Optimized Flurbiprofen Cationic Liposomes *In Situ* Gelling System of Thermosensitive Polymers for Ocular Delivery

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ABSTRACT: A Flurbiprofen (FP) cationic liposomes *in situ* gelling system (CLIGS) of thermosensitive polymers was proposed; we investigated its *in vitro* and *in vivo* properties, and its potential use in ocular drug delivery was evaluated. This system, optimized via center composite design, was conceived from a combination of polymer- and lipid-based delivery systems. Therefore, the system could integrate the advantages of both cationic liposomes and *in situ* gels and further improve the poor stability of cationic liposomes. Cationic liposomes were characterized for their particle size, shape, entrapment efficiency, ζ potential, and photograph of transmission electron microscopy. The *in vitro* penetration capability and precorneal retention time of the FP CLIGS were evaluated by a vertical Franz-type cell method and γ scintigaraphy, respectively. The FP CLIGS showed an improved stability during a 30-day storage period over than of FP cationic liposomes. In conclusion, CLIGS serves as a means to overcome a major limitation of cationic liposomes with a prolonged precorneal retention time, enhanced stability, and convenient administration due to the modified gelatinization temperature; this justifies their use as a carrier adjuvant for ocular delivery behaviors. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 123: 3363–3374, 2012

Key words: block copolymers; drug delivery systems; gelation; modification; self-assembly

INTRODUCTION

Ocular drug-delivery systems are one of the most interesting and challenging endeavors faced by pharmaceutical scientists for the critical and pharmacokinetically specific environment that exists in the eye.[1–4] The anatomy, physiology, and biochemistry of the eye render this organ exquisitely impervious to foreign substances.[5,6] In the eye, the inner and outer blood–retinal barriers separate the retina and the vitreous from the systemic circulation, and the vitreous body reduces convection of molecules because it has no cellular components.[7]

Liposomes are small artificial vesicles that can be produced from natural nontoxic phospholipids and cholesterol (CH). Because of their size, amphiphilic properties, and biocompatibility, liposomes are promising systems for drug delivery. The properties of liposomes vary substantially with lipid composition, size, surface charge, and method of preparation. The behavior of liposomes as an ocular drug-delivery system has been proven to be relevant to their surface charge. Cationic liposomes seem to be preferentially captured at the negatively charged corneal surface compared with neutral or negatively charged liposomes. Cationic vehicles are expected to slow down drug elimination by lacrymal flow by increasing the viscosity of the solution and interacting with the negative charges of the mucus.[8] The binding affinity of liposomes to the cornea indicates that liposomes taken up by the cornea are greatest for positively charged liposomes, less for negatively charged liposomes, and least for neutral liposomes; this suggests that the initial interaction between the corneal surface and liposome is electrostatic in nature.[9] However, the poor stability and aggregation of the particles largely limit its use for ocular delivery. In this study, in an effort to resolve the disadvantages of cationic liposomes as novel carriers, our research group developed a novel ocular drug-delivery system: the cationic liposomes *in situ* gelling system (CLIGS) of thermosensitive polymers. This system was conceived from a combination of the polymer- and lipid-based delivery systems and could, thus, integrate the advantages and avoid the drawbacks of the two systems.

The CLIGS system was based on the temperature-dependent gelling properties of poly(ethylene oxide-*b*-propylene oxide–bethylene oxide) (poloxamer). The

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sol–gel transition temperature depends strongly on the concentration of poloxamer407.[10] If the concentration of poloxamer407 ranges from 20 to 30%, the temperature of the sol–gel phase translation changes between 25 and 40°C.[11] So a conventional solution of poloxamer407 solution would become a gel at room temperature; this would make it hard to be dropped into the eyes. Therefore, it has to be stored in refrigerator to make administration easier. Nevertheless, the potential irritation of low temperatures to sensitive ocular tissues must be taken into consideration. So it is meaningful to modify the phase-translation temperature by the addition of a certain amount of poloxamer188 and polyethyleneglycol6000 (PEG6000) to make sure that the dosage is a free-flowing liquid at room temperature and a gel at eye temperature.

Flurbiprofen [FP; 2-fluoro-α-methyl(1, 1′-biphenyl)-4-acetic acid] is one of the few nonsteroidal anti-inflammatory agents used to treat ocular inflammatory conditions (e.g., to prevent the myosis induced by surgical trauma, as during cataract extraction).[12]

The major purpose of this study was to develop the CLIGS of thermosensitive polymers with FP as the model molecule. The modified system was a free-flowing, droppable liquid at ambient temperature and would covert to a gel at body temperature with minimal syneresis. Furthermore, cationic liposomes were stabilized by the thermosensitive polymers, and the system showed an enhanced transcorneal penetration and stability compared with cationic liposomes and eye drops. When administrated topically, this system exhibited improved efficiency in ocular drug delivery.

EXPERIMENTAL

Materials

Poloxamer407 (PluronicF127) and poloxamer188 (PluronicF68) were gifts from BASF Corp. (Ludwigshafen, Germany). PEG6000 was bought from Haidebei Biochemical Corp. (Shandong, China). Soybean phospholipid, the purity of which was over 97%, was provided by Lipoid Corp. (KG, Ludwishafen, Germany). CH was kindly supplied by China National Medicine Corp., Ltd. Stearylamine (SA) was provided from Jiangxi Technological Co., LTD (Jiangxi, China). FP was purchased from the Wuhan Basto Pharmachem Co., Ltd. All of the other chemicals and solvents were reagent grade or better.

Preparation of FP cationic liposomes

FP cationic liposomes was prepared and optimized in the previous research by our research group as follows: a mixture of FP (6 mg), soybean phospholipid (300 mg), CH (20 mg), and SA (30 mg) was dissolved with 5 mL of ethanol in a 10-mL vial under ultrasonic conditions. Then, the solution was injected slowly into the 20 mL of distilled water with stirring in a water bath at 55°C. After the solution was injected completely, the mixture was consistently agitated (300 rpm) at room temperature for 1 h to remove the ethanol. The crude cationic liposome suspension was then treated with a probe-type ultrasonic for 5 min (active every 3 s for a 3-s duration at 400 W).

