thin layer of lipid was then hydrated with 4 mL of HEPES buffer (pH 7.4), so that the total lipid concentration was 40 mM. The particle size of the crude liposomal dispersion was further decreased by 10 cycles of extrusion through 600-nm-pore polycarbonate filters and additional 6 cycles through 100-nm-pore filters using an extrusion device operated with compressed nitrogen (Lipex[TM], Northern Lipids Inc., Canada). Loading capacity of liposomes was evaluated by changing the drug:

phospholipid molar ratio (3.6:40; 4.3:40; 5.0:40; 5.8:40; 7.2:40; 14.3:40 mM of VOR:PC). After choosing two VOR:PC ratios, cholesterol (10 mM) was added to the formulations to evaluate its ability to enhance the formulations' stability and entrapment efficiency.

Cationic liposomes with the basic composition of VOR:DOTAP:PC at a molar ratio of 7.2:20:40 and 7.2:20:20 were also obtained.

Formulations composed of VOR/PC containing cholesterol or not were represented by the code LPC. The code LPCD was used for formulations also containing DOTAP.

Liposomes further submitted to lyophilization process were produced by the same method presented with the addition of trehalose as cryoprotectant in the ratio of 1:5 (lipid:trehalose).

2.3. Characterization of liposomes

2.3.1. Particle size and zeta potential

Particle size and polydispersity index were determined by dynamic light scattering (DLS) (3 measurements/batch; the software automatically determined the number of runs in each measurement, 25 °C) after adequate dilution of an aliquot of the suspensions in purified water (Zetasizer Nanoseries, Malvern Instruments, Worcestershire, UK). Zeta potentials were determined using the same instrument at 25 °C after the dilution of the samples in 10 mM NaCl aqueous solution to assure conductivity values of approximately 2 mS/cm (3 measurements/batch; 10 runs/measurement, 25 °C).

2.3.2. Entrapment efficiency and drug recovery

VOR entrapment efficiency (EE) was determined by indirectly calculating the amount of entrapped drug inside the liposomes. Separation of free drug (FD) in liposomal dispersion from encapsulated VOR was performed by centrifuging 1 mL of the dispersion for 10 min at 3000 rpm (centrifuge model 3–18 K SIGMA, Osterode am Harz, Germany) using Amicon Ultra filtration tube (Millipore, USA, 300 kDa cutoff). The filtrate was collected and analyzed for free drug content by high-performance liquid chromatography (HPLC) (section 2.9). Filtration parameters were validated allowing for 100% drug recovery in the filtrate. Total VOR (TD) in liposomal dispersion (free + entrapped drug) was obtained by diluting 100 μL of liposomal dispersion in 900 μL of methanol, therefore rupturing the liposomes and releasing all VOR in solution. Entrapment efficiency was calculated according to Eq. (1).

$$EE\% = \frac{(TD - FD)}{TD} \times 100 \qquad (1)$$

Drug recovery (DR) was obtained relating the total VOR in liposomal dispersion with the amount of drug added (AD) at the beginning of the process, according to Eq. (2).

$$DR\% = \frac{TD}{AD} \times 100 \qquad (2)$$

2.3.3. Morphological analysis

The morphological analysis of the VOR liposomes was performed in a TEM (JEM 1011 Transmission Electron Microscope, JEOL, Tokyo, Japan – 100 kV) and the images were captured with a GATAN BioScan camera, model 820 (GATAN, PA, USA) using the Digital Micrograph 3.6.5 software (GATAN, PA, USA). Liposomal dispersion was diluted 100-fold with purified water. An aliquot of 20 μL was deposited on a Formvar-coated copper grid (300 mesh, Electron Microscopy Sciences, PA, USA) and air-dried for 10 min. The excess of formulation was absorbed with filter paper and the sample was then stained with a drop of a uranyl acetate solution 2% (w/v). The sample was air-dried for 10 min and the excess of reagent removed with filter paper.
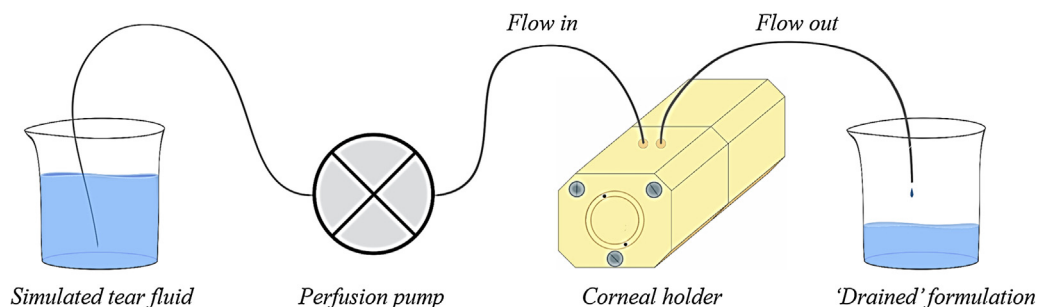
Fig. 1. Scheme from *ex vivo* permeation trough bovine cornea with simulated tear flow. The cornea is placed in the middle section, inside the corneal holder.

