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Self-assembled liquid crystalline nanoparticles as a novel ophthalmic delivery system for dexamethasone: Improving preocular retention and ocular bioavailability

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ABSTRACT

The object of this study was to design novel self-assembled liquid crystalline nanoparticles (cubosomes) as an ophthalmic delivery system for dexamethasone (DEX) to improve its preocular retention and ocular bioavailability. DEX cubosome particles were produced by fragmenting a cubic crystalline phase of monoolein and water in the presence of stabilizer Poloxamer 407. Small angle X-ray diffraction (SAXR) profiles revealed its internal structure as *Pn*3*m* space group, indicating the diamond cubic phase. In vitro, the apparent permeability coefficient of DEX administered in cubosomes exhibited a 4.5-fold (F1) and 3.5-fold (F2) increase compared to that of Dex-Na phosphate eye drops. Preocular retention studies revealed that the retention of cubosomes was significantly longer than that of solution and carbopol gel, with $AUC_{0\rightarrow 180\,min}$ of Rh B cubosomes being 2–3-fold higher than that of the other two formulations. In vivo pharmacokinetics in aqueous humor was evaluated by microdialysis, which indicated a 1.8-fold (F1) increase in $AUC_{0\rightarrow 240\,min}$ of DEX administered in cubosomes relative to that of Dex-Na phosphate eye drops, with about an 8-fold increase compared to that of DEX suspension. Corneal cross-sections after incubation with DEX cubosomes demonstrated an unaffected corneal structure and tissue integrity, which indicated the good biocompatibility of DEX cubosomes. In conclusion, self-assembled liquid crystalline nanoparticles might represent a promising vehicle for effective ocular drug delivery.

1. Introduction

Ocular diseases are usually treated with topical application of drug solutions (eye drops). However, the rapid and extensive precorneal losses caused by drainage and high tear fluid turnover limit drug ocular bioavailability. Meanwhile, for drugs entering the ocular tissue, the cornea is the major route of anterior drug absorption. The lipophilicity and tight conjunction of the corneal epithelium make it the major limiting barrier in corneal drug absorption; consequently, lipophilic (log *D* 2–3) drugs have a higher permeability than hydrophilic ionized drugs (Mannermaa et al., 2006).

To improve ocular bioavailability, several ophthalmic drug delivery systems have been proposed, such as emulsions (Yamaguchi et al., 2005), nanoparticles (Zimmer and Kreuter, 1995) and liposomes (Meisner and Mezei, 1995). These systems might be able to enhance drug bioavailability by facilitating transcorneal/transconjunctival penetration (Tamilvanan and Benita, 2004). Nevertheless, their potential in ocular drug delivery is limited by rapid clearance from the precorneal region, as the same rapid drainage has been observed as for aqueous eye drops. In order to enhance adherence to the corneal/conjunctival surface, dispersion of these vesicular systems into mucoadhesive gels has been proposed (Aggarwal and Kaur, 2005; Gan et al., 2009). However, the high gel viscosity might adversely accelerate the blinking frequency, leading to a feeling of discomfort.

Monoolein (MO), as a nontoxic, biodegradable and biocompatible material classified as GRAS (generally recognized as safe), show the mesomorphic phase, important in making more comprehensible the potential pharmaceutical application of the lipid. It may exist in several different phases depending on temperature and hydration. The phase sequence at room temperature when adding water is as follows: lamellar crystalline phase (Lc) in coexistence with a L2 phase, lamellar liquid crystalline phase (Lα phase) and the inverted bicontinuous cubic phase (C). What is perhaps the most intriguing is the ability of cubic phases to exist in equilibrium with excess water and can be dispersed to form cubosomes.

Liquid crystalline phases of MO, such as cubic phases, present interesting properties for a topical delivery system (Carr et al., 1997; Lee and Kellaway, 2000a,b), as they (i) are bioadhesive, (ii) present a permeation enhancer as the structure forming lipid (MO), and

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(iii) afford the ability to incorporate compounds independently of their solubility, protecting them from physical and enzymatic degradation, and to sustain their delivery (Shah et al., 2001). The emulsification of cubic lipid phases in water results in the production of cubosomes, which can be defined as nanoparticulate dispersion systems. It has been demonstrated that the dispersed particles retain the internal structure of the bulk phase and its properties (Nakano et al., 2002; Siekmann et al., 2002; Boyd, 2003). In comparison with the bulk gel, the dispersions present some advantages, such as larger surface area and high fluidity (low viscosity), and can be incorporated into other product formulations (Lopes et al., 2006).

Despite the amazing properties of cubosomes as innovative drug carriers, little research has thus far been performed to demonstrate their potential as ophthalmic drug delivery systems (Lee et al., 2004; Leesajakul et al., 2004; Esposito et al., 2005). The aim of this work was to study the performance of cubosomes as innovative ocular delivery systems for dexamethasone, chosen as a model drug.

DEX is a lipophilic glucocorticoid steroid, which is similar to the natural steroid hormone made by the adrenal glands in the body. It is known to be an effective anti-inflammatory drug for the treatment of acute and chronic posterior segment eye diseases such as uvetis (Phillips and Katz, 2005). In the generally used clinical product, Dex-Na phosphate eye drops, DEX exists in a hydrophilic ionized form which cannot effectively penetrate the lipophilic corneal epithelium. Therefore, it needs to be instilled 3–4 times per day. It is reported that continuous application of eye drops of 0.1% dexamethasone for extended periods of time could cause glaucoma accompanied by optic nerve damage, defects in visual acuity and fields of vision, and posterior subcapsular cataract formation and thinning of the cornea or sclera (Kim and Chauhan, 2008).

In this study, DEX-containing monoolein cubosomes were prepared, and their internal structure was further characterized by SAXR and cryo-TEM. An in vitro penetration study was performed using freshly excised rabbit cornea. Ocular tolerance was evaluated by corneal cross-sections after incubation. A noninvasive fluorescence imaging system was utilized to assess the preocular retention of the cubosomes. Finally, in vivo aqueous humor pharmacokinetics was investigated using the microdialysis method.