Entrapment efficiency (EE) and characterization of FP cationic liposomes

To determine EE, nonentrapped FP was separated by a dialysis method and quantified by High Performance Liquid Chromatography (HPLC) spectrometry at its maximum absorbance wavelength of 247 nm. The HPLC system was composed of a model LC-20AT pump (Shimadzu, Kyoto, Japan) and a model SPD-20A UV detector (Shimadzu). The analysis column, ultimate XB-C18 5 μm, 250 L × 4.6, was kept at 30°C. The mobile phase was a methanol–0.1*M* phosphoric acid solution (60 : 40 v/v, pH 7.0). The flow rate was 1.0 mL/min. Briefly, the coarse FP cationic liposomes (5 mL) was dialyzed (molecular weight cutoff = 12,000) against 500 mL of distilled water. Complete equilibrium of nonentrapped FP across the dialysis membrane was assumed when the concentration of FP in the dialysis medium did not increase for 2 h. After equilibrium, the concentration of nonentrapped FP was equal to that of free FP, which could go through dialysis membrane. The total FP was measured by directly dissolution of FP cationic liposomes in 80% ethanol. EE was calculated by the following formula:

$$\mathrm{EE}(\%)\frac{\mathrm{FP_{total}}-\mathrm{FP_{free}}}{\mathrm{FP_{total}}}\times 100\% \quad (1)$$

The particle size distribution of the prepared FP cationic liposomes was measured by a laser particle size analyzer (LS 230 Beckman-Coulter) at 25°C by the dilution of the liposomal dispersion to the appropriate volume with ultrapure water. The ζ potential (an indirect measurement of the vesicle surface charge) was obtained with a Delsa ζ potential analyzer (440 SX Beckman-Coulter). Samples for transmission electron microscopy were prepared at room temperature by a conventional negative staining method with 0.2% phosphotungstic acid. Samples were viewed on a transmission electron microscope (JEM-1200 EX, JEOL).

Preparation and characterization of FP CLIGS

FP CLIGS was prepared by the cold process according to the Schmolka.[13] First, an appropriate amount of PEG6000 was added to the prepared FP cationic

TABLE I
Coded and Real Levels of the Independent Variables Used in CCD

Variable	Symbol	Range and level				
		−1.682	−1	0	1	1.682
PEG6000 (mg)	X1	0	30.4	75.0	119.6	150.0
Poloxamer407 (mg)	X2	1000.0	1070.9	1175.0	1279.1	1350.0
Poloxamer188 (mg)	X3	0	60.8	150.0	239.2	300.0

liposomes. Then, poloxamer188 and poloxamer407 were added to the FP cationic liposomes slowly with stirring at 4°C after the PEG6000 was dissolved completely in the FP cationic liposomes. The FP CLIGS was obtained after a storage of dispersion at 4°C for about 12 h. The EEs of the FP CLIGS were determined by the method described in the section on Entrapment Efficiency (EE) and Characterization of FP Cationic Liposomes.

The gelatinization temperature (GT) was measured as followed:[14] A flat-bottomed glass phial, which contained a magnetic stirrer and 5 mL of FP CLIGS, was placed in a water bath (10°C). A thermometer with an accuracy of 0.1°C was immersed in the liquid to measure the GT. The stirring rate was kept at 300 rpm. Along with the temperature increasing gradually, the viscosity of the FP CLIGS became more and more sticky. The temperature when the magnetic bar completely stopped moving was considered to be the GT. The FP CLIGS lyophilized samples were viewed on a scanning electron microscope (SEM; SSX-550 Shimadzu). The viscosity of the FP CLIGS at different temperatures was measured by a rotation viscometer.

The rheological properties were studied with an AR2000ex rheometer (TA Instruments, Leatherhead, United Kingdom) with a plate geometry of 40-mm diameter samples about 1 mm in thickness. The sol–gel transition temperatures of the FP CLIGS and the blank *in situ* gelling system (IGS of thermosensitive polymers, with only gel polymer without FP cationic liposomes) were determined from oscillation at a shear rate 0.01 Hz. The temperature increased at a rate of 5°C/min. The elastic modulus (G') and the loss modulus (G'') were determined as a function of temperature. The shear rate sweeps measurements were carried out at 20.0°C (room temperature) and 35.0°C (the temperature in the conjunctival sac of the eye) by increases in the shear rate from 0 to 100 rad/s.

Optimization of the FP CLIGS formulations and statistical analysis

Response surface methodology is a statistical method that uses quantitative data from appropriate experiments to fit regression model equations and operating conditions.[15] Center composite design (CCD), which is a very commonly used experimental designs used in response surface methodology, was applied in this study to optimize the formulation of the FP CLIGS.

In this experiment, the CCD had five levels and three factors. The volume (5 mL) and component (PC/CH/SA 15/1/1.5) of FP cationic liposomes were kept constant. The amounts of PEG6000, poloxamer407, and poloxamer188 were the three significant factors that affected GT, and the corresponding CCDs are shown in Table I.

To fit a quadratic polynomial model, the software named Design-Expert (Stat-Ease, Inc., Minneapolis, USA) was used to analyze the experimental data. The polynomial model for GT was given as follows in terms of the coded factors to predict the prime formulation with a GT at 32.0°C:

$$\mathrm{GT} = A + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_1X_2 + a_5X_1X_3 + a_6X_2X_3 + a_7X_1^2 + a_8X_2^2 + a_9X_3^2 \quad (2)$$

where X_1 is the amount of PEG6000, X_2 represents the dosage of poloxamer407, X_3 is the amount of poloxamer188, A is the constant, and a_1–a_9 are the equation coefficients. GT was the gelatinization temperature which was calculated according to equation.

In vitro transcorneal penetration

Six male New Zealand rabbits (all weighing 2.50 ± 0.10 kg) were supplied by the Animal Experiment Center of Shenyang Pharmaceutical University. All studies were conducted in accordance with the publication *Guide for the Care and Use of Laboratory Animal* (US NIH publication No. 92-93, revised in 1985) and were approved by the Department of Laboratory Animal Research at Shenyang Pharmaceutical University.

After the rabbits were sacrificed, fresh rabbit corneas were sheared immediately, weighed, and reserved in glutathione bicarbonate ringer (GBR) buffer.[16] The vertical Franz-type cells were applied in the cornea penetration study. FP eye drops (2 mL), FP cationic liposomes, and FP CLIGS were added to the supply chamber, respectively. The receiving chamber was filled with 7.8 mL of GBR buffers. Each formulation contained 0.6 mg of FP. The temperature of the cells was kept at 35°C with

stirring at the bottom of the receiving chambers. The cornea was installed into the Franz-type cells within 0.5 h after excision, and the available area for diffusion was 0.70 cm^2. At time intervals of 0.5, 1, 1.5, 2, 3, 4, 5, and 6 h, 1 mL of media solution were withdrawn from the receiving chamber. An equal amount of GBR buffer was added to the receiving chamber. The final result was the mean value of three replicates. After that 0.2 mL of the filtered media solution was mixed with 10 mL of 80% ethanol, and 20 μL of the mixture was injected into the HPLC system after it was filtered with a 0.45-μm organic cellulose nitrate membrane.

