In Situ Liquid Crystal Gel as a New Ophthalmic Drug Delivery System for Pilocarpine Nitrate: Improving Preocular Retention and Ocular Bioavailability

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Running title: Application of in-situ liquid crystal gel in ocular drug delivery

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In situ liquid crystal gel as a new ophthalmic drug delivery system for pilocarpine nitrate: improving preocular retention and ocular bioavailability

Abstract: The purpose of this article is to develop an in-situ liquid crystal gel that can be used as a novel ocular delivery system for pilocarpine nitrate (PN). The phytantriol (PT) -based in situ liquid crystal gels were prepared by a vortex method using PT, PEG400, Triglyceride (TAG) and water (in the ratio of 61.15:30:3.85:5, w/w). Firstly, the internal structure of the PN-loaded liquid crystal gel was characterized by polarizing microscope (PLM), small-angle X-ray scattering (SAXS), differential scanning calorimetry (DSC) and rheology. In vitro drug release behavior and ex vivo corneal permeation were investigated. Finally, eye irritation test, preocular residence time evaluation, were studied in vivo and compared with eye drops. Based on various characterization techniques, it is proved that the internal structure of the gel is a hexagonal phase. In vitro release results identified that PN could be released continuously from HII gel over a period of 24 h. The in vitro obvious permeability coefficient of HII gel was 3.19-fold (P < 0.01) higher than that of the eye drops. Compared with eye drops, the HII gel had good bioadhesion and displayed longer residence time on the eyeballs surface using fluorescent labeling technology. In addition, through Corneal hydration level and eye irritation test ., it is conjectured that HII gel will not cause eye irritation. In short, the formulation has the advantages of high efficiency, slow release and non-toxicity, and will become a promising pharmaceutical strategy to improve the efficacy of glaucoma.

Key words: Pilocarpine nitrate, Liquid crystal gel, Ophthalmic administration, Corneal penetration, Glaucoma
In situ gel

37°C

Pre-Corneal Residence Time

A(PLM)  B(SAXS)  C(DSC)  D(rheology)
1. Introduction

Topical ophthalmic administration is the main method of ophthalmic treatment. However, due to the high sensitivity of the eye and unique physiological barriers (including corneal and conjunctival barriers, blood-water barriers and blood-retinal barriers), the drug has low bioavailability and poor efficacy. Commonly used ocular dosage forms are topical eye drops and ophthalmic ointments. However, most of the liquid is discharged from the lacrimal duct after the eye drops are administered, and may cause systemic toxicity after being absorbed through the nasopharynx. Due to its greasy properties and blurred vision, eye ointment has poor patient compliance when applied to eye ointment[1].

Over these years, a growing number of innovative drug delivery systems have been applied to the eyes. Such as liposome nanoparticles and microemulsion, to extend the retention time of the eyes, thus reducing the frequency of medication and increasing bioavailability. However, their drug delivery potential in ophthalmology is limited by the rapid clearance of the anterior cornea due to the same rapid clearance as the water-soluble eye drops. Therefore, considering the unique physiological structure of the eye, effective ocular drug delivery still faces many challenges and needs to find a more effective ocular delivery system.

In recent years, in-situ gel has become a new type of sustained release system[2,3]. It is a precursor for administration and then transforms into a gel at the drug delivery site. Compared with other ophthalmic preparations, the precursor preparation of liquid crystal gel has a longer residence time in the eye and good curative effect. In addition, in situ gel is in the form of ordinary solution, similar to eye drops, so the drug is simple and good compliance[4-8]. Conversion from solution to gel is usually caused by some physiological or external stimuli, such as variances in pH value, ionic strength or temperature between the formulation outside the body and the internal tissues[9]. When the formulation is exposed to artificial tears, the precursor solution is transparent and can spontaneously become a gel. Since the triggering factor of this phase change is due to the existence of the water environment, it can also be realized
directly in the physiological fluid. Compared with other methods, such as ultraviolet light and temperature, this method can cause phase transition more mild. According to the literature, it is found that many kinds of in-situ gels have been used in eye administration to improve ocular bioavailability like use of temperature-dependent in-situ gelation polymer (Poloxamer), pH-dependent in-situ gelling polymers (Carbopol and Hypromellose), and ionic strength-dependent in-situ gelling polymer (Gellan gum). However, there are few reports on in situ liquid crystal gel for ocular drug delivery.

