A novel cationic nanostructured lipid carrier for improvement of ocular bioavailability: design, optimization, *in vitro* and *in vivo* evaluation

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ABSTRACT

The objective of this study was to prepare, optimize and evaluate a novel cationic nanostructured lipid carrier (CNLC) system, which can enhance the ophthalmic bioavailability of curcumin (CUR). CUR-loaded CNLC (CUR-CNLC) was prepared using a film-ultrasonic technique. A three-level Box-Behnken design was applied for optimization. The physicochemical properties, *in vitro* release and corneal permeation of the delivery method were evaluated. Preocular retention capacity study was conducted using a non-invasive fluorescence imaging system. Finally, a pharmacokinetic study in the aqueous humor was performed using a microdialysis technique. The optimal formulation had a mean particle size of 158.1 nm with a homogeneous distribution, a positive surface charge of 36.5 mV, a high entrapment efficiency of 99.12% and drug loading of 1.677%. Transmission electron microscope (TEM) morphology depicted a spherical morphology, and differential scanning calorimetry (DSC) indicated an imperfect crystalline lattice for the formulation. The CUR-CNLC formulation exhibited a sustained drug release and a 1.25-fold increase in *in vitro* corneal permeation ($P<0.05$). The results from *in vivo* studies indicated the formulation had a high tolerance and a prolonged drug retention capacity in ocular tissues. A pharmacokinetic study of rabbit aqueous humors demonstrated a 2.36-fold increase in ocular bioavailability compared to a CUR solution (CUR-SOL, $P<0.01$). The amphipathic octadecyl-quaternized carboxymethyl chitosan (QACMC) was used as a novel cationic material to prolong the residence time and improve the ocular bioavailability. Therefore, CNLC system is a promising approach for ocular delivery of CUR.

Keywords

cationic nanostructured lipid carriers; microdialysis; pharmacokinetics; amphipathic octadecyl-quaternized carboxymethyl chitosan; ocular delivery; curcumin

1. Introduction

The topical instillation of an active compound is the first-choice delivery method for ocular therapy. However, the physiological constraints imposed by the protective mechanisms of the eye lead to low absorption of drugs and result in a short duration of therapeutic effect [1]. When drug solution is dropped into the eye, the effective tear drainage and blinking action cause a 10-fold reduction in the concentration of the drug within 4-20 min [2,3]. Most of the administered dose passes through the naso-lacrimal duct into the gastrointestinal tract, which may cause side effects [4]. In addition, the limited permeability of the cornea also contributes to the poor absorption of ocular drugs [1].

Nanostructured lipid carrier (NLC) systems are second generation lipid-based nanoparticles obtained by substituting part of the solid lipids used in solid lipid nanoparticles (SLN) formulations with liquid lipids. Compared with other traditional carriers, NLC have many excellent characteristics, including high bioavailability, high active ingredient payload, biodegradability, good tolerance, prevention or minimization of active ingredient expulsion, avoidance of organic solvent use during preparation, improvement of physicochemical stability, and suitability for large-scale production using existing technologies and sterilization [5]. A cationic nanostructured lipid carrier (CNLC) system combines the positive surface charge of the particles and the negative surface charge of the ocular mucosa by means of electrostatic attraction [6]. Cationic materials are able to improve the bio-adhesion of NLC and prolong the retention time of drugs in the eyes. The biodegradability of the cationic materials is a basic requirement to avoid their accumulation in the ocular tissues because various clinical applications using drug delivery to the eye

require chronic administration. Chitosan, known as a biodegradable and biocompatible polymer, is suitable for delivery via mucosal routes. Early studies by Baldrick [7] and Fulgêncio [8] presented chitosan as a promising cationic biomaterial with excellent biocompatibility for both ophthalmic and intravenous administration. In addition, a number of studies both *in vitro* and *in vivo* showed great biocompatibility and tolerance when using chitosan-based nanoparticles [9-11].

Curcumin (CUR) is isolated from the rhizomes of *Curcuma* [12]. A growing body of experimental evidence suggests the various beneficial pharmacological effects of CUR, including its anti-oxidant, anti-inflammatory, anti-carcinogenic, anti-bacterial, and anti-coagulant features [13]. CUR capsules are useful for diabetic patients, aiding them in obtaining better control over blood glucose and decreasing the incidence of eye disease [14]. CUR has been proven to be effective for ophthalmic use in various ocular fundus pathologies. It inhibits the proliferation of human lens epithelial cells and protects retinal cells, retinal ganglion cells, and corneal epithelial cells [15,16]. However, owing to drawbacks such as low solubility and bioavailability, the clinical application of CUR is limited.