The apparent permeability coefficient (P_{app}) and the flow rate of the steady state (J_{ss}) were calculated as follows:[17]

$$P_{app} = \Delta Q/\Delta t \times C_0 A \times 3600 \quad (3)$$

$$J_{ss} = C_0 P_{app} \quad (4)$$

where the term $\Delta Q/\Delta t$ is the steady state of the linear portion of the plot of the amount of drug in the receptor chamber versus time, A represents the effective cornea area for diffusion (0.70 cm^2), C_0 stands for the initial concentration of drug in the donor cell, and 3600 is the conversion of units from hours to seconds.

In vivo retention time

The *in vivo* precorneal retention of each formulation was determined by the γ-scintigraphy technique (Toshiba GCA 602A). An amount of 50 μL of radiolabeled preparations was instilled onto the left cornea to detect the radiation of Technetium-99m (^{99m}Tc; MBq) and fitted with a 4-mm pinhole.[18] The radioactive activity of the instilled what ranged from 2.1 to 2.9 MBq per 50 μL. A small plastic injector containing 50-μL aliquot solutions was placed near the left eye of the rabbit as a position tracer. After instillation, the eyelids were kept closed for 5 s to prevent loss of the instilled preparation. The rabbit was kept on a table with its head being supported by the experimenter's hand with its left eye in front of the probe at a distance of 6 cm.

Recording was started 5 s after instillation, and frames were recorded over a period of 10 min with a 128 × 128 pixel matrix. Each formulation was tested on three rabbits. A total of 63 frames of dynamic images were recorded in a sequence of 36 × 5 s followed by 12 × 10 s then 15 × 20 s frames.

Stability

The stability of the FP CLIGS was investigated for 1 month at 25°C, compared to the cationic liposomes with the EE and the average particle size as indices. The particle sizes of the FP CLIGS and blank IGS were measured with the method discussed in the section on Entrapment Efficiency (EE) and Characterization of FP Cationic Liposomes.

Ocular irritation studies

Ocular irritation studies were performed with 6 New Zealand rabbits. The blank CLIGS was instilled into the left cornea, and the FP CLIGS was instilled into the right one. An amount of 100 μL of the these two gels was administrated five times a day (at 2-h intervals).The irritation studies were carried out for 7 days. During that period, rabbits were observed periodically for ocular redness, swelling, and watering according to the scoring system of guidelines for ocular irritation testing.[19] After a 7-day examination, the rabbits were euthanized by air embolism, and the eye tissues (cornea, conjunctiva, and iris) were fixed by 4% formaldehyde, embedded in paraffin, and made into histological sections for histopathology microscopy.

RESULTS AND DISCUSSION

Preparation and characterization of FP cationic liposomes

To compare the ethanol injection–ultrasonic method, several other preparation methods were investigated, including the thin-film dispersion method and the reverse-phase evaporation method. These methods caused either low EE or unstable liposomes with a large particle size distribution. The EE of the optimized FP cationic liposomes, which was optimized previously, could reach 84.9 ± 3 (95%). The results of particle size distribution show that the mean particle size was about 136 ± 2.98 nm. The ζ potential was +33.4 ± 9.68 mV. The positive surface charge was due to the distribution of SA on the surface of liposomes during formulation.

Liposomal vesicles represented morphologically unilamellar spherical shapes (transmission electron microscopy image, Fig. 1) and a narrow particle size distribution, which was in accordance with the results of the mean particle size.

Preparation and characterization of FP CLIGS

The viscosity against temperature curve of the optimized FP CLIGS is shown in Figure 2. There was a sharp increase from 25.0 to 31.9°C, which was followed by a dramatic decrease when the temperature changed from 40.0 to 50.0°C. However, the viscosities of the FP CLIGS were only 4.67 Pa s at 25.0°C

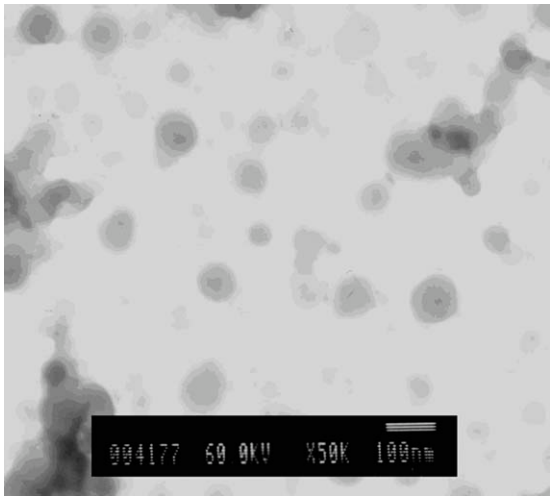

Figure 1 Transmission electron micrographs of the FP cationic liposomes at 40,000× magnification.

and 10.02 Pa s at 50.4°C; these values were much lower than that of 90.39 Pa s at 31.9°C.

The studies of the rheological properties of FP CLIGS and blank IGS are shown in Figure 3. As could be observed, G' was lower than G'' below 30°C. From 30°C onward, the G' values of the FP CLIGS and blank IGS increased drastically from 31.0 to 33.0°C as a result of the gelatinization process. After the sol–gel transition, G' and G'' became independent of the temperature in the range 33.0–50.4°C. G' was one order of magnitude greater than G''; this revealed that the FP CLIGS and blank IGS were more elastic rather than viscous. Above 50.4°C, however, G' decreased more significantly than G'' with temperature because of the peptization process.

The measurements of shear stress were carried out at 20.0 and 35.0°C (the temperature in the conjunctival sac of the eye) with the shear rates ranging between 0 and 100 rad/s, respectively (Fig. 4). At 20°C, the FP CLIGS and blank IGS were in sol status and displayed a Newtonian behavior with a linear relationship between the shear rate and shear stress. The slope of the regression line showed the dynamic viscosity of the solution. The dynamic viscosity was 0.4028 Pa s. As the temperature was increased to 35.0°C, the preparations transformed into gel status, and the rheological behavior became non-Newtonian (Fig. 5). The rheograms were fitted by the Herschel–Bulkley equation, which described a plastic behavior with the viscosity at 90.34 Pa s.

The rheological data showed that the FP CLIGS had good mechanical properties, which might help prolong their ocular retention time. An increased retention time may lead to an enhanced duration of pharmacological response, which is discussed in the section on *In Vivo* Precorneal Retention. Furthermore, the results of the rheological properties illustrate that the FP CLIGS showed behavior similar to that of the blank IGS. Therefore, we could make a conclusion that the addition of cationic liposomes did not change the thermosensitive properties.