2. Materials and methods

2.1. Materials

Dexamethasone was purchased from Zhejiang Xianju Pharmaceutical Co. Ltd. (Zhejiang, China). Monoolein (MO, RYLOTM MG19) was kindly gifted by Danisco Ingredients (Brabrand, Denmark). Poloxamer 407 (Lutrol® F 127) was obtained from BASF (Ludwigshafen, Germany) and CMC-Na (600–1000 mPa s) from Shanhe Medicinal Excipients (Anhui, China). Carbopol 974 was kindly donated by Lubrizol Specialty Chemicals Manufacturing Co. Ltd. (Shanghai, China). Ethyl Rhodamine B was bought from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Bidistilled water was made using Milli-Q (Gradient). All other reagents were of analytical grade. The microdialysis probe (CMA/20, 10 mm) used for aqueous humor sampling was purchased from CMA/AB Microdialysis (Stockholm, Sweden).

Male New Zealand albino rabbits weighing 2–3 kg were provided by the Animal Experimental Center of Shanghai Institute of Materia Medica. The animals were housed in standard cages in a light-controlled room at 19 ± 1 °C and 50 ± 5% RH and were fed a standard pellet diet and water ad libitum. All studies were approved by the Department of Laboratory Animal Research at Shanghai Institute of Materia Medica. Procedures involving animals were reviewed and approved by the Animal Ethics Committee at Shanghai Institute of Materia Medica.

2.2. Preparation of DEX cubosomes, micelles and suspension

Production of the dispersion was based on the emulsification of MO and Poloxamer 407 in water as described by Cortesi et al. (Esposito et al., 2005; Kuntsche et al., 2008). In all experiments, the MO/Poloxamer ratio was 9:1 (w/w). MO and Poloxamer were firstly heated to 70 °C, and then DEX (0.05%) was added to the molten MO/Poloxamer solution and solubilized before adding to the aqueous phase. Afterwards, the oil phase was dropped into the water phase and the components were emulsified using a high-shear dispersing emulsifier (T25 basic, IKA Guangzhou, China) at 10,000 rpm for 5 min. After equilibration for 12 h at room temperature, the crude emulsion was homogenized 7 times (Panda2000, GEA Niro Soavi S.P.A., Italy) at 350 bar. Finally, glycerol was used to adjust the osmotic pressure to physiological conditions.

DEX micelles (0.05%) were prepared by dissolving 10 mg DEX in 20 ml Poloxamer 407 solution (5%, w/v), with glycerol being used to adjust the osmotic pressure.

To prepare a suspension, 10 mg DEX were manually milled with a pestle in 20 ml 0.1% (w/v) CMC-Na solution for 30 min until uniform, and then the osmotic pressure was adjusted with glycerol.

2.3. Characterization of DEX cubosomes

2.3.1. Particle size analysis

The particle size (PS) and polydispersity index (PI) of the cubosomes were analyzed at 25 °C using a dynamic light-scattering particle size analyzer (Particle Sizing System, Nicomp388/ZetaPALS, Santa Barbara, USA) after 100-fold dilution with bidistilled water.

2.3.2. Viscosity

The viscosity of the prepared formulations was determined at different angular velocities at 25 °C using a rotary viscometer (DV-III, Brookfield, USA). The rotation speed was 20 rpm, with spin 18 #. The average of two readings was used to calculate the viscosity.

2.3.3. Drug encapsulation efficiency

The drug encapsulation efficiency was determined by ultrafiltration. A 500-μl aliquot of DEX cubosomes was transferred to the upper chamber of a centrifuge tube fitted with an ultrafilter (Vivaspin500, Sartorius, MWCO 10 kDa), which was then centrifuged at 4000 rpm for 30 min. The amount of DEX loaded in the cubosomes was calculated as the difference between the total amount used in preparation of the cubosomes and the amount in the filtrate, as determined by HPLC. The drug encapsulation efficiency was calculated according to:

$$Q_w = \frac{W_{total} - W_{free}}{W_{total}} \times 100\% \approx \frac{C_{total} - C_{free}}{C_{total}} \times 100\%$$

where Q_w is the drug encapsulation efficiency; W_{total} is the total amount of drug in the cubosomes; W_{free} is the amount of drug in the filtrate; C_{total} is the concentration of drug in the cubosomes; C_{free} is the concentration of drug in the filtrate (Gan et al., 2009).

2.3.4. Small angle X-ray scattering (SAXS) measurement

The SAXS measurements were carried out using NanoStar (Bruker AXS GmbH, Germany), which consisted of a 'Hi-Star' 2D-detector, 3 collimator SAXS system, 3 kW high voltage generator, cross-coupled Goebel mirrors, copper X-ray radiation at 5000 W (λ = 0.154 nm) and 1 mbar vacuum chamber. The scattering angle (2θ) ranged from 0.2° to 2.8°. The sample was transferred to a 0.5-mm diameter quartz capillary and sealed. The measurement was

carried out in a vacuum at 25 °C, with exposure time of 0.5 h and sample-to-detector distance of 10.7 cm.

2.3.5. Cryo-transmission electron microscopy (cryo-TEM)

For cryo-TEM, 4 μl of sample were applied to a holey carbon film grid (R1.2/1.3 Quantifoil Micro Tools GmbH, Jena, Germany), and were absorbed by filter paper (#1, Whatman) for about 3 s. After absorption, the grid was immediately plunged into pre-cooled liquid ethane to fast freeze it. The cryo-grid was held in a Gatan 626 Cryo-Holder (Gatan, USA) and transferred into a JEOL JEM-2010 (200kv LaB6 filament) TEM at −172 °C. The sample was searched and observed under minimal dose conditions at −172 °C. The micrographs were recorded by a Gatan 832 CCD camera at a magnification of 10,000–50,000× and at a defocus of 1.80–4.46 μm.