The response surface methodology (RSM) is useful in simultaneously analyzing process variables when variable interactions are very complicated. Box-Behnken design is one of the techniques in RSM. It is a well-established approach for pharmaceutical formulation development and optimization, allowing the extraction of maximal information out of a few well-designed experiments [17, 18]. This design is a convenient approach for experimental optimization, which has a large number of variables. It is more efficient and economical than other designs [19].

Therefore, it is necessary to develop a novel ophthalmic drug delivery system not only to increase the aqueous solubility of CUR but also to overcome the shortcomings of conventional dosage forms. The objective of the present study was to prepare, optimize and

evaluate a novel CNLC system to enhance the ophthalmic bioavailability of CUR. A CNLC formulation was developed using the film-ultrasonic method. A well-established three-level Box-Behnken design was used to optimize the CUR-CNLC formulation. The CUR-CNLC formulation was further characterized for particle size, zeta potential, morphology and crystallinity. *In vitro* drug release testing was conducted, and corneal permeation was performed using excised rabbit cornea. Additionally, *in vivo* tolerability and retention capability were assessed with ocular irritation and preocular retention time studies. Finally, ocular bioavailability was evaluated using the microdialysis technique. To our knowledge, this is the first integrated report on the enhancement of the ocular bioavailability of CUR following the topical administration of a CNLC delivery system.

2. Materials and methods

2.1. Materials

CUR was purchased from Zelang Medical Technology Co., Ltd. (98%, Nanjing, Jiangsu, China). Glycerin monostearate (GMS), Tween-80, stearic acid and tristearin were provided by the Tianjin Chemical Reagent Company (Tianjin, China). Compritol® 888 ATO, Labrasol®, Gelucire® 44/14, Transcutol® P and Transcutol® HP were gifts from Gattefosse S.A. (Saint-Priest, France). Miglyol® 812, Miglyol® 840, Myri 52 and Super Refined® Oleic Acid were generously provided by Fengli Jingqiu Commerce and Trade Co., Ltd. (Beijing, China). Hydroxypropyl chitosan, carboxymethyl chitosan, and amphipathic octadecyl-quaternized carboxymethyl chitosan (QACMC) were supplied by Nantong Lushen Biological Engineering Co., Ltd. (Nantong, Jiangsu, China). All other reagents were of analytical grade.

2.2. Production of CUR-CNLC

In the present work, two chemically different lipids (GMS and Miglyol® 812) were selected from a list of solid and liquid lipids. The selection rationale was to choose lipids that could better dissolve CUR and produce CUR-CNLC. Myri 52 was chosen as a surfactant. QACMC was used as a cationic material. CUR-CNLC was prepared using a film-ultrasonic technique. Briefly, ethanol was added to the lipid phase containing 2 mg CUR, 25.45 mg GMS and 14.55 mg Miglyol® 812. Then, the lipid phase was heated to 80 °C to melt the lipids. Subsequently, a rotary evaporator (RE-52AA, Ya Rong Biochemical Instrument Co., Ltd., Shanghai, China) was used to remove ethanol in the lipid phase at 80 °C. Meanwhile, 50.10 mg Myri 52, 21.21 mg Gelucire® 44/14 and 10.00 mg QACMC were added to 50 mL of distilled water heated to 80 °C. The solution was stirred using a magnetic heated stir plate (SZCL-4B, Yu Hua Instrument Co., Ltd., Henan, China) to obtain a homogeneous lipid solution. A small amount of ethanol was added to the hot lipid phase and then it was dispersed in the aqueous phase to form a pre-emulsion. After stirring at 80 °C for 0.5 h, the pre-emulsion was treated with an ultrasonic cleaner (KH 2200B, He Chuang ultrasonic Co., Ltd., Shanghai, China) at the same temperature for 0.5 h. The resulting hot O/W nano-emulsion was cooled to 0 °C and then restirred to form CUR-CNLC. CUR-SOL was prepared by adding the same quantity of CUR with the optimal CUR-CNLC formulation and appropriate amount of Tween-80 into 50 mL distilled water. CUR-NLC was produced using the same formulation without QACMC and employed for comparison against CUR-CNLC and CUR-SOL in *in vitro* and *in vivo* studies.