The remarkable distinction of the rheological properties at different temperatures demonstrated the significant gelation behavior of the thermosensitive polymers. The character depended on the special structure of the thermosensitive polymers. At low temperature (<32.0°C), polyethylene oxide (PEO) and polypropylene oxide (PPO) blocks were independent, and the viscosity of polymers was too low to transfer into a gel. When the ambient temperature reached the GT, the hydrophobic PPO block inside started to dehydrate and contract; meanwhile, the hydrophilic PEO blocks outside began to hydrate and distend. Then, the poloxamer407 molecule aggregated to form spherical micelles because of the effect of tangling and stacking. If the temperature was above 40°C, the gel would recover to a free-flowing solution again.[20]

Figure 6 shows the surface morphology of the FP CLIGS powders (after lyophilization), which was examined at 20,000× magnification. It was obvious that the spherical shapes (cationic liposomes) were dispersed within the reticular structure (thermosensitive polymers), as shown in Figure 6. It was caused by the cationic liposomes encapsulated by the thermosensitive polymer. The thermosensitive polymers fracture surface was slightly rough, showing very small particles. This special structure of the FP CLIGS could prevent the liposome particles from aggregation; meanwhile, it would affect the release behavior of the FP (discussed further in the section on *In Vivo* Precorneal Retention).

Optimization of FP CLIGS and statistical analysis

The final result was the mean value of three replicates. The experiments were carried out in a randomized order according to Table II. The three-dimensional response surface shown in Figure 7 was obtained by with Oringin8.0 software according to the following equation.

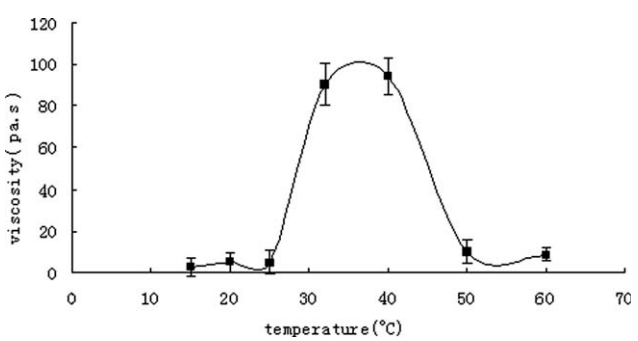

Figure 2 Viscosity against temperature of optimized FP CLIGS.

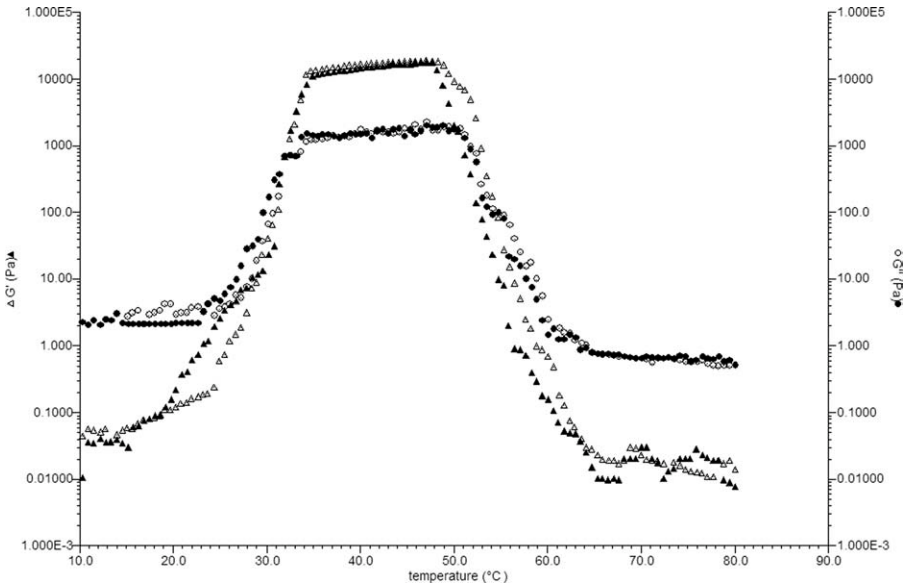

Figure 3 Graphs of the sol–gel transition temperature for FP CLIGS and blank IGS. G' as a function of temperature at 0.01 Hz: (●) G'' of FP CLIGS, (○) G″ of blank IGS, (▲) G' of FP CLIGS, and (△) G' of blank IGS.

The equation for the GT was fitted by the software Design-Expert and is shown here in terms of coded factors:

$$\mathrm{GT} = 33.7 + 3.77X_1 - 4.88X_2 + 2.87X_3 + 0.41X_2X_3 + 0.56X_1^2 - 0.89X_3^2$$

The p values of X_1X_3, X_1X_2, X_1^2, and X_2^2 were over 0.3, so they were deleted because the smaller the p value is, the more significant the corresponding coefficient will be. The coefficient adjusted R^2 value was 0.9723, which implied an excellent correlation between the independent variables.[21,22]

According to Figure 7, we could infer that the amount of the poloxamer407 was the vital factor that influenced the GT. The solution containing less than 18% (w/v) poloxamer407 did not form a gel in the tested temperature range. However, if the concentration of poloxamer407 was higher than 27%, it could cause difficulty in preparation and administration.[23] As illustrated in Figure 7(a,b), GT of the FP CLIGS decreased proportionally with the concentration of poloxamer407 (20–27% w/v). Figure 7(b,c) implies that the GT would rise with increasing amount of poloxamer188. This was because an FP CLIGS with a sufficiently high concentration would convert into gel at or above GT because of the interaction between the hydrophilic PEO and hydrophobic PPO blocks, although the addition of poloxamer188 might change the ratio of PEO/PPO, which would also