An amphiphilic substance containing a hydrophilic head group and a hydrophobic hydrocarbon chain domain self-assembles after adding water to form a long-range ordered structure called a lyotropic liquid crystal phase. For example, lamellar phase (Lα), reverse bicontinuous cubic phase (QII) and reverse bicontinuous hexagonal phase (HII) have received more and more attention due to their unique internal frame and broad drug capacity. Among them, the hexagonal liquid crystal as a drug carrier has attracted widespread interests due to its good stability, potential drug slow-release ability. Phytotriol (PT) (shown in Fig.1) are generally considered safe drug matrix and amphiphilic lipids with good mucosal adhesion and biocompatibility, which was often utilized to form liquid crystals. The lyotropic liquid crystal delivery system has many advantages, such as simple preparation, easy to use, reduced dose, and sustained release effects, PT-based liquid crystal gels have been used in the treatment of rheumatoid arthritis and postoperative analgesia. However, there are few studies on PT in ocular delivery systems.

Glaucoma is one of the major public health problems in the world. This is a chronic progressive eye disease caused by the apoptosis of retinal ganglion cells and subsequent degeneration of nerve tissue. According to the survey, more than 70 million people in the world suffer from glaucoma, and about 10% of them are blind, which makes it the main cause of irreversible blindness in the world. Pilocarpine nitrate is a drug that directly acts on the parasympathetic nerve. It can directly stimulate muscarinic receptor M of iris and ciliary body, cause the contraction of iris and ciliary body, open
the anterior chamber angle, promote the outflow of aqueous humor through trabecular meshwork structure, and produce the effect of lowering intraocular pressure\[17\]. It has been used in the treatment of chronic open angle glaucoma and acute closed angle glaucoma for more than 100 years\[18\]. At the same time, PN has been used the longest as a mainstay drug for glaucoma therapy, and is one of the least-expensive and the most readily available medications\[19\]. Applied in the eye, PN can penetrate the eye wall with miosis beginning in 15-30 min which last up to 4-8 h\[20\]. Due to poor corneal permeability, short anterior corneal retention time and poor anterior corneal tear flushing, PN eye drops need to be administered frequently, generally 3-6 times a day\[21\]. Therefore, the bioavailability is extremely low (less than 5% or even less than 1%). At the same time, frequent daily administration can cause a series of side effects, such as pupil contraction and myopia, and even a series of gastrointestinal reactions. In addition, our previous research has developed a liquid crystal gel. Based on this, we made the gel into a precursor to improve compliance\[22\].

Fig. 1 The pores assemble into hexagonally packed columnar mesophases\[23\] (A), Molecular structure of PN (B) and PT (C)
1. Materials and methods

1.1 Materials

PT was acquired from Shanghai Aladdin Biotechnology Co., Ltd. (Shanghai, China). PN was bought from Shanghai TargetMol Biotechnology Co., Ltd. (Shanghai, China). Triglyceride (TAG) was bought from Sigma-Aldrich Trading Co., Ltd. (Shanghai, China). PEG400 was bought Beijing Solarbio & Technology Co., Ltd (Shanghai, China).

The weight of adult New Zealand white rabbit was in the range of 2.5-3.0 kg, which is supplied by the Animal Experimental Center of Anhui University of Chinese Medicine (Hefei, China). All animal experiments were performed in accordance with the guidelines approved by the ethics committee of Anhui University of Chinese medicine (Hefei, China).

2.2 Prescription Screening

2.2.1 Preparation

According to the literature\cite{24} and our former research (Fig.2), the formulating method was as follows. Firstly, appropriate amount of PT and TAG were mixed and heated to 60 ± 0.5 ℃ to prepare oil phase. Secondly, PN was placed in water at 60 ± 0.5 ℃ to prepare aqueous phase (PEG400 and water). Finally, the water phase in the same temperature was added to the oil phase rapidly, and the mixture was immediately rotated for 5 minutes to obtain a liquid crystal gel precursor.

Then, PT-TAG (oil phase) and PEG400-water (water phase) systems were used to gradually change the weight ratio of the prescription from 10%: 90% to 90%: 10%. The prescription was screened by the above-mentioned preparation method, and four blank formulas were prepared, namely P 0, P 1, P 2 and P 3. The optimal proportion of stable self-assembled liquid crystal system was determined. Considering the solubility of the drug and the particularity of ophthalmic administration, three preparations were made with drug contents of 0% (F0), 0.5% (F1), 1% (F2) and 1.5% (F3), respectively. After 72 h of stabilization, the appearance of the preparations was observed.
Fig. 2 Temperature composition phase diagram of the phytantriol/water binary system [25].