2.3. Drug analysis

The quantitative determination of CUR was performed using a Waters e2695 high-performance liquid chromatography (HPLC) system (Waters Ltd., Milford, U.S.). The detector was set at 425 nm. A reverse-phase column (Diamonsil C18 (2), 5 µm, 200×4.6 mm)

was used, and the column temperature was set at 35 °C. The mobile phase consisted of methanol and 0.05% (v/v) methanoic acid (80:20). The flow rate of the mobile phase was 1 mL·min⁻¹.

2.4. Optimization design of CUR-CNLC

During the preliminary study, the influence of each parameter on the physicochemical properties of CUR-CNLC was assessed. To assess the optimal condition of the technical procedure, a four-factor, three-level Box-Behnken design set up by Design-Expert 8.06 (Stat Ease, Inc., Minneapolis, U.S.) was undertaken as described in Table 1. The independent variables were dosage of lipid (X₁), the ratio between solid lipid and liquid lipid (X₂), dosage of surfactant (X₃) and dosage of cationic material (X₄). The established dependent variables were the geometric mean (OD), which can be calculated by the following formulas 1 and 2:

$$d_n = \frac{Y_i - Y_{\min}}{Y_{\max} - Y_{\min}} \quad (1)$$

$$OD = (d_1 d_2 \cdots d_n)^{\frac{1}{n}} \quad (2)$$

where Y_i is the observed values of drug loading (DL), entrapment efficiency (EE) and appearance score of CUR-CNLC; Y_{max} and Y_{min} are the acceptable maximal and minimal values of the response variable, respectively; and n is the number of response variables [20, 21].

2.5. Entrapment efficiency and drug loading

The EE and DL of CUR in CUR-CNLC was assessed indirectly. Non-entrapped CUR was removed with a filtration-centrifugation technique using UFC 510024 centrifugal filter devices (Amicon® Millipore Corporation, Bedford, MA, U.S.) at 8000 rpm for 10 min [22].

2.6. Mean particle size and Zeta potential analysis

Mean particle size (PS), polydispersity index (PI) and zeta potential (ZP) were determined by a Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) after appropriate dilution with ultra-purified water at 25 °C.

2.7. Differential scanning calorimetry

DSC analysis was performed using a Mettler Toledo DSC (1/500, Mettler-Toledo Ltd., Shanghai, China) equipped with a refrigerated cooling system. Bulk materials, a physical mixture of bulk materials, CUR and a freeze-dried powder of CUR-CNLC were put into aluminum pans. Their thermal analysis profiles were obtained as the temperature increased from -40 °C to 220 °C at a scanning rate of 5 °C·min⁻¹ under a nitrogen atmosphere. An empty pan was used as a reference.

2.8. Morphological study

The optimized CUR-CNLC formulation was examined morphologically in a TEM (HT7700, Hitachi, Tokyo, Japan). After diluting the sample 50-fold with double distilled water, a drop of the sample was immediately spread on a film-coated copper grid, and the excess liquid was absorbed by filter paper. Then, the sample was dried at ambient atmosphere.

2.9. *In vitro* drug release test

In vitro release of CUR from CUR-CNLC was carried out with a dialysis membrane method [23]. A total of 2 mL of CUR-SOL, CUR-NLC and CUR-CNLC were put in dialysis bags with a molecular weight cut off of 8000-14,000. The release medium was 50 mL of 40% ethanol (v/v, pH=4.0) at 34 ±1 °C [24]. The dialysis bags were suspended in release medium on a magnetic heated stir plate and stirred at 150 rpm. A total of 1 mL of sample was withdrawn at predetermined time intervals and replaced with fresh medium immediately for

12 h. Finally, the samples were analyzed by RP-HPLC. The experiments were conducted in three repeats.

2.10. *In vitro* corneal permeation evaluation and hydration level

The *in vitro* corneal permeation profiles of CUR-SOL, CUR-NLC and CUR-CNLC formulations were evaluated using excised corneas of rabbits according to the previous method by Hao [25]. Freshly excised rabbit corneas were immediately mounted on Franz-type diffusion cells, which were maintained at a constant temperature 34 ± 1 °C, under stirring conditions using a transdermal diffusion machine at a rotating speed of 200 rpm (YB-P6, Tianjin, China). Each formulation (500 μ L) was added to the donor chamber, and 4.5 mL fresh glutathione bicarbonate Ringer (GBR) buffer with 3% Tween-80 (v/v) was placed in the reception cell. GBR solution is reported to preserve tissue integrity of excised corneas over 6 h [26]. Tween-80 was used as a solubilizer to increase the solubility of curcumin. At regular time intervals (40, 80, 120, 160, 200 and 240 min), samples (4.5 mL) from the receptor chamber were withdrawn and replaced with an equal volume of fresh GBR. At the end of the corneal permeation experiment, each cornea was desiccated at 60°C for 16 h and weighed to calculate the corneal hydration level. Each experiment was performed in triplicate.