2.4. Stability study

The stability of four developed formulations (LPC6, lyophilized LPC6, LPC7 and LPCD1) was evaluated as a function of time on liposomal mean diameter, PdI and loss of encapsulated drug. This last parameter was demonstrated by the remaining entrapped drug (Rem %), which was calculated according to the Eq. (3), where EEi stands for the initial EE recorded for the sample and EEs for the EE calculated in the subsequent periods of the stability test. The liposomal dispersions were kept in an airtight container, protected from light and at 4 °C for 30 days. Lyophilized formulation was kept at room temperature for 90 days.

$$\text{Rem\%} = \frac{EEs}{EEi} \times 100 \tag{3}$$

2.5. Ex vivo *static permeation through porcine cornea*

Corneas used in the experiments were obtained from pig eyes collected immediately after the animal slaughter (at Frigorífico Sol Nascente, Goiânia, Brazil). The eyes had not been heat treated in the abattoir in any way. They were kept at 4 °C while transported to the laboratory and used within 1 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 [25]. Immediately after corneal preparation, the tissue was mounted in a Franz modified diffusion cell. In order to fit the concave cornea appropriately in the cell, the opening intended for the cornea placement had its edges raised by 2 mM. The area of the cornea exposed to drug diffusion was 0.785 cm². The receiving medium consisted of 15 mL of 25 mM HEPES buffer containing 133 mM NaCl, pH 7.4 at 32 °C. Donor compartment was filled with 250 μL of either formulation LPC6 or LPCD1. As VOR solubility in the medium was determined to be approximately 600 μg/mL, sink conditions were assured. The cell was placed under magnetic stirring to ensure homogeneity of the receptor solution. Samples were collected from the receptor compartment at either 30 or 60 min after the beginning of the assay, when the diffusion cell was dismantled and the corneas submitted to the extraction procedure. Extraction of VOR from the corneas was made by cutting the corneas in small pieces and adding 3 mL of methanol followed by 60 min of stirring at 600 rpm. Samples were then filtered and analyzed by HPLC. Extraction procedure was validated and drug recovery rate was 86.09 ± 6.20%.

2.6. Ex vivo *permeation trough bovine cornea with simulated tear flow*

Cattle corneas were also obtained immediately after the animal slaughter (Frigorífico Sol Nascente, Goiânia, Brazil). They were transported to the laboratory and mounted in the corneal holders following the Bovine Corneal Opacity and Permeability (BCOP) assay guidelines according to OECD TG 437 [26]. First, the posterior part of the corneal holder containing the cornea was filled with 25 mM HEPES buffer containing 133 mM NaCl, pH 7.4. Then the anterior part was filled with 5 mL of either LPC6 or LPCD1 liposomal formulation. Immediately, the perfusion pump (Manostat Carter Multi-Channel Cassette Pump, Thermo Scientific, USA) was turned on to allow simulated tear fluid to flow over the cornea surface at a rate of 550 μL/min (Fig. 1).

After 30 min, all liposomal formulation still remaining in the anterior chamber was washed off with HEPES buffer and corneal opacity was measured using an opacitometer. Opacity values were compared to basal values obtained from control corneas that remained for 30 min in contact with just the buffer. After opacity measurements, the corneas were submitted to the same extraction procedure described above. VOR extraction procedure from cattle corneas was also validated and drug recovery rate was 84.79 ± 1.34%. Samples collected from the posterior compartment at the end of the experiment were also analyzed by HPLC according to Section 2.9.