2.2.2 Phase Conversion

2.2.2.1 The Minimum Volume ($V_m$) and Time ($T_g$) for Phase Conversion

In the rotor method [26], 200 mg of the formulation is accurately weighed and added to a tube with a rotor (10 mm × 6 mm). The test tube was immersed in a water bath at 37 °C with a stirring speed of 30 rpm for 5 minutes. Then, 10 μL of artificial tears were added to the centrifuge tube every 1 minute until the rotor was completely stopped due to gelation. The time when the rotor stops moving due to gelation is determined as $T_g$, and the total volume of water added at this time is determined as $V_m$.

2.2.2.2 Determination of the gelling capacity

In order to simulate the gelation ability in the human environment, the precursors were placed in a centrifuge tube containing 2 mL fresh simulated tears (pH 7.4, 37±1 °C) to determine the gelling ability, the time taken for its gelling formation then dissolution of the gels were visually observed and the gelling capacity were evaluated [27] as follows:
| (-) | No gelation |
| (+) | The gel formed after few minutes and dissolved rapidly |
| (+++) | Immediate gelation and remains for few hours |
| (++++) | Immediate stiff gelation which remains for extended period of time |

2.2.3 Determination of the pH and osmotic pressure

pH was measured at 25 °C by a Model PHS-3C pH-meter (Shanghai Precision Scientific Instrument Co., Ltd). The freezing point method of a Model FM-9X Osmometer (Shanghai Precision Scientific Instrument Co., Ltd) was used to evaluate the osmotic pressure.

2.3 Characterization of structure

2.3.1 PLM

Polarized light microscope observes the internal structure of the precursor and the liquid crystal gels (CK-500, Shanghai Caikon Optical Instrument Co., Ltd, Shanghai, China)\(^{[28]}\). Observed by PLM, the isotropic liquid and Q\(_{II}\) have no birefringence, and the background is dark. The characteristic of L\(_{α}\) and H\(_{II}\) is a birefringent structure, that is, H\(_{II}\) has a fan-shaped structure. Greasy streak texture or Maltese cross shape can be observed in the L\(_{α}\) phase\(^{[29]}\).

2.3.2 SAXS

Further structure analysis and phase identification of LC gels were carried out by SAXS measurement (Anton Paar, Graz, Austria) at room temperature. The specimen were tested at 40 kV and 50 mA for 10 min using an X-ray source (Cu Ka radiation, \(k=0.154\) nm). After the sample was equilibrated for 10 min, the scattering pattern was collected, and the scattering intensity was plotted against the q value, which enabled the identification of the peak position. The type of liquid crystal was determined by the peak scattering vector ratio\(^{[30]}\).

The relevant parameters of the liquid crystal structure are calculated by the following formulas\(^{[31]}\):

\[
q = \frac{4\pi \sin \theta}{\lambda}
\]
$d = 2\pi/q$

$\alpha = (h^2 + k^2 + l^2)^{1/2}/d$

Where $\theta$ is the scattering angle, $q$ is the position of the first peak, $k$ is the wavelength of 0.154 nm, $d$ is the distance between the reflecting interplanar space of the liquid crystalline phase, $\alpha$ is the LP which indicates the size of aqueous channels in the LC internal structure, and $h$, $k$, and $l$ are the Miller indices and have no dimension.

2.3.3 DSC

DSC is used to evaluate temperature and enthalpies of phase transitions during melting and crystallization. 5 to 15 mg samples (PN, PT, TAG, PEG400, Physical mixture of PT, PEG400, PN, TAG, Blank in situ liquid crystal and drug loaded in situ liquid crystal gel) were weighed by Mettler M3 microbalance in a standard 40 μL aluminum pot and sealed immediately. The scanning speed was 10 °C/min. The scanning range was 20-220°C. The atmosphere was nitrogen (flow rate was 20 mL/min), an empty pot is used as a reference. The instrument determines the melting temperature of the solid components and the total heat transferred during any observed thermal process.

2.3.4 Rheological characterization

A DHR-2 rheometer (TA Instruments, New Castle, DE) was used to evaluate the rheological characteristics of LC gels and incorporate a cone-plate sensor with a cone angle of 1° and a diameter of 20 mm. In the rheological test, the flow rate of the liquid crystal gel was studied by controlling the shear rate from 1 to 100 s$^{-1}$ during the experiment, and the temperature was maintained at 37 ± 2°C. Meanwhile, frequency scanning was used to further evaluate the rheological behavior of the preparation. Finally, a temperature scan was used to study the viscosity change with increasing temperature, and the temperature setting range was 25–45 °C.