2.11. *In vivo* study

2.11.1. Animals

New Zealand white rabbits, with weight of 2.5-3.0 kg, were used in the present study. The animals were maintained at 25 ± 1 °C and $50 \pm 5\%$ RH in a light-controlled room with *ad libitum* access to food and water. The procedures involving animals were reviewed and approved by the Animal Ethical Committee at Tianjin University of Traditional Chinese

Medicine.

2.11.2. Ocular irritation study

An ocular irritation study was performed with six rabbits according to a modified Draize technique [27]. CUR-NLC and CUR-CNLC formulations were instilled into the left eyes, and PBS (pH 7.4) was instilled into the right eyes at a rate of 0.1 mL every 4 h, four times a day for a period of 7 days. The condition of the ocular tissue was monitored at 4, 12, 24, 48, and 72 h after the end of the last instillation.

2.11.3. Preocular retention time study

The preocular retention of CUR-CNLC was assessed using a non-invasive fluorescence imaging system. CUR was replaced with Rh B in the oil phase and was then processed via the same method as for the preparation of CNLC. The albino rabbits were maintained in a sober state. Just before the time of imaging, they were anesthetized with chloral hydrate (injection 2.5 ml·kg⁻¹) by ear vein. Exactly 20 µL of the formulation was instilled directly into the conjunctival sac of the right eye, and the left eye was left as a control group. The eyes were manually closed for 10 min to allow distribution over the cornea. At 0, 15, 30, 60, 90, 120 and 150 min, imaging was carried out. The region of interest (ROI) for the residual fluorescence was around the ocular and non-ocular areas [28,29]. The remaining intensity (R) was calculated according to the following formula 3:

$$R\% = \frac{A - B}{C} \times 100 \quad (3)$$

where A was the intensity of ROI, B was the background fluorescence intensity, and C was the intensity of the ROI at 0 min [28].

2.11.4. Pharmacokinetic study in aqueous humor

A pharmacokinetic study in the aqueous humor was carried out using microdialysis. The experimental operation was performed as previously reported by Liu [30]. Briefly, three rabbits were locally anesthetized with 20 mg·kg⁻¹ of lidocaine hydrochloride injection. A microdialysis probe (MD 2000, Bioanalytical Systems, Inc, West Lafayette, Indiana) was implanted into the aqueous humor using a 20 G needle. The needle was removed, leaving the probe with the microdialysis membrane in the middle of the anterior chamber. The probe was perfused with GBR containing 0.06% Tween-80 at a flow rate of 3 μL·min⁻¹ using a microdialysis pump (CMA106, CMA Microdialysis Co., Ltd, Stockholm, Sweden). The corneal wound surfaces were treated with 0.3% (w/v) ofloxacin ophthalmic solution. Animals were allowed to stabilize for 1 day before topical drug administration.

Probe recovery was evaluated using a retrodialysis method. Different concentrations of standard CUR solutions were perfused, and dialysates were collected 15 min after 30 min of perfusion. *In vivo* recovery (*R*) was calculated by the following formula 4:

$$R\% = \frac{C_d - C_p}{C_m - C_p} \times 100 \quad (4)$$

where *C_d*, *C_p* and *C_m* are the concentrations of the drug in the dialysate, perfusate and aqueous humor, respectively. *R* is the value of the slope for the plot of *C_d* - *C_p* versus *C_p*.

The CUR-SOL, CUR-NLC and CUR-CNLC formulations (0.2 mL, 1.0 mg·mL⁻¹) were instilled into different groups of rabbit eyes. Dialysates were collected every 20 min for the first 2 h and every 30 min thereafter. The study was repeated for three times, and all samples were analyzed using HPLC.

2.12. Statistical analysis

The optimization design of CUR-CNLC and the data were analyzed by Design-Expert software (8.06 version, Stat Ease, Inc., Minneapolis, U.S.) [31]. The results were presented as the mean ± standard deviation (SD). Statistical analysis of the results was performed by

two-way analysis of variance (ANOVA) and then one-way ANOVA using *SPSS* software (17.0 version, Spss Inc., Illinois, U.S.). The statistical significance was set at $p < 0.05$ [32].