2.7. In vitro *mucoadhesion*

In vitro mucoadhesion was performed as previously described [27]. Commercially available porcine mucin was hydrated in demineralized water at 4 °C overnight. Then the mucin solution was adjusted to pH 7.4 with 1 M NaOH and diluted with 0.1 M phosphate buffer (pH 7.4) to a final concentration of 1% (m/v). The solution was ultrasonicated until mucin particles size was smaller than 1 μm. Then it was centrifuged at 4000 rpm for 20 min and the supernatant portion was filtered through 0.45 μm of cellulose acetate membrane filter. The filtrate was collected and the particle size of mucin was measured. The filtered liquid with mucin particle size lower than 500 nm was utilized. Equal volumes of each liposomal dispersion and mucin particles suspension were mixed by vortexing for 1 min. The mean particle sizes of the mixture were determined by DLS.

2.8. HET-CAM irritation test

Irritancy levels of best liposomal formulations were assessed using a slightly modified version of the hen's egg test-chorioallantoic membrane (HET-CAM) test previously reported [28]. Fertilized hen's eggs were purchased from a poultry farm and used on the 10th day of fertilization. The eggs were placed in a stand with the equatorial side up where a 2 × 2 cm window was opened to expose the CAM. 300 μL of the testing sample were placed directly onto the CAM's surface. After 20 s, the sample was discarded and the CAM was carefully washed with HEPES buffer (pH 7.4) to ensure the total removal of the tested substance. The CAM was visually observed for five minutes (on the times 0.5, 2 and 5 min) regarding the appearing of any of the following phenomena: hyperemia, hemorrhage and coagulation for which a score was given. HEPES buffer

Table 1
Size, polydispersivity index (PdI), zeta potential (Zeta), entrapment efficiency (EE) and drug recovery (DR) of VOR liposomes composed of PC (40 mM).

Sample code	VOR (mM)	Chol (mM)	Size (nm)	PdI	Zeta (mV)	EE (%)	DR (%)
LPC1	3.6	–	114.4 ± 3.5	0.08 ± 0.01	−1.16 ± 3.64	87.3 ± 0.5	97.6 ± 4.1
LPC2	4.3	–	116.6 ± 4.7	0.12 ± 0.05	−4.35 ± 3.17	83.1 ± 0.2	99.2 ± 1.4
LPC3	5.0	–	110.7 ± 2.2	0.05 ± 0.01	−2.20 ± 3.51	91.1 ± 0.8	103.3 ± 8.7
LPC4	5.8	–	109.1 ± 7.8	0.05 ± 0.04	−5.15 ± 2.30	92.3 ± 0.3	97.8 ± 3.7
LPC5	5.8	10	111.4 ± 3.2	0.06 ± 0.01	−8.03 ± 2.30	74.3 ± 0.6	86.6 ± 1.6
LPC6	7.2	–	116.6 ± 5.9	0.17 ± 0.06	−7.35 ± 4.12	86.8 ± 0.1	105.6 ± 0.1
LPC7	7.2	10	110.9 ± 1.4	0.01 ± 0.01	−9.32 ± 2.33	76.7 ± 0.2	92.9 ± 0.2
LPC8	14.3	–	104.8 ± 5.5	0.02 ± 0.05	−3.44 ± 2.22	87.3 ± 0.5	43.8 ± 1.0

All data shown is expressed in means ± standard deviation (SD) with $n = 4$.

at pH 7.4 and a 10% sodium dodecyl sulfate (SDS) were used as the negative and positive controls, respectively.

2.9. Drug assay

VOR was assayed by a spectrophotometric (HPLC) method. For this, a C8 reversed-phase column (Agilent ZORBAX Eclipse XDB-C8 – 5 μm, 250 × 4.6 mM) was used, with water:acetonitrile (50:50 v/v) as the mobile phase at a flow rate of 1.0 mL/min, detection at 255 nm, and an injection volume of 20 μL. The method was validated presenting a linear calibration curve ($r = 0.999$; $y = 18.044x + 12.562$) over a drug concentration range of 0.4–400.0 μg/mL. Intra- and inter-day precision and accuracy of the method showed a coefficient of variation (% CV) and a relative error (% E) not greater than 5% and 3%, respectively. The limit of detection (LOD) and limit of quantitation (LOQ) was found as 0.1 and 0.4 μg/mL, respectively. The selectivity was investigated (formulation components and cornea homogenate) and no interference was observed in VOR retention time.