2.4. In vitro corneal penetration study

In vitro corneal penetration studies were carried out using a modified Franz diffusion cell with a diffusion area of 0.785 cm^2. Rabbits were killed by injection of an overdose of air into the marginal ear vein. The corneas were excised from the globes and mounted onto the ring of the perfusion apparatus. The corneas were gently rinsed with saline, and extreme care was taken not to produce any wrinkles or folding of the membrane before mounting. Glutathione bicarbonate ringer (GBR) buffer (2 ml) preadjusted to a temperature of 37 °C was placed into the receptor chambers of the apparatus with magnetic stirring throughout the entire experiment. DEX formulation (100 μl) was placed in the donor chamber, which was then sealed to avoid evaporation. A mixture of O_2:CO_2 (95:5) was bubbled through the chambers.

Samples (200 μl) were taken at 30, 60, 90, 120, 150, 180, 210, 240, 270 and 300 min after beginning the experiment and replaced with fresh GBR buffer. The amount of drug that permeated across the cornea was assayed by HPLC.

The HPLC system (Agilent 1100 series) used comprised an autosampler (G1313A ALS), a pump (G1311A Quatpump), a column oven (G1316A Column), a UV detector (G1314A VWD) and data processing software (HP Chemstation Rev.A.10.01). A C_{18} column (Zorbax® SB, 150 mm × 4.6 mm, 5 μm) was used for DEX analysis with acetonitrile–water (40:60) as the mobile phase at a flow rate of 1.0 ml/min at 25 °C. Detection was performed at 240 nm.

The hydration level of the cornea, which indicates the corneal condition, was measured according to a method previously reported (Suhonen et al., 1991).

The amounts of drug that permeated the corneal epithelium were plotted versus time for each formula and the slope of the linear portion of the graph was calculated. The apparent corneal permeability coefficient (cm/s) was determined according to Muchtar et al. (1997):

$$P_{app} = \frac{\Delta Q}{\Delta t \cdot C_0 \cdot A \cdot 60}$$

where $\Delta Q/\Delta t$ is the linear portion of the slope (μg min^{-1}), 60 is the conversion of minutes to seconds, A is the corneal surface area (in this study, 0.785 cm^2), and C_0 is the initial drug concentration (μg cm^{-3}).

2.5. Ocular tolerance evaluation

To examine the effects on corneal structure and integrity, the corneas were removed from fresh rabbit eyes and incubated at 37 °C for 2 h in the DEX cubosome formulations. PBS and a sodium dodecylsulfate (SDS) solution in PBS 0.1% (w/w) were taken as references.

After incubation, the corneas were washed with PBS, and immediately fixed with a formalin solution 8% (w/w). The material was dehydrated with an alcohol gradient, put in melted paraffin and solidified in block form. Cross-sections (<1 μm) were cut, stained with haematoxyline and eosine (H and E) and microscopically observed for any pathological modifications (Baydoun et al., 2004).

2.6. Preocular retention of cubosomes

Preocular retention of the DEX cubosomes was evaluated using a noninvasive fluorescence imaging system (Fx Pro, Kodak In-Vivo Imaging System, USA). Albino rabbits (n = 9) were used in the study and each rabbit was restrained and positioned in front of the camera.

The cubosome formulation was labeled by adding a specified amount of ethyl rhodamine B (Rh B) in the oil phase and then processed via the same method as for preparation of DEX cubosomes. Rh B carbopol gel was prepared by firstly dissolving Rh B in water, and then carbopol 974 (0.25%, w/v) was added to the solution and stirred overnight. Finally, pH (6–7) and osmotic pressure (0.27–0.33 osmol/kg) were adjusted. The viscosity of Rh B carbopol gel was 88.6 ± 0.59 mPa s (DV-III, Brookfield, USA). Rh B directly dissolved in PBS as Rh B solution was taken as reference.

Exactly 5 μl of fluorescence-labeled test formulation (final Rh B concentration was 0.2 μmol/ml) were instilled directly into the lower fornix of the conjunctival sac of the left eye and the eye was manually closed for 10 s to distribute the formulation over the cornea. Rabbits were under conscious condition. Just before the time of imaging, they were anesthetized with i.v. injection of pentobarbital (30 mg kg^{-1}) through ear vein. Imaging was carried out at 0, 5, 10, 30, 60, 90, 120, 150 and 180 min after administration. Regions of interest (ROI) were created around the resulting images of the ocular and non-ocular areas to estimate residual fluorescence activity using a data acquisition, processing and quantification program (Kodak MI SE 4.5). The amount (%) of activity remaining in the ROI versus time profile was evaluated with regard to different formulations. Remaining intensity (R) was calculated according to the equation: $R = (A - B)/C \times 100\%$. Where A was the intensity of ROI, B was the background fluorescence intensity and C was the intensity of ROI at 0 min.

2.7. Aqueous humor pharmacokinetics

Aqueous humor sampling to assess the ocular absorption of DEX was carried out using microdialysis.

The rabbits were kept under anesthesia throughout the experiment using sodium pentobarbital (30 mg/kg) injected into the marginal ear vein. Pupils were dilated by topical instillation of 1% tropicamide prior to probe implantation. The microdialysis probe (CMA/20) was implanted in the anterior chamber using a 22G needle. It was inserted carefully across the cornea, preventing any damage to the iris–ciliary body. The needle was then removed and the probe was placed immediately and adjusted such that the membrane resided in the anterior chamber. The probe was perfused with PBS buffer with a flow rate of 2 μl/min using a microinjection pump (MD-1001, BAS, USA). After probe implantation, the animals were allowed to stabilize for at least 2 h before administering any agent. This time period has been shown to be sufficient for the restoration of intraocular pressure and replenishment of the aqueous humor lost during probe implantation (Anand et al., 2006).

2.7.1. Probe recovery

Probe recovery was evaluated by the retrodialysis method. A series of DEX solutions of known concentrations (20, 85, 150, 650 and 1500 ng/ml) were used as the perfusates with a flow rate of 2.0 μl/min. All dialysates were collected for 15 min at 0.5 h after changing the perfusate.

Table 1
Physico-chemical properties of DEX cubosomes ($n = 3$).

