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Liposome coated with low molecular weight chitosan and its potential use in ocular drug delivery

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ABSTRACT

In this study liposome coated with low molecular weight chitosan (LCH) was proposed and investigated its in vitro and in vivo properties, and its potential use in ocular drug delivery was evaluated. LCH with a molecular weight of 8 kDa was prepared and coated on liposome loaded with diclofenac sodium. The LCH coating changed the liposome surface charge and slightly increased its particle size, while the drug encapsulation was not affected. After coating, the liposome displayed a prolonged in vitro drug release profile. LCH coated liposome also demonstrated an improved physicochemical stability at 25 °C in a 30-day storage period. The ocular bioadhesion property was evaluated by rabbit in vivo precorneal retention, and LCH coated liposome achieved a significantly prolonged retention compared with non-coated liposome or drug solution. The LCH coating also displayed a potential penetration enhancing effect for transcorneal delivery of the drug. In the ocular tolerance study, no irritation or toxicity was caused by continual administration of LCH coated liposome in a total period of 7 days. In conclusion, the LCH coating significantly modified the properties of liposome and brought a series of notable advantages for ocular drug delivery.

1. Introduction

Ocular drug delivery system requires a series of specified characteristics according to the physiological structure of the eye. Human eye is an organ highly sensitive to exogenous substances such as debris, microorganisms and drugs. To treat the local ophthalmic diseases, liquid eye drop is the most desirable dosage form when considering convenience of administration and clinical compliance of the patients. However, conventional eye drops, most of which present in the drug solution form, usually have quite a limited therapeutic efficiency due to the low bioavailability. In clinical use of eye drops, frequent instillations are often required to get the expected therapeutic effect, and this leads to rising inconvenience and adverse effects.

The low bioavailability of eye drops is due to the quick elimination in the extraocular area. After instillation, the formulation is quickly diluted by the tear film and drained into the nasolacrimal duct. On the other hand, corneal and conjunctival epithelia of human eye, along with the tear film, construct a compact barrier preventing the drug absorption into the intraocular area.

In recent years, studies on novel ocular drug delivery systems have been reported, such as in situ gel, microemulsion, microspheres, solid lipid nanoparticles (SLN) and liposomes (Qi et al., 2007; Chan et al., 2007; Gavini et al., 2004; Cavalli et al., 2002). Generally, ocular drug delivery systems are expected to prolong the pre-ocular retention and promote the absorption of the drug. Meanwhile, the adverse effects, such as ocular toxicity, irritation, or vision interference of the delivery system should be taken into serious consideration. Liposomes serving as ocular drug delivery systems have been a promising perspective in the last decade of years. Liposome is a highly biocompatible and biodegradable drug carrier. In ocular drug delivery, it offers advantages such as prolonged drug retention and improved drug absorption (Kaur et al., 2004). Nevertheless, efforts are still needed to improve the drug delivery efficiency and to extent the application range of ocular liposomes. It has been reported that positively charged liposomes had a higher binding affinity to the corneal surface than the neutral and negatively charged vesicles as a result of interaction of positively charged liposomes with the polyanionic corneal and conjunctival surfaces, and therefore increase the drug retention and absorption (Fresta et al., 1999). However, positively charged liposomes for ocular delivery were commonly using cationic lipid such as stearylamine as positive charge substance which may lead to irritation and potential toxic effect to the eye (Taniguchi et al., 1988).

Chitosan is a natural-sourced cationic polymer with unique biological properties including favorable biocompatibility and mucoadhesiveness, and has been extensively studied in drug

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delivery research. However, chitosan is water-insoluble under physiological pH value, which largely constrains its application. When the molecular weight (MW) of chitosan is decreased by physical, chemical or enzymatic depolymerization, a marvelous improvement of its water-solubility is achieved as a result of the decrease in intramolecular hydrogen bonds (Kubota et al., 2000). Solubility of low molecular weight chitosan (LCH) increases with the decrease in molecular weight (Li et al., 2005). In recent years LCH has attracted much interest in the field of life and health sciences for not only its favorable water-solubility but also a series of biological properties which are distinct from its high molecular weight precursor (Seyfarth et al., 2008).