3.3 Evaluation of in vitro properties

3.3.1 In vitro release study
The in vitro release of PN from liquid crystal was studied by dynamic dialysis.[33]

In short, 50 mg fresh PN liquid crystal gels were transferred into dialysis bags (MWCO 8KD-14KD Aladdin, USA) to meet the dialytic capacity of the drug. 150 μL of eye drops were used as a control. Both eye drops and PN liquid crystal gel contain 1.5 mg of PN. Then, the dialysis bag was immersed in 12 mL of artificial tears at 37 ± 1°C under agitation at 100 rpm.

At predetermined time intervals, 1 mL samples were taken at 0.17, 0.5, 1.00, 1.50, 2.00, 4.00, 8.00, 12.00, 24 h. Simultaneously, the STF of the same volume was replaced. The samples were filtered by 0.45 μm microporous membrane, and the drug content was determined by HPLC. The cumulative release percentage (%) was plotted against the time (T, h). The cumulative drug release (Qn, μg / mL) was calculated by the following formula:

$$Q_n = C_n \times V_0 + \sum_{i=1}^{n-1} c_i \times v_i$$

$C_n$ represents the concentration of PN at each sampling time. $V_0$ stand for the volumes of the dissolution medium. $V_i$ represents sample volume. $C_i$ represents the PN concentration of the ith sample.

The dynamics and mechanisms of PN release in the gel were evaluated by fitting the zero order, first order, Higuchi equation and Ritger-Peppas model to evaluate the dynamic model with the highest correlation coefficient. The release kinetics and mechanism of liquid crystal gel were fitted by the following formula:

- Zero order model equation: $y = k_1 t + a_1$
- First order model equation: $\ln(100 - y) = k_2 t + a_2$
- Higuchi equation: $y = k_3 t^{0.5} + a_3$
- Ritger peppas model equation: $y = k_4 t^n$

In the above formulas, $y$ is the cumulative release percentage. $t$ is the sampling time; $K_1, K_2, K_3$ and $K_4$ are the release rate constants of the equation; $a_1, a_2$ and $a_3$ are constants; and $n$ is the release index describing the release mechanism.

3.3.2 In vitro corneal permeability study
Remove the eyeballs of New Zealand rabbits that have been derived, and immediately remove the cornea into glutathione bicarbonate ringer (GBR) buffer. The modified Franz diffusion cell was used for in vitro corneal penetration studies, and the effective diffusion area was 0.5024 cm$^2$. Then, the corneas were fixed between the receptor and the donor compartments with the epithelial side facing the donor chamber. Each formulation (50 mg) with concentration of 1.50 mg PN was transferred to the donor compartment, and then the receptor chamber was introduced into 5 mL GBR solution pre-adjusted to a temperature of 37 °C. Simultaneously, 100 μL PN eye drops (containing 1.50 mg PN) was used as the control group. The samples (0.4 mL) were collected at specified intervals (30, 60, 90, 120, 180, 240, 300 and 360 min) and replaced with fresh GBR of the same volume$^{[34]}$. The corneal penetration test for each formulation was repeated three times. The samples were filtered by 0.22 μm microporous membrane. The PN concentration of samples was determined by HPLC as described above with correction for the volume replacement. The amount of PN that permeated the corneal epithelium was plotted versus time and the slope of the linear portion of the graph was calculated. The steady-state flux $[\text{J}_{\text{ss}}, \mu g/(\text{cm}^2.\text{s})]$ and apparent permeability coefficient $(\text{P}_{\text{app}}, \text{cm/s})$ were determined as follows:

$$\text{J}_{\text{ss}} = C_0 * \text{P}_{\text{app}}$$

$$\text{P}_{\text{app}} = \frac{\Delta Q}{\Delta t \cdot C_0 \cdot A \cdot 60}$$

$\Delta Q/\Delta t$ represents the slope of the linear part of the drug content $(\text{Qn}/\mu g)$ in the receiving pool versus time; $C_0$ represents the initial concentration of the drug in the donor cell (g/mL); $A$ represents the corneal surface area (0.5024 cm$^2$ in this study), and 60 is the factor used to convert minutes into seconds.