Formulations		Mean diameter (nm)	Polydispersity index	Viscosity (mPa s)	Encapsulation efficiency (%)
F1	10% oil	214.1 ± 41.1	0.144 ± 0.021	0.96 ± 0.22	98.8 ± 2.6
F2	20% oil	226.3 ± 55.6	0.176 ± 0.014	10.0 ± 1.53	98.5 ± 5.4

Recovery (R) is the ratio between drug concentration in the dialysate (C_d) and in the tissue (C_m), which is calculated according to the following equation (Burngay et al., 1990):

$$R = \frac{C_d - C_p}{C_m - C_p} \quad (1)$$

where C_p is the drug concentration in the perfusate. R is the value of the slope for the plot of $C_d - C_p$ versus C_p.

2.7.2. Aqueous humor sample

50 μl of each formulation was instilled into the eye. Dialysates were collected every 10 min for the first hour, every 20 min during the second hour and every 30 min during the third and fourth hours after instillation. All dialysate samples were kept frozen −20 °C until analyzed.

2.8. Statistical analysis

Statistical analysis of the results was performed using one-way analysis of variances (ANOVA), referring to a level of $p < 0.05$. This statistical analysis was computed using Origin® software.

3. Results

3.1. Characterization of DEX cubosomes

3.1.1. Particle size, viscosity and encapsulation efficiency

As can be seen in Table 1, the mean diameter of F1 was 214.1 ± 41.1 nm (PI 0.144 ± 0.021) and of F2 was 226.3 ± 55.6 nm (PI 0.176 ± 0.014). There was no significant effect of oil content on the particle size and polydispersity index of the DEX cubosomes. The viscosity of DEX cubosomes F2 was about 10 mPa s, 10-fold higher than that of DEX cubosomes F1, which might be due to the higher oil content. As for the DEX micelles and suspension, the viscosities were 1.03 ± 0.15 and 1.58 ± 0.26 mPa s, respectively. Because of its lipophilicity, the encapsulation efficiency of DEX in the cubosomes was above 98%, which meant most of the drug was encapsulated in the cubic nanoparticles.

3.1.2. Small angle X-ray diffraction

SAXR was performed to determine the structural organization of the cubosome formulations. Several Bragg peaks can be seen in Fig. 1. The spacing ratio of reflections was $\sqrt{2} : \sqrt{3} : \sqrt{4} : \sqrt{6} : \sqrt{8} : \sqrt{9}$, in accordance with the *Pn*3*m* space group, indicating a diamond cubic phase (Siekmann et al., 2002).

Fig. 2 shows the diffraction profiles of blank cubosomes (A) and DEX cubosomes F1 (B). Noticeable is the fact that, in the absence and in the presence of DEX, the samples exhibit similar cubic unit cell dimensions, which indicates that the addition of DEX does not substantially affect the cubic structure.

3.1.3. Cryo-transmission electron microscopy (cryo-TEM)

Cryo-TEM observations were in full agreement with the *SAXR* qualitative results, which revealed mostly square cubosomes with a clear inner periodicity. The diameters observed in the cryo-TEM images were about 200 nm, which was also consistent with those determined by dynamic light-scattering particle size analyzer (Fig. 3).

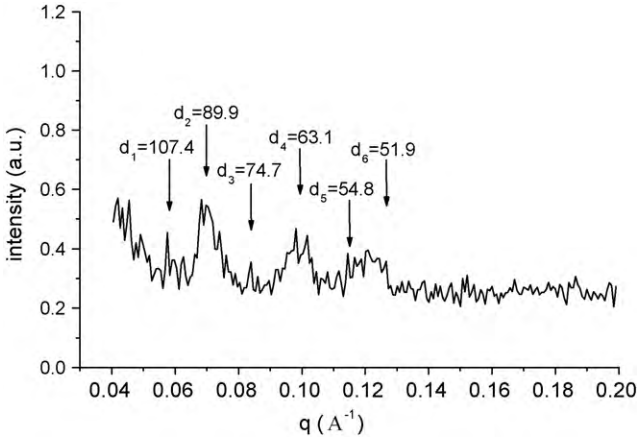

Fig. 1. Small angle X-ray diffraction profiles of ophthalmic DEX cubosomes F1.

3.2. In vitro corneal penetration

Fig. 4 illustrates the in vitro corneal penetration experimental results of the three different dosage forms, DEX cubosomes, DEX micelles and Dex-Na phosphate eye drops. Following a lag time, a linear relationship between accumulative permeated DEX and time could be seen. The apparent permeability coefficients (P_{app}) of DEX in cubosomes F1, cubosomes F2, DEX micelles and Dex-Na phosphate eye drops were 2.16×10^{-6}, 1.67×10^{-6}, 1.27×10^{-6} and 0.48×10^{-6} cm/s, respectively. Compared with Dex-Na phosphate eye drops, DEX formulated in cubosomes exhibited 4.5-fold (F1) and 3.5-fold (F2) increase in P_{app}, which indicated the enhanced penetration achieved with cubosomes. P_{app} of DEX in cubosomes F1 was also 1.7-fold higher than that in DEX micelles.

Careful handling of the isolated cornea and maintenance of its physical activity throughout the experiment is very important for reproducibility of the results. The corneal hydration level is a parameter frequently used to evaluate damage of this tissue. Generally, the normal cornea has a hydration level of 76–80%, while an 83–92% hydration level denotes damage of the epithelium and/or endothelium (Saettone et al., 1996). In this study, the corneal

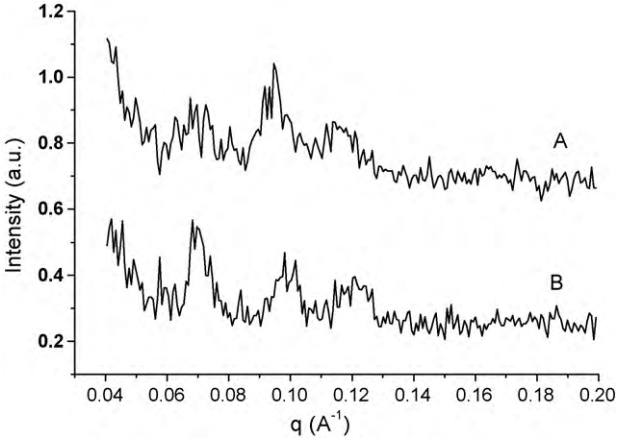

Fig. 2. SAXS profiles from (A) the blank cubosomes and (B) the DEX cubosomes F1.