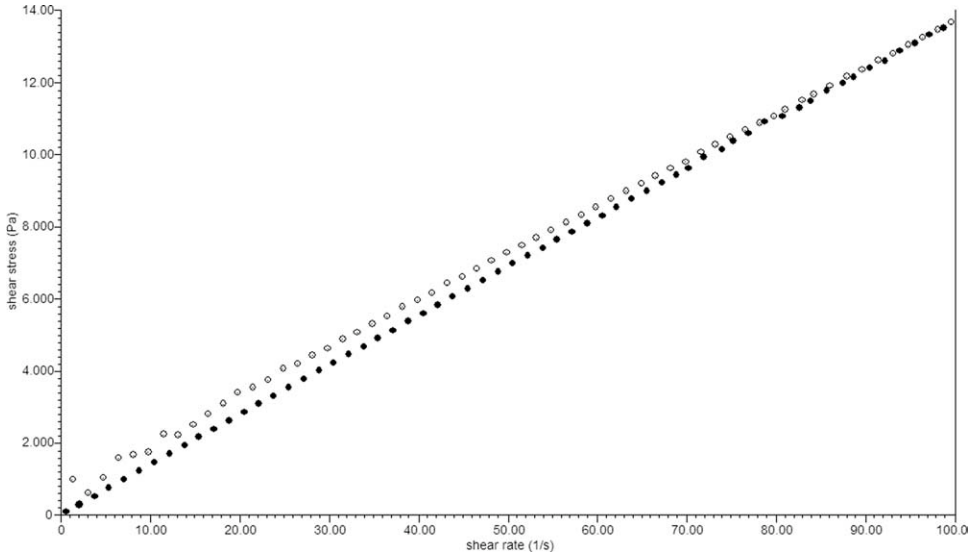

Figure 4 Rheogram of a FP CLIGS and blank IGS at 20°C: (●) FP CLIGS and (○) blank IGS.

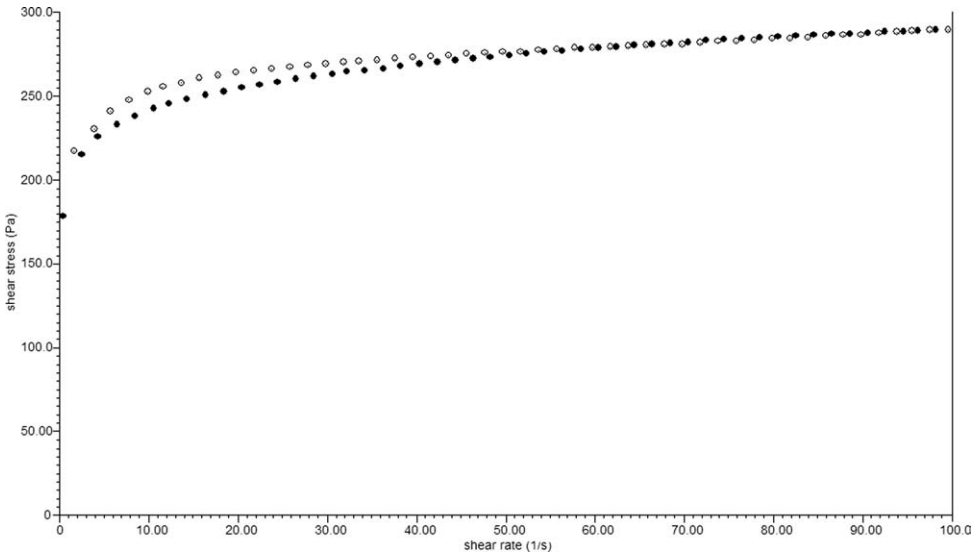

Figure 5 Rheogram of a FP CLIGS and blank IGS at 35°C: (●) FP CLIGS and (○) blank IGS.

affect the gelling mechanism.[24] As Figure 7(a,c) illustrates, GT increased with increasing amount of PEG6000, which was added to the FP CLIGS as a solubilizer. Furthermore, the effect of PEG6000 was to reduce the viscosity and increase the tolerance of the patients.[25] The predicted GT of the optimized FP CLIGS formulation was 32.0°C with the equation fitted previously, and the optimized formulation was PEG6000, 63 mg (1.26%); poloxamer407, 1250 mg (25%); and poloxamer188, 286 mg (5.72%). The actual GT could reach 31.9°C, which indicated good predictability of the model equation.

In vitro transcorneal penetration

The transcorneal penetration of FP CLIGS in the simulated ocular circumstances (35°C, pH 7.4) was studied. P_{app}, J_{ss}, and the lag time values are shown in Table III. Compared to the FP eye drops, the FP cationic liposomes and the FP CLIGS represented a significant increased permeation over a period of 6 h. The P_{app} value of the FP CLIGS (30.75×10^4 cm/s) was increased by almost 15 times over that of the FP eye drops (2.24×10^4 cm/s). The J_{ss} value of the FP CLIGS (9.22×10^4 μg s^{-1} cm) was increased by 13 times over that of the FP eye drops (0.67×10^4 μg s^{-1} cm). Additionally, according to Figure 8, the gradually rising curve of the FP CLIGS suggested that the novel gel showed a nearly sustained zero-order drug release and prevented the fluctuation of drug concentration. The possible reason might have been that the FP cationic liposomes were distributed

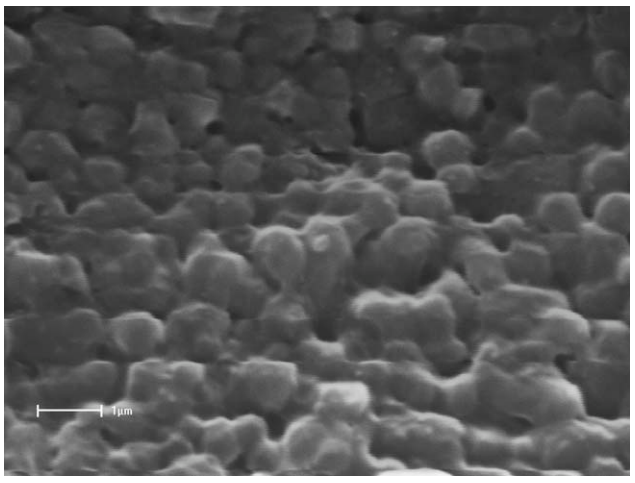

Figure 6 Scanning electron micrographs FP CLIGS powders at 20,000× magnification.

TABLE II
Observed Values for the Different Levels of CCD

PEG6000 (mg)	Poloxamer407 (mg)	Poloxamer188 (mg)	GT (°C)
−1	−1	−1	31.5
1	−1	−1	41.1
−1	1	−1	23.1
1	1	−1	29.8
−1	−1	1	36.9
1	−1	1	43.7
−1	1	1	28.7
1	1	1	35.5
−1.682	0	0	28.3
1.682	0	0	41.1
0	−1.682	0	42.6
0	1.682	0	24.4
0	0	−1.682	24.7
0	0	1.682	36.5
0	0	0	33.6
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0	0	0	33.5
0	0	0	33.7
0	0	0	33.5
0	0	0	33.6