2.10. Statistical data analysis

Statistical analysis was carried out by two-way analysis of variance (ANOVA) followed by Tukey's or Student–Newman–Keuls' posttests, when appropriate. Unpaired *t*-test was used for individual comparisons. *p*-values ≤ 0.05 were considered significant. Results are expressed as mean ± SD (standard deviation) when appropriate. Analyses were performed by using GraphPad Prism Software 5.03 (GraphPad Inc., USA).

3. Results and discussion

3.1. Obtention and characterization of VOR loaded liposomes

Both LPC and LPCD formulations evaluated by DLS demonstrated a unimodal distribution and nanometric particle sizes, regardless of the drug:lipid ratio. All formulations presented particle sizes around 100 nm and low polydispersity index (below 0.22), demonstrating their particle size homogeneity (Tables 1 and 2).

The slightly negative zeta potential values of LPCs (around −5 mV) are characteristic of soy phosphatidylcholine [29]. Such slightly negative zeta potential values help stabilizing nanoparticles sterically at the particle/water interface. As expected, LPCDs

presented positive zeta potentials values (approximately +40 mV) as DOTAP is positively charged at pH 7.4 [30,31]. Besides sterical stabilization, residual positive charges are expected to confer mucoadhesivity properties to the liposomal particles. As such systems are intended for topical ocular drug delivery, it is extremely desirable that they reach intimate contact with the mucous layer covering the corneal tissue, which could prolong residence time and increase bioavailability.

None of the VOR concentrations tested have affected on the physicochemical parameters of the formulations ($p > 0.05$). However, when 14.3 mM VOR was used (drug:lipid ratio 14.3:40) less than 50% of the drug was recovered. This low DR probably reflects liposomal system saturation, which might have led to drug retention in the filtering membrane during extrusion process, or it is also possible that part of the drug may remain adhered along with the lipid film in the round-bottom flask wall after the solvent evaporation and film hydration process and it is likely to believe that a higher drug:lipid ratio could result in a higher drug loss due to these reasons. In this way, 7.2 mM VOR were used in subsequent studies.

Cholesterol is often included in liposomal formulations to give further rigidity to the bilayer that may improve formulation stability and *in vivo* performance [32]. Conversely, in this study cholesterol addition significantly reduced EE of LPC5 and LPC7 in comparison to LPC4 and LPC6, respectively ($p < 0.05$). Since VOR has intermediary lipophilicity (log P 1.8), it is possible that drug molecules are in a dynamic equilibrium between internal aqueous core, lipid bilayers and external aqueous environment. Therefore, part of drug molecules could be competing with cholesterol for incorporation in the lipid lamella. The increase in mechanical rigidity of the bilayers, due to cholesterol addition, also decreases their lateral diffusion, which could affect drug aqueous/lipid environment equilibrium, reducing EE. However, when DOTAP was present, such effect was not relevant ($p > 0.05$). Lipid acyl chain ordering and mobility can have important effects on the accommodation of drug molecules. Previous studies of membrane phase domain behavior have demonstrated that DOTAP is miscible with the phosphatidylcholine phase, and when added in such formulations it exerts a "fluidizing" effect or "disordering of the membrane," as would be expected from inclusion of the unsaturated alkyl chains of DOTAP [31]. Among other more complex mechanisms, such fluidizing effect could increase drug incorporation and mask the drug/cholesterol competition.

Table 2
Size, polydispersivity index (PdI), zeta potential (Zeta), entrapment efficiency (EE) and drug recovery (DR) of 7.2 mM of VOR cationic liposomes prepared with different ratios of PC and DOTAP (cationic lipid) adding or not 10 mM of cholesterol.