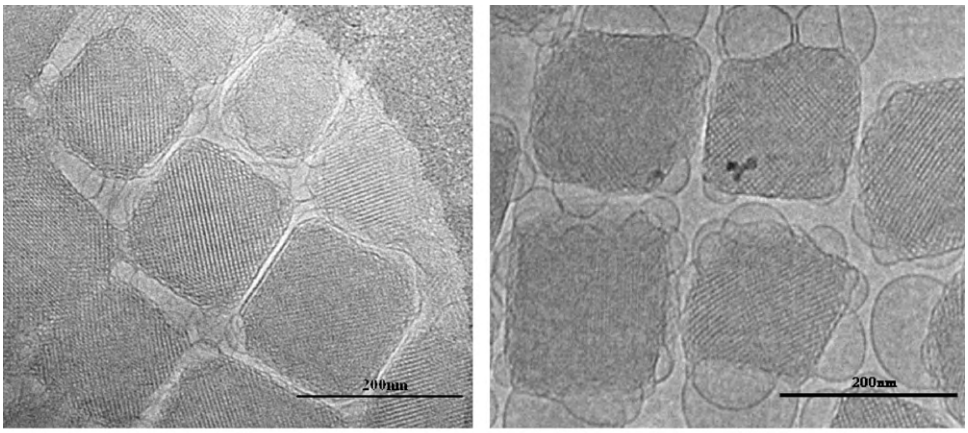

Fig. 3. Photographs of DEX ophthalmic cubosomes by cryo-TEM: (1) F1, (2) F2.

hydration level of all corneas ranged between 79.01 ± 0.01% (DEX cubosome F2) and 82.76 ± 1.09% (Dex-Na phosphate eye drops), and did not exceed 83.0%, which indicated the integrity of the corneas throughout the experiments.

3.3. Ocular tolerance evaluation

Fig. 5 presents corneal cross-sections after incubation of freshly excised rabbit corneas with various preparations to investigate their influence on corneal cell structure and tissue integrity. After incubation in a PBS solution (Fig. 5A), the epithelium (EP) and stroma (ST) structure is maintained. A typical stratified epithelial layer can be recognized by the appearance of a bulge at the nuclei of the basal columnar cells and the squamous surface cells. When the corneal epithelium is exposed to SDS (Fig. 5B), previously narrow intercellular spaces are clearly widened, cells and nuclei are deformed and superficial epithelial cells are detached from tissue assembly. Treatments of corneas with DEX cubosome formulations are exemplified in Fig. 5C and D, showing a cornea cross-section after incubation in F1 and F2, which leaves the corneal structure and integrity almost visibly unaffected. The above results indicated the good corneal biocompatibility of DEX cubosome formulations.

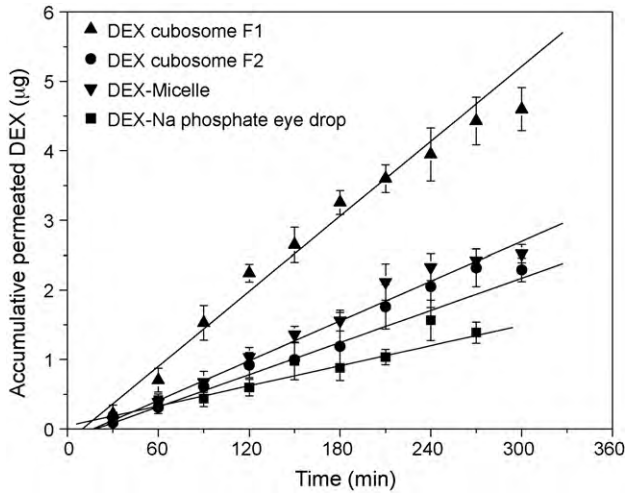

Fig. 4. In vitro transcorneal permeation profiles of DEX in various dosage forms ($n = 3$).

3.4. Preocular retention of cubosomes

After administration, rapid clearance could be seen in the Rh B solution group (Fig. 6A). Up to 90 min, almost no fluorescence intensity resided in the ROI, with only a little intensity remaining in the nasolacrimal duct. As for the Rh B carbopol gel, the images were very similar to that of Rh B solution, therefore it is not illustrated in Fig. 6. When instilled with Rh B cubosomes, a stable liquid film was formed at the cornea/conjuctiva surface (Fig. 6B), which indicated the good wettability and spreadability of the cubosomes. At 90 min, a relatively strong intensity still could be seen in the ROI.

Fig. 7 illustrates the amount (%) of activity remaining in the ROI versus time profiles for Rh B solution, Rh B carbopol gel and cubosomes F1. For all formulations, the profiles consisted of a rapid initial clearance phase followed by a slower basal drainage phase.

A summary of statistical analysis is shown in Table 2. The $AUC_{0\rightarrow 180\,min}$ of cubosome formulation was significantly higher than that of solution and carbopol gel. In fact, there was about a 3.5-fold increase in $AUC_{0\rightarrow 180\,min}$ of Rh B cubosomes F1 compared to that of Rh B solution, with a 2.5-fold increase compared to that of Rh B carbopol gel. The clearance of Rh B cubosomes in the initial phase was much slower, as the clearance rate (k) was significantly lower than that of the other two formulations. Activity remaining in the ROI at 90 min after administration was defined as A_{90}. A_{90} of Rh B cubosomes was about 40%, which was significantly higher than that of Rh B solution/Rh B carbopol gel. Moreover, no significant differences in precorneal clearance parameters between the Rh B carbopol gel formulation and Rh B solution was found in Table 2. It was therefore not evaluated as reference dosage forms in the in vivo aqueous humor pharmacokinetics study (Table 3).

3.5. Aqueous humor pharmacokinetics

In vivo probe recoveries were usually determined before the experiments to ensure the proper functioning of the probe, the value of which ranged from 10% to 40% as reported (Rittenhouse et al., 1998; Katragadda et al., 2008). In this study, it was approx-

Table 2
Precorneal clearance parameters ($n = 3$).