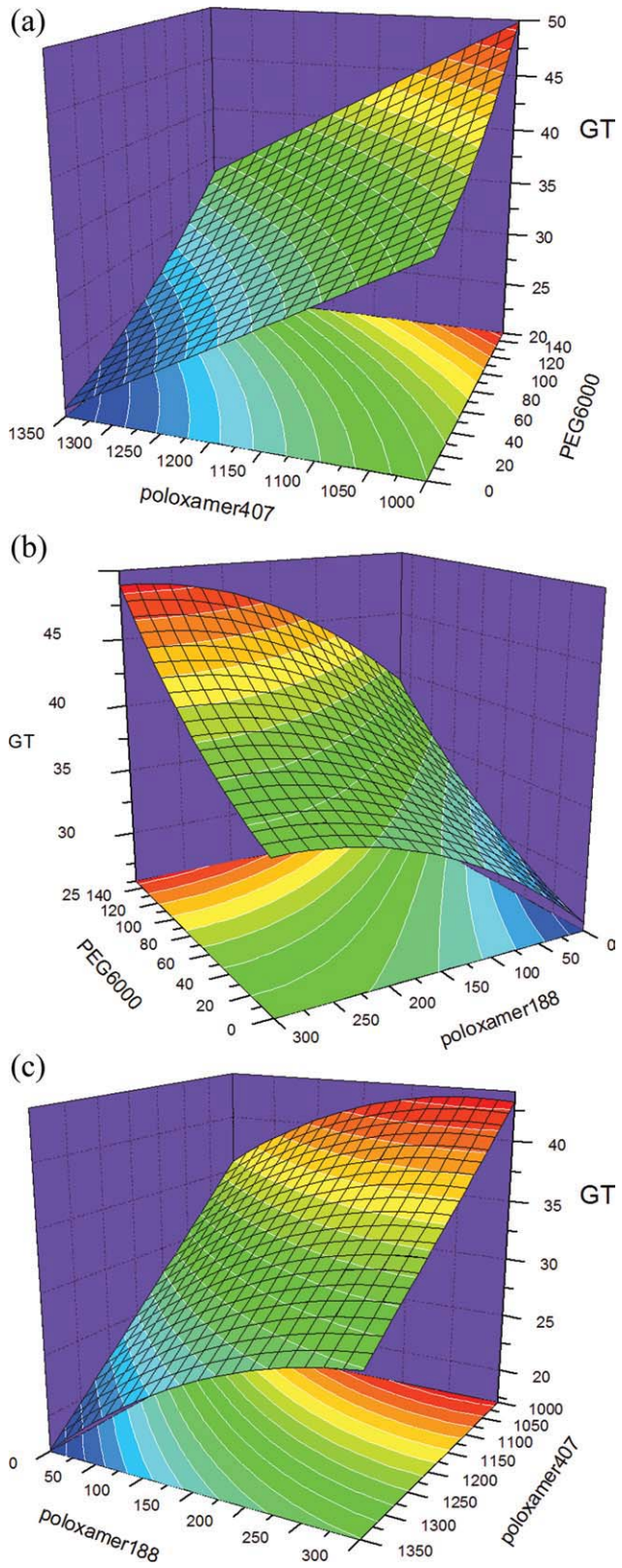

Figure 7 Response surface for the GT: (a) the amount of the PEG6000 was fixed at 75 mg, (b) the dosage of the poloxamer407 was fixed at 1175 mg, and (c) the amount of the poloxamer188 was fixed at 150 mg. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE III
Permeation Parameters of FP Through the Excised Corneas

Sample	$P_{app} \times 10^4$ (cm/s)	$J_{ss} \times 10^4$ (μg s^{-1} cm)	Lag time (min)
CLIGS	30.75	9.22	36.76
FP cationic liposomes	36.10	10.83	21.76
FP eye drops	2.24	0.67	1.57

$n = 3$.

in the hydrophilic channels among the PEO; then, the release of FP cationic liposomes from the *in situ* gel was controlled by the corrosion of the thermosensitive gel with a mode of passive diffusion.[26–29] Figure 8 also shows that the curve of the FP cationic liposomes rose sharply during the first 2 h and increased slightly throughout the next 4 h. However, there was not an obvious distinction between the two formulations in the results of the accumulated drug after 6 h. An amount of 43.3% of the drug contained in the FP CLIGS had penetrated the cornea after 6 h, which was 8.84 times higher than that of the FP eye drops (4.89%). As can been seen, the CLIGS could also enhance the penetration of FP. However, the effect was a little weaker compared to that of the cationic liposomes. Nevertheless, the results of the penetration *in vitro* could not represent the real ocular absorption because of the quick elimination in the extraocular area and the eye blink response. So with only presentation of both long precorneal retention time and high penetration could the system be a proper ocular delivery system.

In vivo precorneal retention

Precorneal retention can be used to evaluate the bioadhesion of ophthalmic formulation, and it may provide useful information for the prediction of bioavailability in the intraocular section. Noninvasive γ scintigraphy is a well-established technique for the evaluation of ophthalmic drug delivery *in vivo*. Images were analyzed by with an eNTEGRA

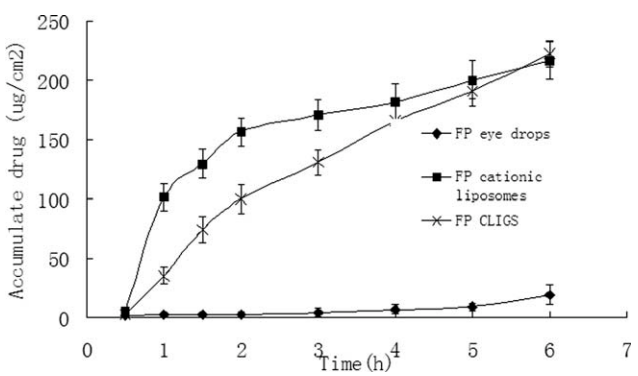

Figure 8 Transcorneal penetrations of various formulations *in vitro* of the corneas of rabbits.

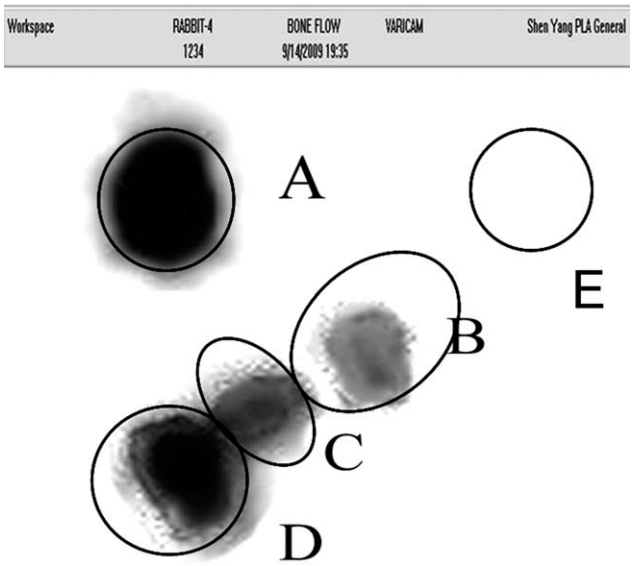

Figure 9 Typical scintigraphic images divided into five ROIs: (A) the position reference, (B) the lachrymal duct, (C) the inner canthus, (D) the precorneal surface, and (E) the background.