In this study, LCH coated liposome (LCHL) was prepared and evaluated for ocular drug delivery. Diclofenac sodium (DS) was encapsulated in the liposome as model molecule. Aqueous solution containing 0.1% (w/v) DS (used as eye drops) is clinically effective to treat postoperative ocular inflammation and pain after photorefractory keratectomy and cataract surgery (Diestelhorst et al., 1996). LCH with an appropriate molecular weight was coated on negatively charged liposome. The linking of LCH on the surface of lipid bilayer could presumably modify the action mechanism of liposome and improve its efficiency in ocular drug delivery.

2. Materials and methods

2.1. Materials

Chitosan (deacetylation degree 97% and molecular weight 540 kDa) was purchased from Haidebei Biochemical Corp. (Shandong, China). Hydrogenated soy phosphatidylcholine (PC) was supplied by Lipoid (Ludwigshafen, Germany). Phosphatidylserine (PS) was a product of Avanti Polar Lipids (USA). Cholesterol was produced by Shanghai Guoyao Corp. (Shanghai, China). Diclofenac sodium (DS) was purchased from Wujing Pharm. Corp. (Hubei, China). All other reagents were of analytical grade.

2.2. Preparation of LCH

LCH with different MWs was prepared by oxidative degradation with hydrogen peroxide (H_2O_2) (Qin et al., 2002). Briefly, 1 g of chitosan was dissolved in 80 mL of 0.05 M hydrochloride to form a uniform solution, and then added 10 mL of 30% H_2O_2. The solution was kept at 70 °C with magnetic stirring for an appropriate time to process the degradation. Subsequently the solution was concentrated at 50 °C under reduced pressure to a volume of 10 mL. The resulting solution was neutralized by 5% NaOH to pH 7.0 and then mixed with 40 mL ethanol to precipitate the product. The precipitate was collected by filtration, washed thoroughly with ethanol and dried in vacuum. Its average MW was determined by a viscosity method (Kumar et al., 2007) using an Ubbelohde viscometer (Shenli Instrument Co., Shanghai, China). The solubility of LCH in pure water was evaluated as follows: an excess amount of LCH was dispersed in water and sonicated in a bath-type sonicator (Kunshan Electronics, Jiangsu, China) for 20 min. Afterward, the solution was centrifugated at 4000 rpm for 20 min, and the precipitate was dried in vacuum and weighed to calculate the amount of dissolved LCH.

2.3. Preparation of DS loaded liposomes

Liposomes containing 0.1% (w/v) DS were prepared by an injection method. Briefly, PC, cholesterol, PS and DS in a molar ratio of 3:1:0.1:0.2 were dissolved in an appropriate amount of ethanol. The solution was injected slowly by a 21-gauge syringe needle into an appropriate amount of 105 mM calcium acetate solution, which was kept stirring at 60 °C under a nitrogen flow. After the injection, the suspension was kept under stirring for 20 min at 60 °C and then for another 20 min at 25 °C to evaporate the ethanol. The obtained suspension was homogenized under 10^4 psi (700 bar) for 6 circles by a high pressure homogenizer (NS1001L, Niro, Italy). Then the liposome was dialyzed with 0.9% NaCl for 12 h to create a calcium acetate gradient across the lipid bilayer. The resulting suspension was incubated at 50 °C for 10 min to complete the drug loading.

2.4. Coating of liposome

LCHL with different LCH concentrations was prepared as follows. Firstly LCH was dissolved in 0.9% NaCl to form a 5% (w/v) solution. Then the above mentioned DS liposome was slowly added to an appropriate portion of 5% LCH solution, followed by magnetic agitation at 25 °C for 20 min, and the volume was adjusted by adding 0.9% NaCl in order to keep the DS concentration (0.1%). Then it was sonicated for 5 min in a bath-type sonicator for particle homogenization.