Sample code	DOTAP: PC (mM)	Chol (mM)	Size (nm)	Zeta (mV)	PdI	EE (%)	DR (%)
LPCD1	20:40	–	96.5 ± 2.2	+42.4 ± 22.7	0.12 ± 0.06	85.1 ± 1.5	108.4 ± 4.0
LPCD2	20:40	10	96.4 ± 1.1	+30.3 ± 15.97	0.11 ± 0.02	85.9 ± 0.5	121.1 ± 5.4
LPCD3	20:20	–	99.8 ± 1.6	+47.3 ± 25.5	0.10 ± 0.05	77.9 ± 0.4	105.5 ± 1.1
LPCD4	20:20	10	114.3 ± 4.2	+32.3 ± 17.13	0.22 ± 0.08	81.0 ± 2.5	111.6 ± 1.5

All data shown is expressed in means ± standard deviation (SD) with $n = 4$.
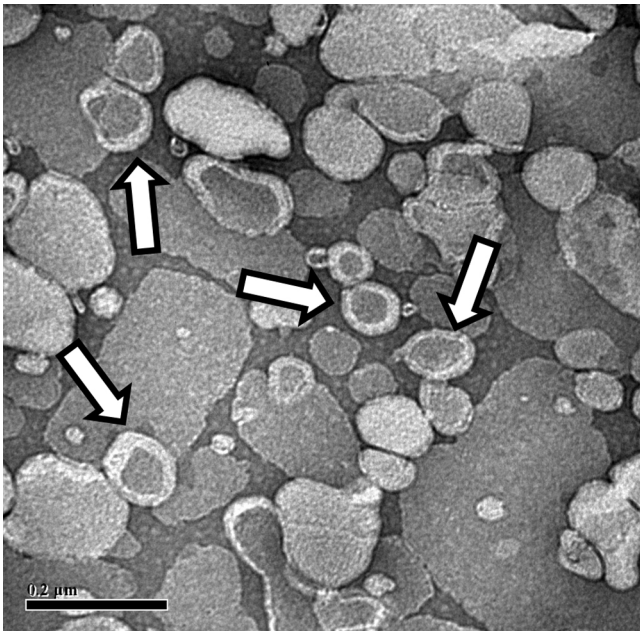
Fig. 2. TEM micrograph of LPC6 liposome dispersion (average vesicle size of 100 nm) stained with uranyl acetate (2%), where it is possible to visualize the lipid bilayer around the liposome core in a lighter color.

It has also been described that incorporation of DOTAP into bilayers composed of saturated chain phosphatidylcholines decreases the diameter of spontaneously forming liposomes and reduces the dispersity of the size distribution. Accordingly, formulations LPCD1, LPCD2 and LPCD3 presented smaller particles sizes than LPCs formulations [33]. However, such effect was not present when molar ratio of PC and cholesterol exceeded DOTAP molar ratio, evidenced by the increased mean particle size of LPCD 4 in comparison to LPCD 3 ($p < 0.05$).

3.1.1. Morphological analysis

Morphological analysis was carried in order to recognize both macro and microscopic characteristics of the liposomal systems.

As seen in Fig. 2, liposomes are presented as unilamellar vesicles, with a non-stained border that represents the lipid bilayer of the liposome. The image shows vesicles with an average size (diameter) of 100 nm, which confirms the data obtained from DLS.

3.2. Stability study

Liposome diameter and PdI of formulations LPC6, LPC7 and LPCD1 remained constant ($p > 0.05$) during the 30 days of stability evaluation. In addition, entrapment efficiency did not change significantly ($p > 0.05$), indicating that the interaction between liposomal membrane and VOR is very stable and no drug loss was observed for up to one month during these storage conditions. The stability study revealed cholesterol addition did not influence in any of the evaluated parameters throughout 30 days (Fig. 3).

The lyophilized VOR-loaded liposomes (lyophilized LPC6) were stable for up to 90 days, kept at room temperature and safe from light exposure. Hence, the cryoprotectant used was capable to protect the liposomes from the stressful process of freeze-drying without interfering significantly in the vesicles' properties, as expected [34].

3.3. Ex vivo *static permeation through porcine cornea*

In *ex vivo* permeation experiments, drug concentration of both donor formulations (LPC6 and LPCD1) were ∼2.5 mg/mL (7.2 mM), considering that DR was close to 100%. From this dosage, ∼86% were entrapped within the liposome and the rest soluble in the HEPES buffer, therefore ready for diffusion. Still, after 30 min experiments VOR could not be quantified in the receptor medium (concentrations were under LOQ). After 60 min, a total of $13.78 \pm 3.04 \, \mu g/cm^2$ of VOR permeated the cornea and was found in the receptor solution from the LPC6 formulation, while no VOR was quantified in the receptor medium when formulation LPCD1 was applied (concentrations were under LOQ).