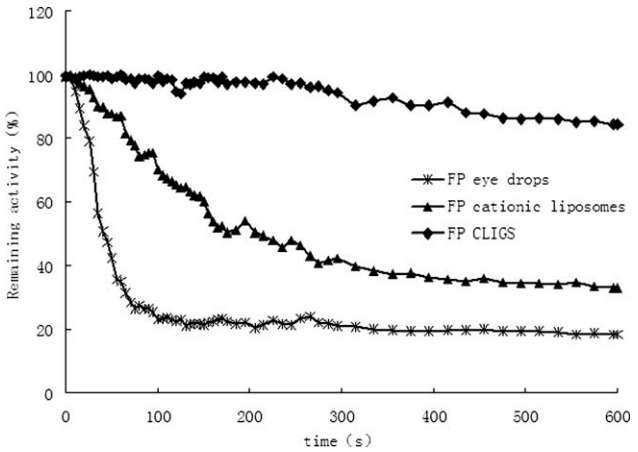

Figure 10 Precorneal clearance of various formulations incorporating ^{99m}Tc–diethylenetriaminepentaacetic acid (DTPA).

workstation 2.0 (General Electric). All of the graphs were divided into five regions of interest (ROIs): (A) the position reference, (B) the lachrymal duct, (C) the inner canthus, (D) the precorneal surface, and (E) the background, and the movement of the γ-emitting material accurately followed within these zones. The radioactivity in the anatomical ROIs (ROIs 2, 3, and 4) in the first frame was assumed to be 100% of the instilled dose (Fig. 9); the remaining activity in the precorneal ROI was plotted against time to evaluate the elimination parameters.

The curve of the remaining activity on the corneal surface versus time (10 min of dynamic imaging) is shown in Figure 10, and the parameters describing the precorneal drainage are summarized in Table IV. The parameters were the remaining activity on the corneal surface at the end of the study at 10 min (RA_{10}); the area under the curve of the percentage activity remaining in the precorneal ROI versus time ($AUC_{0–10\ min}$), which represented the retention time of the formulation tested; and the half-life of elimination ($t_{1/2}$), and the initial fraction of the elimination profile was analyzed to yield a rate constant (k_1). The results were expressed as mean ± standard deviation. SPSS 13.0 was used to identify differences that were considered to be statistically significantly at $p < 0.01$.

According to the $AUC_{0–10min}$ values, the precorneal retention time of the FP CLIGS soared significantly ($p < 0.01$) compared with the eye drops (Fig. 10). More precisely, a 3.6-fold improvement was achieved because the *in situ* gel was formed automatically on the surfaces of the rabbits' eyes. Meanwhile, the remarkable increase of $t_{1/2}$ values, which was 95.9-fold greater than that of the eye drops, suggested that elimination rate of FP was reduced because of the use of thermosensitive polymers (Table IV). Furthermore, the remaining activity of the FP CLIGS after 10 min was 83.65%, which was almost 4.5-fold that of the eye drops ($p < 0.01$). However, the FP cationic liposomes did not increase the $AUC_{0\rightarrow10min}$ and $t_{1/2}$ values significantly over those of the FP CLIGS in the mean precorneal retention time of the formulation on the corneal surface. The curve of the FP CLIGS was nearly constant. However, the other two declined dramatically in the first 100 or 200 s, which was followed by a leveling off until 600 s. These results correlated well to the obtained transcorneal penetration *in vitro*. It was evidence to prove that the FP cationic liposomes had a significantly shorter precorneal retention time than the FP CLIGS. From the

TABLE IV
Precorneal Clearance Parameters

Formulation	RA_{10} (%)	$AUC_{0–10min}$ (%)	k_1 (min^{-1})	$t_{1/2}$ (min)
1	18.40 ± 2.280	255.2 ± 36.98	0.208 ± 0.021	0.434 ± 0.079
2	33.05 ± 7.980	499.3 ± 64.08	0.072 ± 0.004	1.815 ± 0.488
3	83.65 ± 9.870	924.6 ± 72.32	0.029 ± 0.002	41.64 ± 2.085

Statistically significant difference from the reference solution at the level of $p < 0.01$.The values are mean ± standard deviation. 1, FP eye drops; 2, FP cationic liposomes; 3, FP CLIGS.

TABLE V
Stability Within 30 Days at Room Temperature

Sample	Particle size (nm)			EE (%)		
	1 days	7 days	30 days	1 days	7 days	30 days
FP CLIGS	138 ± 3.4	140 ± 4.8	153 ± 7.4	82.4 ± 1.6	80.2 ± 2.3	77.8 ± 4.4
FP cationic liposomes	142 ± 4.6	162 ± 6.3	206 ± 10.8	80.3 ± 2.5	73.8 ± 2.7	64.2 ± 4.7

Each value represents the mean ± standard deviation ($n = 3$).

results of penetration and precorneal retention time, compared with those of the FP cationic liposomes, the FP CLIGS could be considered an efficient system because of the apparently longer precorneal retention time, although its penetration rate was slightly lower than that of the FP cationic liposomes.