2.5. Encapsulation efficiency (EE) measurement

The DS loaded liposome and non-encapsulated drug were separated by a size-exclusion separation. Briefly, Sephadex G-50 flushed with instilled water was loaded in a 5 mL syringe and then centrifugated at 2000 rpm for 5 min to obtain a dehydrated column. Subsequently, 0.5 mL of liposome was applied onto the column and centrifugated (2000 rpm, 5 min), and then the centrifugation process was repeated for 7 runs, before each run 0.5 mL of water was applied onto the column as eluent. After centrifugation, aliquots of DS loaded liposome were obtained, and the amount of encapsulated DS was determined by HPLC. Prior to HPLC analysis, the liposome was dissolved with ethanol. HPLC conditions were as follows: a Diamasil® C18 column (200 mm × 4.6 mm, 5 μm, Dikma, China) was used. The mobile phase was a mixture of methanol, water and glacial acetic acid (80:20:0.5). The flow rate was 1.0 mL min^{-1} and the column temperature was 35 °C.

EE was calculated as: EE (%) = (encapsulated drug/total drug) × 100, where the total drug represents the addition of drug encapsulated in liposome and non-encapsulated in outer water phase.

The recovery of DS in the formulation was calculated as: recovery (%) = (total drug/initially added drug) × 100.

2.6. LCH binding efficiency

The LCH binding efficiency to the liposome was evaluated by ultrafiltration. Briefly, 0.5 mL of LCHL was applied onto an ultrafilter (Amicon Ultra, Millipore Co., USA, MWCO 30 kDa) set in a centrifuge tube, followed by centrifugating at 4000 rpm for 30 min. The amount of LCH in the ultrafiltrate was determined by a colorimetric method (Muzzarelli, 1998), in which an anionic dye, Cibacron Brilliant Red 3B-A (Sigma, USA), was used to react with the amino groups in the LCH molecules. Briefly, a 0.0075% (w/v) dye solution in citrate buffer saline (pH 3.2) was prepared. Prior to analysis, 3 mL of dye solution was mixed with 0.3 mL of the above mentioned ultrafiltrate and then incubated in a 30 °C water bath for 5 min. Then the absorption at 575 nm was measured using a UV–visible spectrophotometer (UV-9100, Shanghai, China). The LCH binding efficiency was calculated as follows:

$$\text{binding efficiency}\,(\%) = \frac{\text{LCH}_{\text{total}} - \text{LCH}_{\text{free}}}{\text{LCH}_{\text{total}}} \times 100\%$$

where $\text{LCH}_{\text{total}}$ was the initial LCH amount added to the formulation.

Table 1
Grading of ocular irritation test.

Grade	Discomfort	Cornea	Conjunctiva	Discharge	Lids
0	No reaction	No alterations	No alterations	No discharge	No swelling
1	Blinking	Mild opacity	Mild hyperemia; mild edema	Mild discharge without moistened hair	Mild swelling
2	Enhanced blinking; intense tearing; vocalizations	Intense opacity	Intense hyperemia; intense edema; hemorrhage	Intense discharge with moistened hair	Obvious swelling

2.7. Particle size and zeta potential measurement

Particle size and zeta potential of LCHL were analyzed by a Nano-ZS zeta sizer (Malvern Instruments, Malvern, UK) at 25 °C. The analyses were carried out on both LCHL and plain liposomes, within 2 h after their preparation. The liposomes were diluted with distilled water prior to analysis, and each measurement was carried out in triplicate.

2.8. In vitro drug release

In vitro drug release experiments were carried out in a ZS501-A shaking bath (Liaoning, China) at 90 rpm and 35 ± 1 °C. Two milliliters of sample was enclosed in a dialysis bag and then dialyzed with 40 mL of PBS (pH 7.4). At intervals the release medium was completely refreshed in order to maintain the concentration contrast. The drug amount in the release medium was determined by HPLC.