After 30 min experiment, 47.85 ± 5.72 and $45.31 \pm 2.02 \, \mu g/cm^2$ of VOR were retained in the cornea from LPC6 and LPCD1 systems, respectively. After 60 min experiment retained amounts were 54.93 ± 3.64 and $62.14 \pm 7.84 \, \mu g/cm^2$, respectively (Fig. 4).

To our knowledge, this is the first description of liposomal VOR formulation in the literature. The simplest formulation tested, composed solely by the phospholipid and the drug (LPC6), showed to
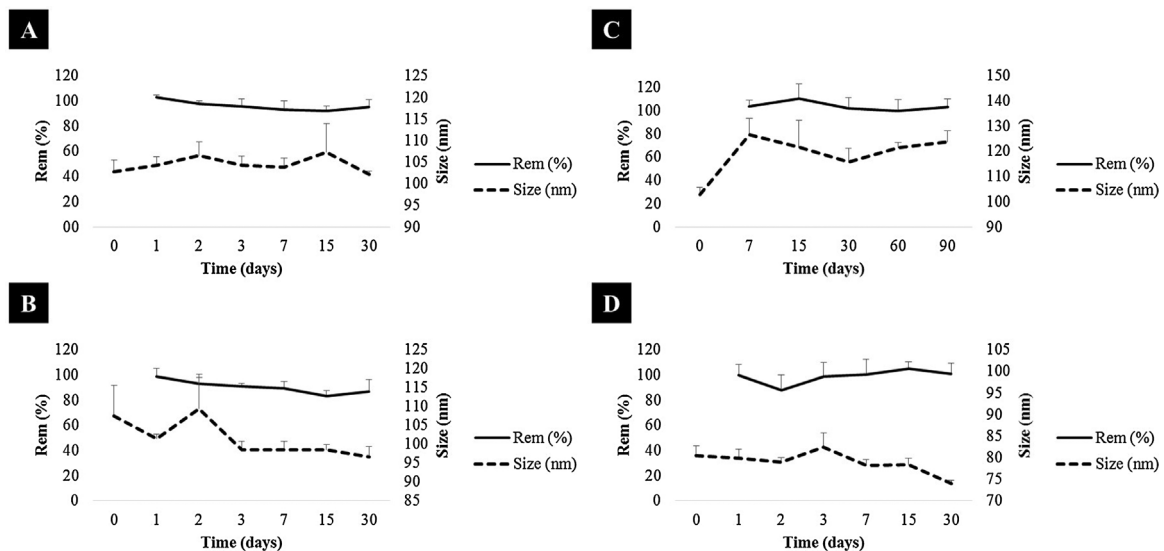

Fig. 3. Size and remaining entrapped drug (Rem) of VOR-loaded liposomes throughout the stability study. Formulations A, B, C and D correspond to formulations LPC6, LPC7, LPC6 lyophilized and LPCD1, respectively. Formulations A, B and D were kept for 30 days under refrigeration (4 °C) and the lyophilized one (C) was kept at room temperature, safe from light exposure for 90 days (mean ± SD, $n = 4$).
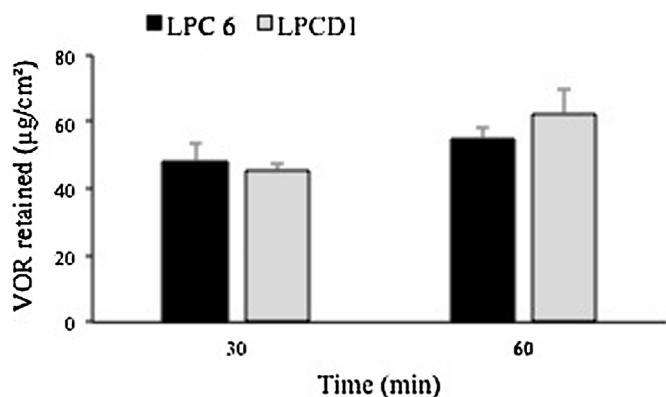
Fig. 4. Amount of VOR retained within the porcine cornea (mean ± SD, $n = 5$).

be more advantageous in terms of enhanced delivery than a previously reported microemulsion [21]. First, liposomal formulation incorporated more drug than the microemulsion (total dose of 2.5 and 1.0 mg/mL, respectively). Second, VOR amounts retained in the cornea after one hour of liposomal contact were superior to VOR amounts retained after 12 h of microemulsion permeation (60 and ~40 μg/cm^2, respectively). Consequently, liposomal formulation was also superior to VOR suspension, which, in the reported study, accounted for less than 5 μg/cm^2 retained [21].