3. Results

3.1. Optimization and validation

The results from preliminary studies indicated that the optimal parameters of the CUR-CNLC formulation are 2 mg CUR, 60 mg total liquid (30 mg GMS, 30 mg Miglyol® 812), 70 mg Myri 52, 20 mg Gelucire® 44/14, 30 mg cationic material and 50 mL water. Each experimental response could be represented by the following formula:

$$Y = aX_1 + bX_2 + cX_3 + dX_4 + eX_1X_2 + fX_1X_3 + gX_1X_4 + hX_2X_3 + iX_2X_4 + jX_3X_4 + kX_1^2 + lX_2^2 + mX_3^2 + nX_4^2 + oX_1^2X_2 + pX_1^2X_3 + qX_1^2X_4 + rX_1X_2^2 + sX_1X_3^2 + tX_2^2X_3 + uX_2^2X_4 + vX_2X_3^2 + w \quad (5)$$

where Y is the response, X_1 is the concentration of lipid, X_2 is the ratio between solid lipid and liquid lipid, X_3 is the concentration of surfactant, X_4 is the concentration of cationic material, w is a constant, a to v are the regression coefficients.

The responses of optimization experiments can be modeled by the two-factor model, the quadratic model and the adjusted cubic model are shown in Table 2. It was demonstrated that the best-fit model for the responses was the adjusted cubic model, which had the maximal r value. ANOVA was carried out to evaluate the significance of the cubic model terms on response and their quantitative effects. Through comparing the associated p -value with the F -value of each term at a 95% confidence level, the appropriate model terms were aliased. Compared to the two-factor model and the quadratic model, the adjusted cubic model had the largest r value for the response. The adjusted cubic model was chosen to describe the effects of the variables thereafter.

The sign and value of the quantitative effect represented the tendency and magnitude of the responses, respectively. EE, DL and the appearance score of CUR-CNLC were calculated

to obtain the OD value and the OD value was input for the construction of RSM. The response surface analyses were also plotted in three-dimensional model graphs. The three-dimensional response surface plots for the OD values are presented in Figure 1. By analyzing these graphs, the optimal parameters of the CUR-CNLC formulation were determined to be 2 mg CUR, 40 mg total liquid (36.36% GMS Miglyol® 812), 50.10 mg Myri 52, 21.21 mg Gelucire® 44/14 (0.04% wt), 10 mg QACMC and 50 mL water. The EE and DL of the optimal formulation were $99.12 \pm 0.01\%$ and $1.677 \pm 0.19\%$, respectively. This formulation was considered to be the most appropriate formulation for ophthalmic application and was employed for further characterization.

3.2. Physicochemical characterization

Figure 2a and 2b depict the unimodal distribution profiles of PS and ZP. The PS of the optimized formulation was 158.1 nm, the PI was 0.29, and the ZP was 36.5 mV.

3.3. Differential scanning calorimetry

The drug entrapment was further characterized by DSC to reveal the state of the encapsulated drug. Figure 3 shows DSC thermograms of QACMC, Myri 52, Miglyol® 812, GMS, CUR, a physical mixture of the bulk materials of the optimal CUR-CNLC formulation and a freeze-dried powder of the optimal CUR-CNLC formulation. The thermogram of the bulk materials physical mixture indicated a melting process of Myri 52, Miglyol® 812 and GMS with a maximal tri-peak at $-3-64\text{ }^{\circ}\text{C}$. The peak of QACMC was at $119\text{ }^{\circ}\text{C}$. The DSC curve of CUR showed a melting endotherm at $178.8\text{ }^{\circ}\text{C}$, whereas it was not observed for CUR-CNLC. This could be due to the presence of CUR in a dissolved state in the lipid matrix [33]. The results illustrated that the CUR in CUR-CNLC was in an amorphous state

[34].

3.4. Morphological study

The TEM image in Figure 4 reveals that the optimized CUR-CNLC formulation was dispersed as individual CNLC with a well-defined spherical shape and a homogeneous distribution of approximately 160 nm in diameter.