2.9. Stability of LCHL

The stability of LCHL in different storage conditions was evaluated, and different coating levels of LCH (0.25% and 0.5%) were compared. The samples were sealed in glass bottles and stored at 4 °C or 25 °C for a period of 30 days, and at intervals aliquots of samples were withdrawn for particle size, zeta potential and EE measurement.

2.10. In vivo precorneal retention

The drug concentration in the precorneal area after instillation in rabbits was determined in order to evaluate the precorneal retention of LCHL, compared with non-coated liposome and a 0.1% DS aqueous solution. The animal study was approved and performed in accordance with the guidelines of the Institutional Animal Ethics Committee. New Zealand albino rabbits (male, weighing 2.5–3.0 kg) were used. The method was modified from a strip technique (Qi et al., 2007). Briefly, the rabbits were given an instillation of 150 μL of samples into the lower conjunctival sac of both eyes. After a certain time interval, a paper strip (2 mm × 7 mm) was gently inserted into the lower eyelid of the rabbit, then the rabbit eye was closed and the strip was kept contact for 10 s before withdraw, and then the weight gain of the strip before and after sampling was recorded. Subsequently the strip was put into a 1.5 mL centrifuge tube, added 250 μL of methanol (containing 2 μg mL^{-1} of aceclofenac as internal standard), and then was extracted by sonication for 10 min in a bath-type sonicator (KQ-100DB, China) followed by vortex for 2 min. The extract fluid was filtered by 0.45 μm membrane and dried under a nitrogen flow and stored at −18 °C. Prior to HPLC analysis, the samples were re-dissolved with 50 μL of methanol. The HPLC conditions were as described in Section 2.5. Pharmacokinetic parameters were calculated by statistical moment method (Schliecker et al., 2004).

2.11. Corneal penetration study

The effect of LCHL on drug corneal penetration was studied using freshly excised rabbit cornea and a diffusion apparatus (Camber, 1985). New Zealand albino rabbits (male, weighing 2.5–3.0 kg) were used. Briefly, 1 mL sample and 7.8 mL glutathione bicarbonate Ringer (GBR) buffer were applied at outer and endothelial side of a cornea, respectively. The apparatus were maintained at 35 °C. At appropriate intervals, aliquots of 1.0 mL samples were withdrawn from the endothelial compartment, and an equal volume of GBR buffer was replaced. The samples were filtered by 0.45 μm membrane and analyzed by HPLC.

In this study, the cumulative penetration quantity at different intervals (Q_n, μg cm^{-2}) was calculated as follows:

$$Q_n = \frac{V_0}{A}\left(C_n + \frac{V}{V_0}\sum_{i=1}^{n-1} C_i\right)$$

where V_0 is the volume of the endothelial compartment (7.8 mL); A is the area of the penetrating region (0.70 cm^2); C_n is the drug concentration in the endothelial compartment at different intervals; V is the sampling volume (1.0 mL).

The rate of drug penetration was measured by apparent permeability coefficient (P_{app}, cm min^{-1}) as follows:

$$P_{app} = \frac{\Delta Q_n}{\Delta t}\cdot\frac{1}{C_0}$$

where $\Delta Q_n/\Delta t$ is the slope rate of the straight line portion on Q_n–t plot; C_0 is the initial drug concentration in the epithelial compartment (1.0 mg mL^{-1}).