Sample	$AUC_{0\rightarrow 180\,min}$ (% min)	k (min^{-1})	A_{90} (%)
Rh B solution	2196.7 ± 920.1	0.031 ± 0.008	10.4 ± 5.7
Rh B carbopol gel	3104.3 ± 1267.9	0.026 ± 0.015	14.2 ± 8.8
Rh B cubosome F1	7715.8 ± 1050.9[a,b]	0.013 ± 0.002[a,b]	37.8 ± 2.8[a,b]

[a] $p < 0.05$, statistically significant difference from Rh B solution.
[b] $p < 0.05$, statistically significant difference from Rh B carbopol gel.

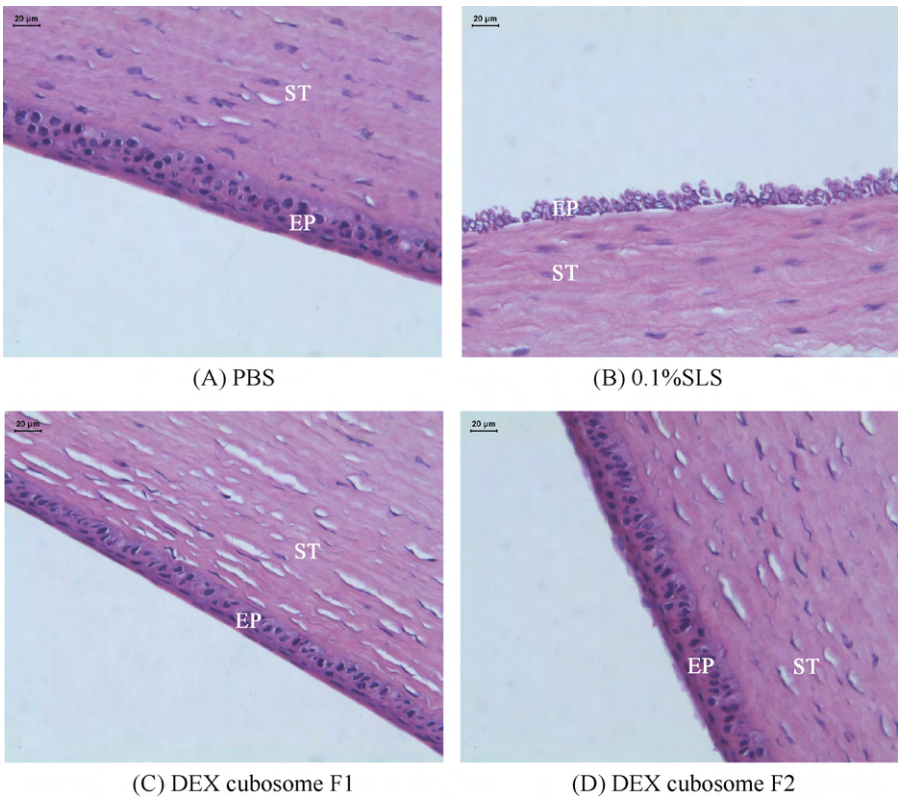

Fig. 5. Histological cross-sections of excised rabbit cornea showing epithelium (EP) and stroma (ST), stained with hematoxylin-eosin (scale bar 20 μm) after incubation at 37 °C.

imately 34.75%, and it remained constant throughout the whole experiment.

Concentration–time profiles of DEX in rabbit aqueous humor are shown in Fig. 8, while the pharmacokinetic parameters have been summarized in Table 2. The area under the curve ($AUC_{0\rightarrow240\,min}$) values of DEX administered in DEX cubosomes F1, micelles, Dex-Na phosphate eye drops and DEX suspension were 24023.5 ± 9899.7, 5362.6 ± 1887.6, 13505.8 ± 3234.3 and 3005.1 ± 1559.6 ng ml^{-1} min, respectively. The $AUC_{0\rightarrow240\,min}$ of DEX administered in cubosomes exhibited a 1.8-fold increase compared to that of the Dex-Na phosphate eye drops, with about an 8-fold increase compared to that of the DEX suspension. Cmax for DEX in cubosomes also exhibited a remarkable increment relative to Dex-Na phosphate eye drops and DEX suspension ($p < 0.05$). However, no significant differences could be seen in Tmax. MRT of DEX administered in cubosomes also increased significantly, compared with DEX suspension.

4. Discussion

SAXR and cryo-TEM are usually used to characterize the internal structure of dispersed cubic particles (Gustafsson et al., 1997). The cryo-TEM images in this study showed a typical ordered cubic texture and inner periodicity which was further confirmed by SAXR. Introduction of guest molecules generally influences the self-assembly structure properties. The more hydrophilic ones would induce a transition to the lamellar phase, while the more lipophilic ones would induce a transition to the hexagonal phase (Sagalowicz et al., 2006). The similarity of SAXR profiles between blank cubosomes and DEX cubosomes F1 indicated that the addition of 0.05% DEX did not change the internal structure of the dispersed cubic particles.

Reports on the nanodispersion of MO and oleic acid as a topical delivery system have shown that it was non-irritant to the skin of hairless mice (Lopes et al., 2006). Therefore, it might be considered feasible to utilize nanodispersions of MO as an ocular drug delivery system. Histologically, cross-sections of rabbit corneas after incubation with cubosome formulations showed that the corneal structure and integrity were almost visibly unaffected. It was therefore concluded that MO cubosomes will not result in histological impairment as vehicles for ocular drug delivery.

Preocular retention of cubosomes was evaluated using a noninvasive fluorescence imaging system. Identifying ROI and defining them as ocular and non-ocular allowed the quantification of the remaining activity in these regions at different time points. The non-ocular ROI represent the inner canthus and nasolacrimal duct

Table 3
Pharmacokinetic parameters of DEX in rabbit aqueous humor after instillation of various dosage forms ($n = 3$).