3.5. *In vitro* drug release test

The *in vitro* release profiles of CUR from CUR-SOL, CUR-NLC and CUR-CNLC are displayed in Figure 5. It was observed that 90.37% CUR was released from CUR-SOL in 12 h. CUR-CNLC showed a biphasic release pattern: a small initial burst release (19.73%) over a period of 20 min, followed by a sustained release (80.94%) over a period of 12 h. The *in vitro* release data were fitted to different kinetic equations, including zero order, first order, Higuchi, Hixson-Crowell, Ritger-Peppas and Weibull models. The fitted equations are shown in Table 3. The best fit kinetic model was the Weibull model ($r=0.9933$). Compared with CUR-NLC, CUR-CNLC displayed a more sustained release phase in the release profiles. This sustained release mainly resulted from the erosion and degradation of the components of CUR-CNLC [35].

3.6. *In vitro* corneal permeation evaluation

Table 4 lists the corneal permeability coefficients and HL values determined for the CUR-SOL, CUR-NLC and CUR-CNLC formulations. The CUR-NLC and CUR-CNLC formulations showed P_{app} values higher than those of CUR-SOL. CUR-NLC and CUR-CNLC exhibited a remarkable respective 1.24-fold and 1.25-fold increase compared with that of CUR-SOL ($P<0.05$). The HL values indicated that the three formulations did not cause any damage to the epithelium or endothelium during this study.

3.7. *In vivo* study

3.7.1. Ocular irritation study

The ocular irritation scores of both CUR-NLC and CUR-CNLC were 2 ± 0 . No signs of toxicity or irritation to the external ocular tissues were observed after the Draize test [27]. Although there was a slight hyperemia in the conjunctiva, no ocular damage or abnormal clinical signs in the cornea or iris were visible.

3.7.2. Preocular retention time study

As shown in Figure 6, rapid clearance could be observed in the Rh B solution group after administration. Fluorescence intensity remaining in the ROI at 30 min after administration was defined as A_{30} . At 30 min, only 37.44% fluorescence intensity remained in the ROI, which was significantly lower than that of Rh B-CNLC, A_{30} of Rh B-CNLC was 51.36%, as displayed in Figure 7. At 90 min, the statistical analysis noted that 24.52% and 31.87% intensity remained in the Rh B solution and Rh B-CNLC group, respectively. This indicated that the CNLC formulation exhibited a relatively strong intensity and slow clearance from the ROI.

3.7.3. Pharmacokinetic study in aqueous humor

In vivo probe recovery was determined before the pharmacokinetic study to ensure that the implanted probes were in good function. The *in vivo* recovery was $50.75 \pm 3.91\%$ ($n=3$).

Aqueous humor pharmacokinetics parameters are summarized in Table 5. As shown in Figure 8, compared with CUR-SOL, the area under the concentration-time curve (AUC) value was enhanced by 2.36-fold for CUR-CNLC ($p < 0.01$), and concentration at peak ($C_{\max}$) value of CUR-CNLC was enhanced by 1.28-fold ($p < 0.01$). The time to peak concentration ($T_{\max}$) of CUR-CNLC was determined to be up to 126.7 min, and the MRT value was

extended. There were significant differences in the $T_{\max}$ and MRT values of CUR-CNLC compared with those of CUR-SOL ($p < 0.01$).

4. Discussion

4.1. Selection of materials and preparation methods

Due to the physiological constraints imposed by the protective mechanisms of the eye, a nanotechnology-based drug delivery system was chosen to improve the bioavailability of CUR. In recent years, drug delivery systems of cationic nanoparticles have shown greater stability and prolonged circulation half-life over traditional drug delivery systems [36]. QACMC was chosen as a promising cationic material for ocular drug delivery for the following reasons. First, QACMC can improve the water solubility of lipid soluble drugs, as well as the EE and DL of the NLC due to its perfect amphiphilicity [37]. Second, steric stabilization of QACMC is able to protect the nanoparticles from undesired attacks in biological media, thereby increasing the stability of the nanoparticles and prolonging their circulation time [38]. Third, its cationic character can be employed as a target for ophthalmic drug delivery. Fourth, the viscosity of QACMC can prolong the retention of the drug and improve its bioavailability in ocular administration [39, 40].

To improve corneal permeability, an absorption enhancer was added to the CUR-CNLC system. Gelucire® 44/14 has been reported to improve the drug bioavailability of poorly water-soluble drugs without causing tissue damage at low concentrations [41]. Therefore, Gelucire® 44/14 was selected for inclusion in the CUR-CNLC formulation as an absorption enhancer to improve ocular drug permeation.

Different techniques can be employed to prepare NLC, including ultrasonication, homogenization, melt-emulsification and low-temperature solidification methods [42]. Film ultrasonic processing does not involve large amounts of surfactants and organic solvents,

which must be avoided in ophthalmic formulations. Therefore, it is considered to be an appropriate technique for the preparation of CNLC for ocular application.