2.12. Ocular irritation test

New Zealand albino rabbits (male, weighing 2.5–3.0 kg) were used to evaluate the ocular tolerance of LCHL. For each single instillation, 150 μL samples were instilled into the lower conjunctival sac. For acute irritation, the rabbits received 3 consecutive instillations with 10 min intervals, and 30 min after the treatment the rabbits were examined for signs of ocular irritation. For long term irritation, the rabbits received instillations 5 times a day for 7 days, and the rabbits were examined at the end of the treatment. Untreated rabbits were used as control. The irritation level was evaluated by the animal discomfort and symptoms and signs in the conjunctiva, cornea, and lids (Table 1), according to the scoring system of guidelines for ocular irritation testing (Diebold et al., 2007). After the examination, the rabbits were euthanized by air embolism, and the eye tissues (cornea, conjunctiva, iris and sclera) were fixed by 4% formaldehyde, embedded in paraffin and made into histological section for histopathology microscopy.

3. Results and discussion

3.1. Preparation of LCH

In this study LCH was prepared by degradation of chitosan using H_2O_2, by which method a steady and sufficient degradation would be achieved while minimum impurity was induced. LCH was presented in the hydrochloride form in order to increase its solubility.

It was reported that chitosan with a molecular weight lower than 50 kDa could be recognized as LCH that is water-soluble (Seyfarth

Table 2
Solubility of LCH with different MWs in water (pH 7.0), each value represents the mean ± S.D. (n = 3).

MW (kDa)	5	8	10	20	30	40	50
Solubility (mg mL^{-1})	123.2 ± 8.5	77.2 ± 5.6	54.0 ± 5.3	14.6 ± 2.0	7.1 ± 0.9	1.6 ± 0.3	0.4 ± 0.1

et al., 2008). However, in our study we found that 40–50 kDa LCH did not demonstrate a sensible solubility in water at neutral pH (Table 2), though its wettability and solubility in acid solution increased significantly. When its MW was cut down to lower than 30 kDa, the solubility increased marvelously. In previous studies (Gonzalez-Rodriguez et al., 2007; Guo et al., 2003; Felt et al., 1999; Henriksen et al., 1996), the chitosan solution was adjusted to pH 5.0–6.0 with acetic acid which was necessary to solubilize chitosan. However, the physiological environment of human eye is at pH 7.4, which might lead to precipitation of chitosan and then further interrupt its pharmaceutical actions. According to our solubility results, when meeting the requirement of liposome coating (an initial LCH solution with concentration of at least 50 mg mL^{-1} was needed), LCHs with MW lower than 10 kDa were appropriate candidates. On the other hand, LCH with higher MW will provide a higher viscosity which could improve the ocular retention. Based on this consideration, in this study LCH of 8 kDa was chosen to carry out the liposome study.

3.2. Preparation of DS liposome

DS is a weak acid molecule with amphiphilic properties which can be encapsulated either in the aqueous phase or in the lipid bilayer of liposomes (Lopes et al., 2004). In this study, a modified calcium acetate gradient method for the active loading technique (ALT) of DS liposome was first reported. This method was different from the previously reported calcium acetate gradient method (Hwang et al., 1999). The EE of DS liposome prepared by our method was determined to be 100%. On the other hand, DS recovery in liposome was determined to be 99.6%, which suggested that in the dialysis process DS was drawn into the inner water phase of liposome vesicles, rather than out of the dialysis bag. Therefore, interactions between DS and LCH in the following coating process could be mostly avoided.

3.3. Effects of LCH coating on liposome

DS liposome was coated with different amounts of LCH, and its effects on liposome physicochemical properties were evaluated, as shown in Table 3. In the liposome formulation, PS was added as a negatively charged lipid which provided the binding force to the positively charged LCH. The non-coated liposome (Non-L) was negatively charged, and with the increasing amount of LCH coating the zeta potential increased from −26.1 to +10.1 mV, as a result of the interaction of LCH with the liposome surface. In the case of LCHL1 (0.1% LCH, w/v), zeta potential was nearly zero (−0.7 mV), and the particle size increased sharply which led to flocculation and precipitation of the liposome. This is because that at this relatively low concentration, LCH could only form a relatively loose coating layer on the liposome surface. On the other hand, at the condition of pH 7.0, LCH (the pK_a of which is 6.3) was only partly protonated and the positive charge was not strong enough. As a result, the negative charge of liposome was neutralized but not reversed by LCH. The flocculation of liposome might be explained that the LCH coating layer was loosely and slowly constructed, and in this process the uncoated region of liposome surface interacted with LCH chains from nearby liposomes through electrostatic force, and then resulted in aggregation of the vesicles with each other.