3.3.3 Corneal hydration level

In order to evaluate the irritation of the preparation on the cornea, the corneal hydration level (HL) was measured after in vitro corneal permeability test. At the end
of the in vitro penetration study, each cornea was rinsed with normal saline to remove
the residual preparation on the corneal surface, and then weighed. Then, after drying
at 70 ± 0.5 °C for 12 h, the sample were weighed. In general, The HL value of
healthy cornea was 76–80%, corneal hydration value of more than 83% indicates
some degree of corneal injury[35]. The HL% value was calculated using the following
equation[36]:

\[
HL\% = \frac{W_t - W_d}{W_t} \times 100
\]

3.4 In vivo performance evaluation
3.4.1 In vivo eye irritation studies

The potential ocular irritancy and/or damaging effects of the formulations
components and eye drops were evaluated according to a modified Draize test on
male New Zealand albino rabbits (n = 6)[37]. All rabbits were randomly divided into
two groups with three rabbits in each group. In the single dose trial, all samples
were dripped into the inferior conjunctival sac of each rabbit's right eyes, the left eyes
of the contralateral side were treated with saline. After administration, gently close the
eyelids for about 10 seconds to avoid loss of preparation. Observe eye reactions
(redness, swelling, conjunctival edema, iris and corneal damage, etc.) at 5, 15, 30
minutes and 1, 2, 3, 5, 9, 12, and 24 hours after administration. In the multiple
administration test, the eye tissue reaction was observed after 1, 2, 3, 4, 5, 6, 7 days
according to the method of single administration. Then, according to the evaluation
criteria in Table 1, the irritation of the samples to the eyes were judged.

| Table 1. Classification of eye irritation |
|-----------------------------------------|
| Score | Stimulus level            |
|-------|---------------------------|
| 0–3   | Nonirritant               |
| 4–8   | Slight irritation         |
| 9–12  | Medium irritant           |
| 13–16 | Severe irritation         |
3.4.2 Pre-Corneal Residence Time Analysis

In this experiment, sodium fluorescein-loaded formulations (i.e. LC gels and solution) were prepared to evaluate their in vivo preocular residence according to a previously reported protocol. Generally, 1% sodium fluorescein was used to replace PN in the preparation, and then the preparation containing sodium fluorescein was prepared by the same method as the liquid crystal gel precursor. Then, 50 mg of the precursor preparation and 50 μL of fluorescein sodium solution were dropped on the lower dome of the rabbit eyes, and finally, the fluorescence intensity was monitored with a fluorescent lamp and excited with blue-light-activated fluorescent lamp.

3 Results

3.1 Prescription screening

3.1.1 Screening and optimization of prescription

In 2003, Barauskas and Landh reported the phase diagram of PYT/water binary system[38]. In addition, we fully considered the solubility of PN and the basis of our previous work in selecting the formula ratio, and prepared four blank liquid crystal gel precursors (P0, P1, P2, P3). The above samples need to be balanced at room temperature for 72 h, and then the appearance of the samples can be observed by naked eyes under good light conditions. As shown in Table 2. P1 (PT: PEG: TAG: water = 61.15: 30: 3.85: 5) has better fluidity and better transparent appearance than other preparations. Therefore, considering the solubility of PN, the preparation contains 0.5% (F1), 1% (F2), 1.5% (F3) PN drug-loaded preparation.

| Formulations | PT(wt%) | PEG400(wt %) | TAG(wt%) | Water(wt%) | PN(wt%) | Appearance |
|---------------|---------|--------------|----------|------------|---------|------------|
| P0            | 61.15   | 32.50        | 3.85     | 2.50       | 0       | Milky      |
|   |   |   |   |   |   |
|---|---|---|---|---|---|
| P1 | 61.15 | 30.0 | 3.85 | 5.00 | 0 |
| P2 | 61.15 | 27.5 | 3.85 | 7.50 | 0 |
| P3 | 61.15 | 25.00 | 3.85 | 10.00 | 0 |
| F0 | 61.15 | 30.00 | 3.85 | 5.00 | 0 |
| F1 | 61.15 | 30.00 | 3.85 | 5.00 | 0.50 |
| F2 | 61.15 | 30.00 | 3.85 | 5.00 | 1.00 |
| F3 | 61.15 | 30.0 | 3.85 | 5.00 | 1.50 |

3.1.2 The Minimum Volume (V_m) and Time (T_g) for Phase Conversion

The appearance before and after phase inversion was shown in Fig.3. When the liquid crystal precursor was exposed to artificial tears, it undergoes a phase change due to the presence of an aqueous environment, and the precursor solution gradually becomes a gel state. Experimental results showed that 80 μL of artificial tears need to be added to 200 mg of the precursor preparation to make the phase change into a gel state, and the time required is about 1.5 to 2 sec
3.1.3 Determination of the gelling capacity

It was observed that the best prescription P1 showed the "++" grade of gelation ability, which is reported to be the most satisfactory grade\(^{[27]}\). The precursor formulation was gelatinization immediately after exposure to PN at 37 °C degrees can remain for a long time.