4.2. Characterization of CUR-CNLC

Much of the published data has suggested that an appropriate particle size and a narrow size range ensure low irritation, adequate bioavailability and good compatibility with ocular tissues [43]. The type and amount of cationic modified material had immense effect on the particle size and its distribution. An increase in the concentrations of the cationic materials increased the viscosity of the aqueous phase, thus hindering the rapid dispersion of the cationic materials in the organic phase. Thus, a larger particle size was obtained for the formulations containing higher concentrations of cationic materials.

4.3. *In vitro* study

Drug release from CUR-CNLC involves multiphases. Burst effect was observed due to initial release of drug absorbed on the surface of nanoparticles [44]. On the one hand, the initial burst release is attributed to the large specific surface portion of the NLC from which the drug molecules desorbed. On the other hand, the sustained release pattern can be associated with the retention capacity of the lipophilic bilayers of the oily nanodroplets [45]. This factor is expected to be incorporated owing to its low aqueous solubility. Both initial burst release and sustained release are of interest for clinical applications. Initial burst is useful to improve drug permeation for a faster onset of action, while sustained release supplies drug over a prolonged period of time. In addition, the positive charge of CUR-CNLC can elicit electrostatic adhesion or interaction with the negatively charged mucin of the corneal epithelium, hence increasing ocular residence time on the corneal surface [46]. Thus, the corneal permeation of the drugs was also enhanced.

4.4. *In vivo* study

Rh B as a fluorescent dye was encapsulated in the nanoparticles, which can reflect retention capacity of LCNPs. Rh B has the characteristics of lipophilic. It may be well trapped into CNLC, so the nanoparticles are labeled by Rh B [28]. Results of the pharmacokinetic study demonstrate that CUR-CNLC can prolong the retention of CUR following topical administration. The enhanced ocular bioavailability provided by the CNLC delivery system may be attributed to the following reasons. First, nanoparticles possess a bioadhesive property due to their extremely small particle size and increased surface area. Second, the encapsulation of CUR embedded into nanoparticles facilitates transport through the corneal route, leading to a maintained corneal gradient of drug concentration. Third, the cationic character of QACMC is able to prolong the retention of a drug and improve its bioavailability in ocular administration. Fourth, Gelucire® 44/14 enhances the absorption of poorly soluble drugs, contributing to improved drug bioavailability [41]. Therefore, all of these factors ensure intimate contact with the epithelial mucosal surface of the eye, preventing tear wash out and consequently providing sustained drug release and a prolonged drug retention time.

5. Conclusions

A novel CUR-CNLC delivery system with the addition of the cationic material QACMC for ocular application was successfully developed and evaluated. The optimized formulation showed an appropriate particle size, a sustained release property, improved corneal permeability, high ophthalmic tolerability, and most importantly prolonged retention capacity. Therefore, CNLC system is a potential approach to improve ocular bioavailability and safe profile of poorly water-soluble drugs.

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Table 1. Initial 3-level design, providing the lower (-1), medium (0) and upper (+1) level values for each variable.

Variable	Level		
	Low level (-1)	Medium level (0)	High level (1)
X ₁ /mg	40	60	80
X ₂	1:4	1:1	7:4
X ₃ /mg	50	70	90
X ₄ /mg	10	30	50

X₁, dosage of lipid; X₂, the ratio between solid lipid and liquid lipid; X₃, dosage of surfactant; X₄, dosage of cationic material.

Table 2. Coefficients of the models in optimization experiment.

Coefficient	Two-factor model	Quadratic model	Adjusted cubic model
a	-2.6×10^{-2}	-4.1×10^{-2}	-1.7×10^{-2}
b	0.28	3.3×10^{-2}	-0.20
c	-1.9×10^{-2}	-9.8×10^{-3}	-0.10
d	-5.2×10^{-3}	-2.1×10^{-2}	-0.23
e	3.6×10^{-3}	3.6×10^{-3}	5.5×10^{-2}
f	2.9×10^{-4}	2.9×10^{-4}	0.12
g	5.9×10^{-5}	5.9×10^{-5}	2.4×10^{-2}
h	-4.9×10^{-3}	-4.9×10^{-3}	-7.4×10^{-2}
i	-8.7×10^{-3}	-8.7×10^{-3}	-0.13
j	-4.3×10^{-7}	-4.3×10^{-7}	-1.7×10^{-4}
k	-	1.3×10^{-4}	5.1×10^{-2}
l	-	0.12	7.1×10^{-2}
m	-	-6.5×10^{-5}	-2.6×10^{-2}
n	-	2.6×10^{-4}	0.10
o	-	-	0.20
p	-	-	-5.0×10^{-2}
q	-	-	1.1×10^{-2}
r	-	-	7.2×10^{-3}
s	-	-	6.4×10^{-2}
t	-	-	0.11
u	-	-	4.9×10^{-2}
v	-	-	0.16
w	2.170	2.6	0.31
R	0.8717	0.9231	0.9829
P	0.0007	0.0012	0.0013