In the case of 0.25% LCH coating (LCHL2), the average particle size was 82.4 nm which was only slightly larger than the non-coated, and the zeta potential turned into positive. This is because that with the increasing level of LCH, a condensed coating layer was formed on the particle surface and transferred the zeta potential into positive. The slight increase in particle size might be due to the liposome aggregation in a small range. But further aggregation in a large range was prevented by the surface charge and steric hindrance from the LCH chain.

In the coating process of LCHL, the sample remained its translucent appearance when the liposome suspension was added into the LCH solution. However, in the contrary order, i.e. the LCH solution was added into the liposome suspension dropwisely, it could be observed that flocculation emerged temporarily in the instillation process but would quickly disappear under agitation. This revealed that a condensed coating layer was constructed as the LCH strength increased, and consequently the liposome particles were re-dispersed from aggregation by electrostatic repelling force. The BE of LCHL2 was 45.0% comparing to 89.5% of LCHL1, i.e., more LCH was virtually coated to liposome, and this is an evidence showing that the coating layer was intensified by the more excess of LCH.

As the LCH amount increased to 0.5% (LCHL3), there was no significant difference in terms of particle size and zeta potential comparing to that of 0.25% LCH. The BE of LCHL3 was 23.8%, which meant that the amount of liposome-attached LCH was virtually the same as LCHL2, while the excess LCH was free in the solution. It can be concluded that the coating layer had reached saturated in the presence of 0.25% LCHL, and further increase in LCH amount would not change the coating strength and zeta potential of liposome.

Regarding EE, it was not significantly affected by LCH layer on the liposome surface. The approximate 100% EE was well conserved after different levels of LCH coating. This ascertained that LCH did not interfere with the drug encapsulated inside the vesicles.

In conclusion, 0.25% was an appropriate LCH coating level which constructed a saturated coating layer and provided the liposome a stable particle size as well as a suitable surface charge. Meanwhile, a LCH level higher than 0.25%, e.g. 0.5%, would not significantly influence the liposome physicochemical properties except that there was more LCH free in solution.

3.4. In vitro drug release

The drug release of LCHL in the simulated ocular circumstances (35 °C, pH 7.4) was studied. In order to evaluate the possible interaction between LCH and DS, a physical mixture of the drug and LCHL

Table 3
Physicochemical properties of LCHL with different LCH concentrations, each value represents the mean ± S.D. (n = 3).

No.	LCH (%)	Particle size (nm)	Zeta potential (mV)	Binding efficiency (%)	EE (%)
Non-L	0	69.0 ± 3.1	−26.1 ± 1.2	–	99.6 ± 0.4
LCHL1	0.1	392.3 ± 12.5	−0.7 ± 0.1	89.5 ± 7.4	99.6 ± 0.2
LCHL2	0.25	82.4 ± 2.2	+9.6 ± 0.6	45.0 ± 5.0	100.0 ± 0.9
LCHL3	0.5	84.0 ± 4.7	+10.1 ± 0.5	23.8 ± 3.6	99.9 ± 0.4

Table 4
LCHL stability within 30 days in different storage conditions, each value represents the mean ± S.D. (n = 3).