This permeation study through porcine cornea was programed to be a 1-h study, since in normal conditions the physiological mechanisms of the eye tend to limit its exposure time to topical administrated drugs. It has indeed been shown approximately 90% of the drug administered on the eye is lost in the first 30 s after the instillation [3]. Hence, penetrated drug amounts in the cornea after static exposure are normally overestimated. In this way, *ex vivo* permeation experiments with simulated tear flow were performed on an attempt to predict formulation performance under precorneal tear drainage conditions, which physiologically washes the drug out of the eye's surface.

3.4. Ex vivo *permeation trough bovine cornea with simulated tear flow*

At physiological conditions, the pre-ocular tear film is subjected to a continuous cycle of production, evaporation, absorption and drainage, which leads to a dynamic equilibrium in the pre-ocular tear film. However, a process of reflex flow is initiated in stress conditions as in response to challenges like instillation of medicines, stimulation of the free nerve endings in the densely innervated cornea and to some degree from stimulation of the conjunctiva, lasting until equilibrium conditions are reestablished [35,36]. Such tear turnover reflex is one of the mechanisms responsible for the low residence time of ophthalmic formulations. Tear flow in healthy subjects in basal conditions have been reported to be 1.1 μL/min [37]. Considering a basal tear volume of 9.7 μL this would represent a tear turnover of approximately 11%/min. It is clear that clearance of a drug formulation cannot be simply extrapolated from the basal tear turnover, since, besides reflex flow, which would decay to basal flow with time, other variables such as formulation dilution by basal tear volume and reflex blinking, need to be considered. Still, an *ex vivo* permeation experiment that takes into account at least the expected basal clearance can provide much information about expected *in vivo* formulation performance and bioavailability, which is remarkably important for an antifungal formulation with already know minimal inhibitory concentrations for most pathogenic species. Hence, permeation experiments were conducted perfusing the donor compartment with simulated tear fluid at a flow rate equivalent to 11%/min turnover.

Cattle corneas are normally used in BCOP measurements because of the larger exposition area. As cattle corneal holders were available in the laboratory, they could be easily adapted for permeation experiments with simulated tear flow.

At the end of experiment there was no significant change in corneal opacity values ($p > 0.05$). VOR was not detected in the posterior chamber of the corneal holders. The amount of VOR retained within the cornea was 21.65 ± 2.01 and 26.48 ± 8.03 μg/cm^2 when LPC6 and LPCD1 formulations were applied to the anterior chamber, respectively ($p > 0.05$).

Cationic liposomes are expected to interact with mucous layer covering the cornea, which would result in better retention in comparison to neutral or negatively charged liposomes. Such interaction is most likely due to electrostatic reasons, since mucin has a negative residual charge, opposing to the positive ones from the cationic liposomes [38,39]. However, it is not possible to draw such conclusions from these *ex vivo* studies, since the mucus layer is very sensitive and is probably lost during operation and cornea storage.

Even though retained values are about half of those obtained from static permeation experiments, drug delivered from both formulations are still higher than the minimal inhibitory concentrations (MIC) for several fungi species isolated from clinical cases of corneal keratitis [10,11], e.g. MIC for *Fusarium* spp. rages from 8 to 12 μg/mL [9]. This information is of great importance for further studies regarding these formulations, since based on the knowledge of the amount of drug that penetrates the cornea we will be able to establish more accurately the dosage concentrations for treatment of fungal keratitis.

3.5. In vitro *mucoadhesion*

Mucin-particle method was used to evaluate the mucoadhesive properties of the VOR-loaded liposomes by measuring the particle size changes upon mucin particles contact using DLS. LPC6 and LPCD1 average sizes before the addition of mucin were 116.6 ± 5.9 nm (PdI 0.17 ± 0.06) and 96.5 ± 2.2 nm (PdI 0.12 ± 0.06), respectively. Mucin was used at an average particle size of 275.1 ± 83.6 nm, with a PdI of 0.51 ± 0.09. There was no interaction evidence between mucin and LPC6 liposomes, particle sizes measured were 131.0 ± 17.5 nm (PdI 0.20 ± 0.07), reflecting presence of both liposomal and mucin particles in suspension. Instead, cationic liposome formulation's (LPCD1) average size after mucin addition was increased to 2441.3 ± 164.5 nm with a PdI of 0.96 ± 0.06. As expected, cationic liposomes adhered to mucin's particles turning into aggregates with irregular sizes (since PdI was almost equals 1).