3.2 Characterization of preparations

3.2.1 PLM

We put the samples under the PLM for observation. The precursor formulations (F0, F1, F2, F3) showed a black background under PLM, the liquid crystal gel (H0, H1, H2, H3) formed after phase inversion had a clear fan-shaped texture-like optical birefringence structure observed under PLM, and it can be confirmed that it is a hexagonal phase liquid crystal. H0, H1 and H2 had no drug crystals, while H3 showed a few crystals (as shown in Fig 4).
3.2.2 SAXS

In order to further confirm the composition of the gels containing PN, SAXS technique was carried out in this paper. The structures of the gels could be determined according to the ratio of the q corresponding to scattering peaks in the SAXS curves. The typical SAXS pattern was displayed in Fig 5, with respective relative ratios of gels as $1: \sqrt{2}: \sqrt{3}$. The results showed that the liquid crystal gels formed after the precursor phase inversion were hexagonal liquid crystal gels.
Table 3. Lattice Parameters (α) and Layer Spacing (d) of liquid crystal gel

| Formulations | Space group | Bragg peaks | α (Å*) | d (Å) |
|---------------|-------------|-------------|--------|-------|
| H0            | HII         | 1:√2:√3     | 47.95  | 41.52 |
| H1            | HII         | 1:√2:√3     | 48.16  | 41.71 |
| H2            | HII         | 1:√2:√3     | 48.58  | 42.07 |
| H3            | HII         | 1:√2:√3     | 47.32  | 40.98 |

* 1 Å = 10^{-10} m

Distinctly, the content of PN may affect the internal symmetry of gels mesophases due to the alterations of the lattice parameter. When PN was added to the formulation, the lattice parameter changed from 47.95Å to 48.58Å. These results could prove that the hydrophilic PN were mainly contained in the water channel, and minimally contained in the lipid-water interface. According to literature research, this may be due to the partial hydration of the polar head group and water in the lyotropic liquid crystal system, which leads to the expansion of the hydrophilic head group of PT[39]. However, the decrease in the value of α in H3 may be due to the dehydration of the polar head group of PT. When the hydrophilic PN binds more water molecules, it will cause the hydrogen bond between the PT head group and water to decrease, that is, the effective size of the PT head group decreases and the α value decreases[40].

3.2.3 DSC

As shown in Fig.7, the thermograms showed a sharp endothermic peak at 190 °C, which corresponded to the melting point of PN in the crystal form. However, the endothermic peaks of PN completely disappeared in the thermograms of PX (Physical mixture) and H3. At the same time, it can be seen from the figure that the melting point of H3 was 165 °C.

Fig.6. DSC thermograms
3.2.4 Rheological characterization

It could be seen from the rheological curve of Figure 7(A) that as the shear rate increases, the viscosity of the precursor formulation gradually stabilizes. Obviously, the viscosity of F2 is lower than F0, so the drug can improve the fluidity of the liquid crystal gel precursor formulation\textsuperscript{[41]}. When the formulation inverts, the viscosity of the system increases significantly. The results showed that the liquid crystal gels had pseudoplastic flow characteristics (shear thinning system) viscosity increased at low shear rate and decreased at high shear rate. One of the advantages of shear thinning agents is that they have high viscosity during eye opening and stabilize tear film. When the blink occurs, the gel becomes thinner to prevent the irritation produced by the high viscosity Newtonian fluid\textsuperscript{[42]}, so that the preparation is well distributed on the surface of the eye.

Fig. 7 (B) showed the relationship between the composite viscosity and the angular frequency. The complex viscosity of the precursor formulations did not change significantly with the angular frequency, but the complex viscosity of the liquid crystal gel decreases with the increase of the angular frequency. These situations were conducive to the uniform dispersion of the gels in the eye.

The experiment adopts the oscillation-frequency test mode. As exhibited in Fig. 7 (C), the precursor preparations (F2 and F0) exhibits \( G'' > G' \) (Viscous modulus)>G' (Elastic Modulus), and the viscous modulus was dominant, showing liquid-like behavior. At the same time, the liquid-like behavior of the precursor formulations were more conducive to the delivery of ophthalmic drugs. When the precursor formulations were transformed into a hexagonal liquid crystal gel, the elastic modulus \( G' \) and the viscosity modulus \( G'' \) are much higher than the formulations before the phase change. Furthermore, after phase inversion to form the liquid crystal gels, \( G' \) increases with increasing frequency, while \( G'' \) first rises and then decreases with increasing frequency. However, in the entire scanning process, the values before and after \( G'' \) did not change significantly. In the frequency range of 0.01-0.05Hz, the viscosity modulus was dominant (\( G' > G' \)), but in the frequency range of 0.05-10Hz, the elastic modulus was dominant (\( G' > G' \)), It could be concluded that the external frequency changes
will affect the viscoelasticity of the liquid crystal gel, and we can infer that the liquid crystal gel had less irritation to the eyes at low frequencies\[^{43}\]. It could be concluded that the external frequency changes would affect the viscoelasticity of the liquid crystal gel, and we could deduce that the liquid crystal gel had less irritation to the eyes at low frequencies. At high frequencies, the preparations could resist the damage of high frequency blinking shear force and had good stability. Therefore, the "liquid" behavior of the precursor formulations were more conducive to the delivery of ophthalmic drugs. Besides, the gel-like behavior of liquid crystal gels could make the drug release slowly.