Samples	$AUC_{0\rightarrow240\,min}$ (ng ml^{-1} min)	Cmax (ng ml^{-1})	Tmax (min)	*MRT* (min)
Dex cubosome F1	24023.5 ± 9899.7[a,b]	336.8 ± 187.6[a,b]	23.3 ± 5.8	74.8 ± 20.8[b]
Dex micelle	5362.6 ± 1887.6	392.1 ± 89.8	13.3 ± 5.7	34.9 ± 10.9
Dex-Na phosphate eye drops	13505.8 ± 3234.3	233.1 ± 56.6	26.7 ± 15.3	61.7 ± 15.6
Dex suspension	3005.1 ± 1559.6	136.7 ± 75.6	11.5 ± 9.3	28.5 ± 19.2

[a] $p < 0.05$ statistically significant difference from Dex-Na phosphate eye drops.
[b] $p < 0.05$ statistically significant difference from Dex suspension.

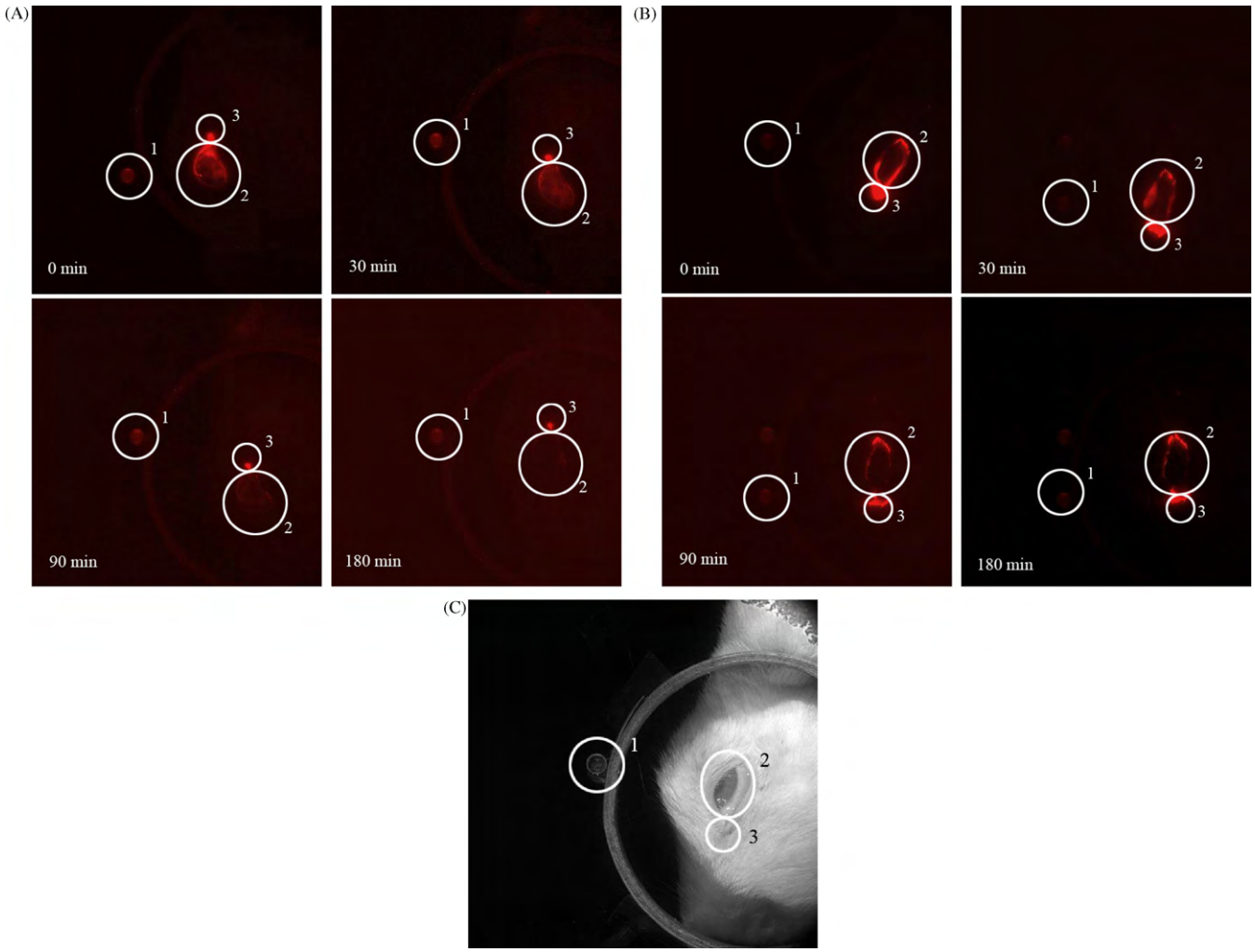

Fig. 6. Fluorescence images of rabbit eyes after administration of (A) Rhodamin B solution, (B) Rhodamin B cubosomes F1 (10% oil content); (C) white light image of rabbit eye. 1: intensity reference standard, 2: ocular ROI, 3: non-ocular ROI (inner canthus and nasolacrimal duct region).

and were therefore not considered relevant to precorneal residence. As illustrated in Fig. 6, only two ROI were created, ocular and non-ocular, with an intensity reference standard used to estimate the residual fluorescence activity. The fluorescence reference in Fig. 6 was used for better identifying the position of rabbit eyes. About 100 μl Rh B solution (10^{-4} μmol/ml) was put in a small glass tube to be used as reference. Therefore, the fluorescence intensity seems far lower than that of the ocular ROI and non-ocular ROI.

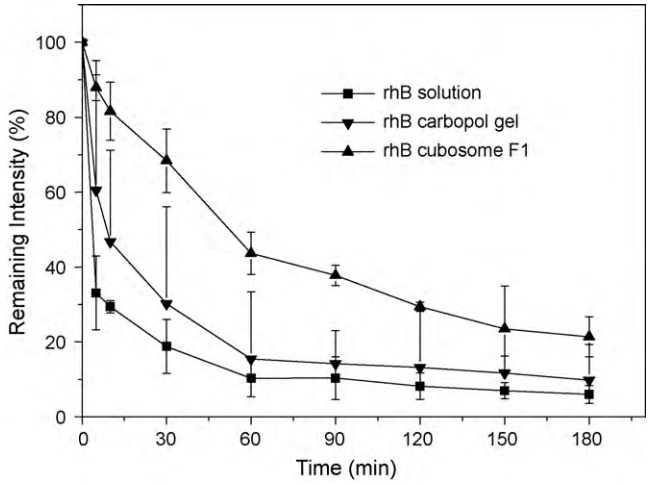

Fig. 7. Precorneal clearance of various formulations ($n = 3$), Rh B cubosomes F1 (10% oil content).