Even though permeation results of negatively and positively charged liposomes were not statistically different, such mucoadhesion could mean a better performance of the cationic formulation in physiological conditions since it indicates that the formulation should have a good affinity with the mucosal layer of eye and, therefore, stay longer in the area, enabling a more efficient release of the drug in the cornea [38–40]. To confirm such *in vivo* different performances it is absolutely necessary that both formulations confirm to be biocompatible and non-irritant.

3.6. HET-CAM irritation test

The HET-CAM test is an alternative to Draize eye test, used in the evaluation of the irritancy potential of a given substance [26,41]. The HET-CAM irritation test demonstrated that the cationic liposome formulation could be slightly more irritant than the LPC6 formulation, as a stronger hyperemia can be visualized in the egg's CAM (Fig. 5).

After 5 min, the final score for the test samples was 0, 0, 3 and 11 for negative control, LPC6, LPCD1 and positive control, respectively. According to the score table previously reported [28],
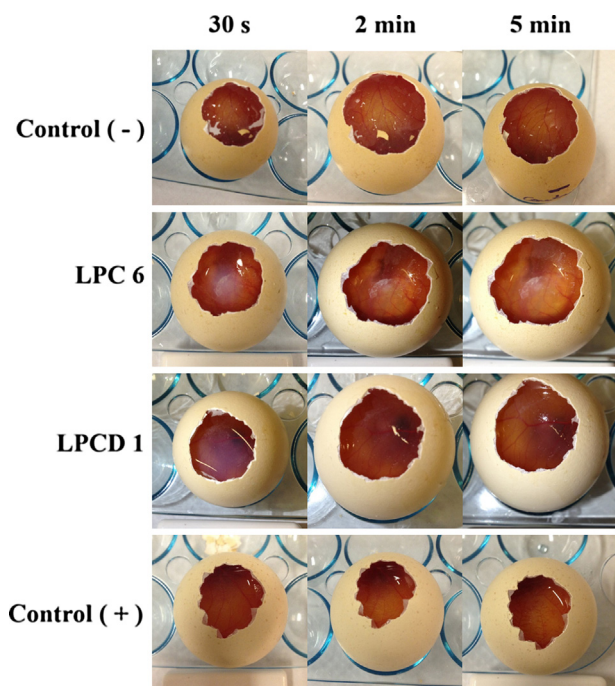
Fig. 5. Irritancy evaluation of the chorioallantoic membrane of hen's eggs after contact with the tested samples. Negative control corresponds to a HEPES buffer (pH 7.4) and positive control consists of a sodium dodecyl sulfate (SDS) 10% solution.

controls responded correctly to the test and LPC6 formulation could be classified as 'non-irritant', while LPCD1 formulations should be classified as 'weak-irritants'. This data complies with the literature that presents liposomes as non-toxic delivery systems [39]. Moreover, in toxicity studies VOR was found non-irritant in dark conditions [41].

More evidence demonstrating the performance of both VOR liposomal formulation under clinical conditions and determining the therapeutic regime require further investigation.

So far, LPC6 formulation seems to be the most promising one for the treatment of fungal keratitis, as it demonstrated to be stable, easily lyophilized and non-irritant. Another advantage of LPC6 formulation is that because of its simple preparation and inexpensive components, the price of the final product may be reduced. Previous reports have suggested VOR could be more cost saving than liposomal Amphotericin B as first line therapy of invasive aspergillosis [42], indicating the drug itself as a viable option. Considering previously cited sociodemographics of fungal keratitis, therapy cost may not be ignored. Development of effective inexpensive formulations would be crucial for therapy success.

4. Conclusion

VOR liposomes composed of solely soy phosphatidylcholine were simply prepared, stable, and capable of delivering a fair amount of drug into the cornea in a short period of time. According to HET-CAM's irritability study, the developed formulation should not bring discomfort to the patient. In summary, this study shows for the first time the feasibility of an ophthalmic formulation based on liposomal VOR for the topical treatment of fungal keratitis.

Acknowledgements

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