Fig.7 (D) described the effect of temperature on the formulations. Whether it was a precursor preparation or a liquid crystal gel, its viscosity would not change significantly with the increase of temperature. Besides, H5 was more stable than H0, indicating that PN could enhance the stability of the internal structure of liquid crystal gel and was suitable for ophthalmology medicine.
Fig.7 (A) Apparent shear viscosity as function shear rate of precursor preparations (F0 and F2) and H\textsubscript{II} gels (H0 and H2). Inset exhibits the viscosity at specific shear rate (0.10 l/s). (B) Complex viscosity of precursor preparations (F0 and F2) and H\textsubscript{II} gels (H0 and H2) at 37 °C as a function of the applied angular frequency. (C) Variation of elastic modulus G’ and viscous modulus G'' as a function of frequency for precursor preparations (F0 and F2) and H\textsubscript{II} gels (H0 and H2). (D) Temperature sweep results displaying the complex viscosity of precursor preparations (F0 and F2) and H\textsubscript{II} gels (H0 and H2). (Strain: 0.5% Frequency: 1 Hz) from 25 °C to 45 °C.

3.3 In vitro studies
3.3.1 In vitro release study

As shown in Fig.8, the Qn of H1, H2, H3 in the previous hour were 43.84%, 34.31% and 39.49%. However, the Qn of eye drops had reached 85.54%, and it had reached 98.59% within two hours. Obviously, the drug release increased by approximately 2-folds from eye drops in comparison to the corresponding H\textsubscript{II} gels. It could be speculated that H\textsubscript{II} gels has a good sustained-release effect compared with traditional eye drops.

Hydrophilic PN is located in the aqueous channels of H\textsubscript{II} mesophase, and the drug release rate is influenced by the lattice parameters and the diameter of water cylinders. Therefore, we can deduce that although H3 contains the largest drug loading, its lattice parameters and water channel diameters are smaller than those of H1 and H2, which was supported by the outcomes of SAXS. The release trends of the three drug loads were similar. However, in terms of cumulative release, H2 was higher than H1 and H3. According to the above results, H2 has the best drug release effect, and we will choose H2 for the following experiments.
Fig. 8. In vitro release curves of H II gels and PN eye drops. All data reported as mean values±SD, (n=3).

As shown in Table 4, through the in vitro release data of liquid crystal gel, the regression coefficient of the Higuchi model was between 0.8420 and 0.8465, and was larger than other models, so it proved to be diffusion controlled release\(^{44}\). The Ritger-Peppas model was used to evaluate the drug release mechanism. The ‘n’ of the three preparations was less than 0.45, so the drug was controlled by Fickian diffusion\(^{45}\).

Table 4. Release kinetics of in situ hexagon liquid crystal and PN eye drops

| Formulations | Zero-order | First-order | Higuchi model | Ritger-Peppas |
|--------------|------------|-------------|---------------|---------------|
|              | R\(^2\)    | R\(^2\)     | R\(^2\)       | R\(^2\)       | n             |
| H1           | 0.6941     | 0.7395      | 0.8432        | 0.9576        | 0.2366        |
| H2           | 0.6331     | 0.7319      | 0.8465        | 0.9583        | 0.3024        |
| H3           | 0.6324     | 0.7373      | 0.8420        | 0.9313        | 0.3151        |
| Eye drops    | 0.3567     | 0.4330      | 0.5247        | 0.8041        | 0.0243        |

3.3.2 Study on corneal penetration
Fig. 9 In vitro corneal permeability curve of H2 gels and PN eye drops

Table 5. In vitro corneal osmotic parameters of in situ liquid crystal and PN eye drops

All the data are representative \( \bar{x} \pm SD, \ n = 3 \)

| Formulations | \( J_{\text{ss}} \times 10^2/ (\mu g\cdot s^{-1} cm^{-2}) \) | \( P_{\text{app}} \times 10^6/ (cm\cdot s^{-1}) \) |
|--------------|---------------------------------|---------------------------------|
| H2           | 3.5 ± 0.021*                    | 0.35 ± 0.18*                    |
| Eye drops    | 0.055 ± 0.018                   | 0.11 ± 0.15                     |

*P < 0.05, which is statistically different from eye drops.