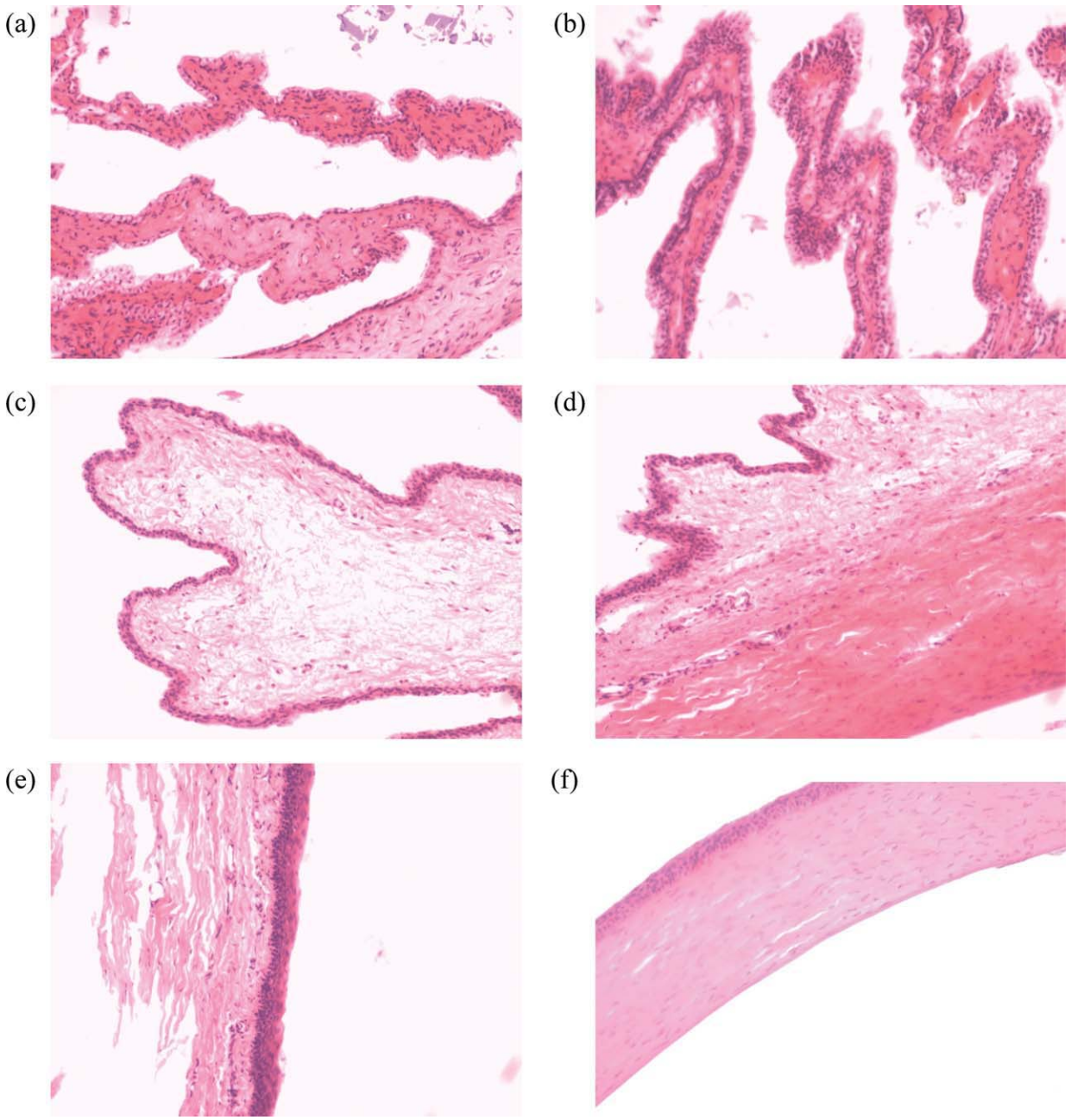

Figure 11 Histopathology slides of rabbit eyes: (a) iris of blank CLIGS, (b) iris of FP CLIGS, (c) conjunctive of blank CLIGS, (d) conjunctivae of FP CLIGS, (e) cornea of blank CLIGS, and (f) cornea of FP CLIGS. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Stability study

The physicochemical stabilities of the FP CLIGS and FP cationic liposomes are shown in Table V. At room temperature, the FP CLIGS displayed superior stability compared with the FP cationic liposomes. After 30 days, the particle size of the FP cationic liposomes increased significantly, and EE decreased to 64.2%. This was due to hydrolysis and oxidation of the lipids at room temperature, which consequently induced decomposition and aggregation of the liposome vesicles. This also resulted in drug leakage. Nevertheless, in the case of the FP CLIGS, the increase in particle size was much lower than that in the FP cationic liposomes, and EE was only slightly reduced. Furthermore, the particle size of the blank IGS was investigated according to the method in the section on Entrapment Efficiency (EE) and Characterization of FP Cationic Liposomes. The average particle size of the blank IGS was about 56 nm at room temperature, which proved that it was too small to affect the particle size measurement of the FP CLIGS. This stability study suggested that the *in situ* gel of thermosensitive polymers probably interfered in the liposome decomposition. It may have performed as isolation clothes on the liposome surface because it had a preferable stability in water. However, the instability of lipids could not be absolutely avoided, and this may explain why the particle size also increased slightly. Meanwhile, as the decomposition happened and the liposome integrity was disturbed, the *in situ* gel of thermosensitive polymers could have acted as linkers among the nearby particles; this would also have led to aggregation. The FP CLIGS had a more stable particle size than the FP cationic liposomes; this indicated the system could be reserved at room temperature instead of being stored in a refrigerator. It made administration easier and prevented the potential irritation of low temperatures to sensitive ocular tissues.

Ocular irritation studies

The average score of both the optimized FP CLIGS and blank of CLIGS was grade 1. Eyes treated with the FP CLIGS and the blank of CLIGS showed mild discharge (grade 1) after several instillations, and this might have been due to the physiological secretion and addition of SA.[30] The micrographs of the excised cornea, iris, and conjunctivae (Fig. 11) indicated that the blank CLIGS was no irritant to the cornea [Fig. 11(a)], iris [Fig. 11(c)], or conjunctivae [Fig. 11(e)]. No inflammatory cell imbibitions or necrosis, hyperemia, swelling, or distension was observed to the cornea [Fig. 11(b)], iris [Fig. 11(d)], or conjunctivae [Fig. 11(f)] after exposure to the FP CLIGS for 7 days. The cornea, conjunctivae, and iris epithelial cells maintained normal morphology and constructed integrated epithelium. Conjunctivae lymphoid tissue was identified in all of the conjunctivas without the abnormality of its size and location. Normal levels of polymorph nuclear cells were observed in the conjunctivae stoma [Fig. 11(e,f)]; this indicated that there was no ocular damage or abnormal clinical signs for the FP CLIGS. In conclusion, the formulation of FP CLIGS was safe and nonirritable for the ocular drug-delivery system.

CONCLUSIONS

The FP CLIGS of thermosensitive polymers has brought a significant modification on its ocular drug-delivery behaviors. The precorneal retention time was significantly prolonged by CLIGS because of the higher bioadhesive profile compared with the FP liposomes and eye drops. Furthermore, the FP CLIGS also demonstrated an improved, sustained transcorneal penetration capability, which was attributed to the advantages of both liposomes and gel. Therefore, the combination of the two significant advantages of the CLIGS makes it superior over any other formulations in real eye conditions. Meanwhile, the FP CLIGS displayed preferable physicochemical stability and pronounced *in vivo* ocular tolerance. Last but not least, the FP CLIGS could convert to gel at eye temperature, whereas it was a free-flowing liquid at room temperature; this could make the administration more comfortable.

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