-, the model does not have this coefficient and relative term.

Table 3. Mathematical models of the regression for *in vitro* release profiles of CUR-CNLC.

Function	Equation	R
Zero order	$Y=0.05634t+0.3172$	0.9062
First order	$\ln(1-Y)=-0.1425t-0.3327$	0.9458
Higuchi	$Y=0.2405t^{1/2}+0.1057$	0.9720
Hixcon-Crowell	$(1-Y)^{1/3}=-0.03435t+0.8881$	0.9351
Ritger-Peppas	$\ln Y=0.4255\ln t-1.105$	0.9919
Weibull	$\ln\ln[1/(1-Y)]=0.6227\ln t-0.8824$	0.9933

Y, the percentage of CUR; t, time point; R, regression coefficient.

Table 4. The corneal permeability coefficients ($\bar{X} \pm \text{SD}$, n=3).

Formulation	$P_{\text{app}} \times 10^7 (\text{cm} \cdot \text{s}^{-1})$	$J_{\text{ss}} \times 10^6 (\mu\text{g} \cdot \text{s}^{-1} \cdot \text{cm}^{-2})$	HL (%)
CUR-SOL	3.778 ± 0.2940	1.889 ± 0.1470	81.09 ± 0.0200
CUR-NLC	$4.693 \pm 0.5485^*$	$2.346 \pm 0.2743^*$	80.21 ± 1.063
CUR-CNLC	$4.733 \pm 0.2895^*$	$2.367 \pm 0.1448^*$	80.28 ± 0.4105

P_{app} , apparent permeability coefficient; J_{ss} , steady-state flux; HL, hydration level.

* $P < 0.05$ versus CUR-SOL.

Table 5. Pharmacokinetic parameters of CUR in aqueous humor after topical administration in the conscious rabbits ($\bar{X} \pm SD$, n=3).

Formulation	$T_{\max}$ (min)	$C_{\max}$ (ng·mL ⁻¹)	AUC (ng·mL ⁻¹ ·min)	MRT (min)
CUR-SOL	30.00±0	23.11±1.915	2161±55.74	65.14±2.299
CUR-NLC	103.3±11.55**	27.01±4.548	3334±103.1**	115.3±2.312**
CUR-CNLC	126.7±14.43**	29.60±1.080*	5093±245.7**	147.0±2.987**

$T_{\max}$, time to maximal concentration; $C_{\max}$, maximal concentration of CUR; AUC, area under the curve; MRT, mean residence time.

* $P < 0.05$ versus CUR-SOL; ** $P < 0.01$ versus CUR-SOL.

Figure captions

Figure 1. RSM showing the influence of the independent variables on OD. (X_1) concentration of lipid, (X_2) the ratio between solid lipid and liquid lipid, (X_3) concentration of surfactant, and (X_4) concentration of cationic material.

Figure 2. Physicochemical characteristics of CNLC. (a) particle size distribution, (b) zeta potential distribution.

Figure 3. DSC thermograms. (a) physical mixture of Cur-CNLC materials, (b) Cur-CNLC, (c) QACMC, (d) Myri 52, (e) Miglyol 812, (f) GMS, (g) curcumin.

Figure 4. TEM morphology of the optimal CUR-CNLC formulation.

Figure 5. *In vitro* release profiles of CUR from CUR-SOL, CUR-NLC and CUR-CNLC (n=3).

Figure 6. Fluorescence images of rabbit eyes after administration. (a) Rh B solution, (b) Rh B CNLC. 1: ocular ROI, 2: intensity reference standard, 3: inner canthus and nasolacrimal duct region.

Figure 7. Preocular retention of various formulations.

Figure 8. CUR concentration-time profiles following a 200 μ L topical administration at dose of 1.0 mg·mL⁻¹ in aqueous humor (n=3).

a

b