Fig.10 showed the corneal penetration curve of H2 gel and eye drops. Obviously, the corneal permeability of the liquid crystal gel group was higher than that eye drops group. It could be seen from Table 5. that the apparent permeability coefficients of H2 and eye drops were \( 0.35 \times 10^6 \) and \( 0.11 \times 10^6 \) cm/s, respectively. Compared with commercially available drugs, the apparent permeability coefficient of H2 was 3.19 times that of eye drops, indicating that the rabbit cornea penetrates much more PN than eye drops.

First of all, we speculate that this difference may be due to the good biocompatibility of biolipid carriers and corneal epithelial cells, which leads to
increased solubility of the drug, which makes it easier to penetrate the corneal barrier\cite{45}. Furthermore, many literatures demonstrated that PT can promote the transdermal absorption of hydrophilic drugs\cite{46}. Therefore, it is inferred that PT itself has permeability promoting properties and can enhance the permeability of the corneal epithelium\cite{47,48}.

3.3.3 Corneal hydration level

By calculating the HL value to assess the tissue damage to the cornea. The HL value of a healthy cornea is between 76~80\%, and the corneal hydration value exceeds 83\%, indicating a certain degree of corneal damage. The HL value of the eye drops was higher than 80\%, while the HL value of the HII gels preparation was 76.91±0.43\%. The results showed that the HII gels would not produce obvious corneal irritation and damage.

4.4 In vivo study

4.4.1 Ocular irritation test

As shown in Table 6, after a single administration, the in situ liquid crystal and eye drops had little effect on the cornea and iris, the cornea was not turbid, and the conjunctiva was free of redness, congestion, swelling and other irritation. After repeated administration, the liquid crystal gel would be located in the subconjunctival sac of the rabbit's eye. The conjunctiva may be slightly reddened due to blinking reaction, but the score was lower than 3. The comprehensive results showed that both in situ liquid crystal and PN eye drops had less irritating to rabbit eyes.

| Location | Physiological saline | Eye drops | F5 |
|----------|----------------------|-----------|----|
|          | Single | Long-term | Single | Long-term | Single | Long-term |
| Cornea   | 0      | 0         | 0      | 0         | 0      | 0         |
| Iris     | 0      | 0         | 0      | 0         | 0      | 0         |

Table 6. Evaluation results of ocular irritation in single and multiple doses of improved Draize test (n=6)
4.4.2 Pre-Corneal Residence Time Analysis

As displayed in Fig. 10, the H\(_\text{II}\) gel could observe a stronger fluorescence intensity. Compared with eye drops, the fluorescent signal of the H\(_\text{II}\) gel was distributed in the subconjunctival capsule for up to 2 h. Obviously, the rabbit eyes coated
with eye drops did not show strong fluorescence intensity and were quickly cleared after 30 min, which indicates that the retention time of eye drops in the eye was very short. This showed that compared with traditional eye drops, HII gel could significantly increase the contact time of the drug in front of the cornea. We speculated that the good bioadhesion of PT led to relatively strong fluorescence intensity and slow clearance. Secondly, PT may interacted with the mucin in the corneal epithelium, thereby shortening the residence time of the drug on the corneal surface\cite{49}, or PT may have a certain degree of biodegradability in the body, causing the fluorescence intensity to gradually decrease over time. Generally, HII gel has good bioadhesion and would not reduce the residence time due to blinking, which has been verified by previous rheological results.

5 Conclusion

In summary, compared with eye drops, the liquid crystal gel precursor formulation significantly improves the bioavailability and biocompatibility of the drug. The in vitro release test results show that the liquid crystal gel had a better sustained-release effect, avoiding the shortcomings of repeated administration. In vitro corneal penetration experiments showed that liquid crystal gel could enhance corneal penetration. In addition, the corneal hydration level and Draize test proved that the preparation was less irritating to the eyes and was suitable for ocular administration. The analysis of the pre-corneal residence time proved that the preparation has good bioadhesion. In short, the liquid crystal gel precursor formulation had good fluidity and convenient administration. It had good bioadhesion, sustained release, good corneal permeability and low irritation. The research results provided a new way and method for the clinical treatment of glaucoma or other eye diseases, and provide a theoretical basis and reference for other topical drug research.

Availability of data and material

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Declaration of interest
The authors declare that there is no conflict of interest.

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**Conflict of interest**

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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