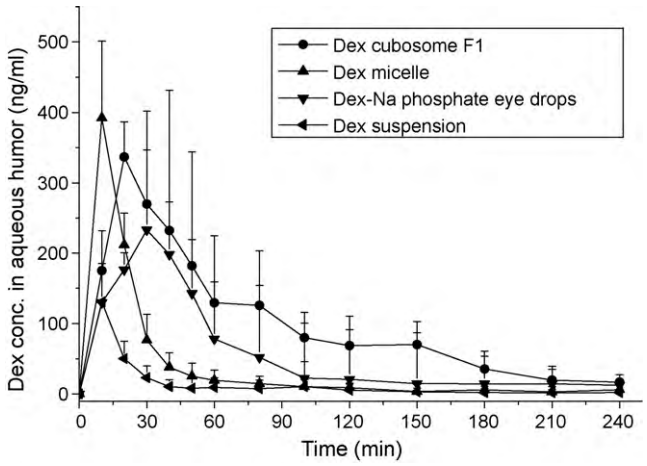

Fig. 8. Concentration–time profiles of DEX in rabbit aqueous humor after instillation of various dosage forms ($n = 3$).

It was found that the cubosomes formulation exhibited a slow clearance and significantly prolonged residence of fluorescence in the ocular ROI (A_{90} was about 40%) compared to solution. The lipid bilayer microstructure may serve to prolong retention. A possible explanation for this could be the non-specific interactions (hydrophobic and van der Waals) of the cubosomes with the superficial oily layer of the tear film (Alany et al., 2006). Moreover, compared with carbopol gel, significant differences were observed in the clearance rate (k) and residence of fluorescence in the ocular ROI at 90 min (A_{90}). As the viscosity of carbopol gel was about 90 mPa s, the relatively high viscosity might adversely accelerate the blinking frequency leading to rapid clearance. It was therefore considered that cubosomes could be of value as vehicles for ocular applications as they appeared to have prolonged preocular residence with low viscosity.

The cornea consists of three primary layers: the cellular epithelium and endothelium are lipophilic, while the gel-like stroma is hydrophilic. The epithelium and endothelium contain 100-fold greater amounts of lipid material per unit weight than the stroma (Suhonen et al., 1998). For most topically applied drugs, passive diffusion along their concentration gradient, either transcellularly or paracellularly, is the main permeation mechanism across the cornea. Physico-chemical drug properties, such as lipophilicity, solubility, molecular size and shape, charge and degree of ionization, affect the route and rate of permeation of the cornea (Jarvinen et al., 1995). It has been shown that the optimal lipophilicity for corneal permeation corresponds to log D values of 2–3 (Mannermaa et al., 2006).

In the in vitro cornea penetration study, P_{app} of DEX formulated in cubosomes exhibited a 4.5-fold (F1) and a 3.5-fold (F2) increase relative to Dex-Na phosphate eye drops. The phosphate derivative of DEX exists in an ionized form, which is too hydrophilic to penetrate through the lipophilic corneal epithelium. Therefore, one of the possible mechanisms for cubosomes in enhancing corneal permeation is to deliver the drug in unionized form. The unionized species usually penetrates the lipid membranes more easily than the ionized form (Suhonen et al., 1998). Compared with DEX micelles, P_{app} of DEX in cubosomes F1 also showed a 1.7-fold increase. Some researchers found the periodically curved lipid bilayer of cubosomes was very similar to the microstructure of the cell membrane (Larsson, 1989; Giorgione et al., 1998). It is reasonable to suppose the formation of a mix of cubosome MO with corneal epithelium lipid, where the cubosomes might act as a depot from which DEX can be continuously released. This might be another possible mechanism for the penetration enhancement effect of cubosomes.

Results of an in vivo aqueous humor pharmacokinetic study also indicated the 1.8-fold and 8-fold increase in $AUC_{0\rightarrow 240\,min}$ of DEX administered in cubosomes compared with Dex-Na phosphate eye drops and DEX suspension, respectively.

Ocular drug absorption from the lacrimal fluid to the anterior ocular tissues via transcorneal absorption is determined by two major factors: ocular contact time of the delivery system and drug permeability in the cornea (Mannermaa et al., 2006). On one hand, an in vitro preocular retention study indicated the prolonged residence of cubosomes in the preocular region, which would improve one of the above-mentioned major factors, ocular contact time. On the other hand, the in vitro cornea penetration study showed that P_{app} of DEX formulated in cubosomes exhibited a 4.5-fold (F1) increase relative to Dex-Na phosphate eye drops. Therefore, as has been discussed above, the other major factor affecting drug permeability in the cornea might also have been improved. Consequently, as illustrated in the in vivo aqueous humor pharmacokinetic study, DEX formulated in the self-assembled liquid crystalline nanoparticles (cubosomes) might exhibit increased ocular bioavailability by improving both of the two important factors.

5. Conclusion

In this study, self-assembled liquid crystalline nanoparticles, named cubosomes, were investigated as an ocular drug delivery system. The apparent permeability coefficient of DEX formulated in cubosomes was significantly enhanced. In addition, the cubosomes (10% oil) with low viscosity were retained in the preocular region much longer. Consequently, the ocular bioavailability of DEX has been greatly improved. On the other hand, MO/Poloxamer cubosomes might have good ocular biocompatibility as they appeared to exert no deleterious influence on corneal structure and integrity in the in vitro ocular tolerance test. In conclusion, cubosomes might represent a promising vehicle for effective ocular drug delivery.

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