T	No.	Particle size (nm)		Zeta potential (mV)		EE (%)	
		15 days	30 days	15 days	30 days	15 days	30 days
25 °C	Non-L	112.8 ± 5.9	151.3 ± 2.8	−26.8 ± 1.3	−28.2 ± 0.5	96.2 ± 0.6	89.2 ± 0.8
	LCHL2	93.5 ± 2.1	96.7 ± 5.3	9.5 ± 0.2	9.1 ± 0.2	98.4 ± 1.4	96.9 ± 0.2
	LCHL3	94.4 ± 1.1	97.0 ± 1.9	10.3 ± 1.0	9.8 ± 0.4	98.1 ± 1.1	97.2 ± 1.0
4 °C	Non-L	75.7 ± 1.5	79.4 ± 3.7	−26.9 ± 0.9	−26.6 ± 0.7	99.6 ± 0.2	99.0 ± 0.5
	LCHL2	86.3 ± 1.2	90.8 ± 4.0	9.6 ± 1.2	9.5 ± 0.8	99.8 ± 0.6	99.5 ± 0.2
	LCHL3	86.4 ± 2.4	89.2 ± 3.8	10.2 ± 0.4	10.0 ± 1.1	99.6 ± 1.8	99.7 ± 1.5

(DS + LCHL) which was prepared by adding DS into a blank LCHL (0.25% LCH) was also included.

DS release from solution, Non-L, LCHL and DS + LCHL, each containing 0.1% DS (w/v), is shown in Fig. 1. For DS solution, about 80% of the drug was released at 2 h, and the release amount reached the maximum at 6 h. In the case of DS + LCHL where both LCHL and free LCH existed, the release profile was unchanged comparing to that of the solution. This suggests that there was no interaction between DS and LCH which would affect the drug release rate.

The release rate of either Non-L or LCHL was significantly lower than that of the solution. The difference between Non-L and LCHL was significant, whereas that between LCHL2 and 3 was not significant. At 6 h, the release percentage of Non-L, LCHL2 and 3 was 38.9%, 25.4% and 23.8%, respectively, and after 24 h, 61.4%, 55.2% and 54.4% of DS were released, respectively. The DS release profile was prominently prolonged by the liposome encapsulation, and further by LCH coating. The drug was completely entrapped in the liposome without any drug free in solution or absorbed on the liposome surface, and therefore there was no burst release at the beginning. In this case, the drug release rate depends on the membrane permeability which is affected by the fluidity of lipid bilayer (Volodkin et al., 2007). The liposome prepared in this study (PC/cholesterol = 3/1, molar ratio) has an approximate transition temperature (T_m) of 32 °C (McMullen et al., 1994). At the experimental condition (35 °C), the lipid bilayer is in liquid-crystalline phase which permits the drug permeation. For the case of LCHL, the drug permeation rate was reduced by the presence of LCH coating. The mechanism might be explained that LCH molecule is closely linked to the liposome surface and constructs an intense shell, which probably restricts the bilayer fluidity. Consequently, the membrane was consolidated and the permeability was decreased.

3.5. Stability

The physicochemical stability of LCHL with different coating levels is shown in Table 4. At 25 °C, LCHL2/3 displayed superior stabilities compared with Non-L. After 30 days, the particle size of Non-L significantly increased, and the EE reduced to 89.2%. This is due to hydrolysis and oxidation of the lipids at room temperature which consequently induce decomposition and aggregation of the liposome vesicles. This also resulted in drug leakage and slight decrease in the zeta potential. However in the case of LCHL2/3, the increase in particle size was much lower than that in Non-L, and the EE was only slightly decreased. This suggests that the LCH layer probably has an inhibition on the liposome decomposition. It may perform as a "shield" on the liposome surface, because it has a preferable stability in water. But the instability of lipids cannot be absolutely avoided, and this may explain that the particle size also slightly increased. Meanwhile as the decomposition happens and the liposome integrity was disturbed, the LCH chains could act as linkers among the nearby particles, this would also lead to aggregation.

On the other hand at 4 °C, both coated and non-coated liposomes demonstrated significantly improved stabilities. The increase in particle size was lower than that of 25 °C, and there were no significant changes in zeta potential or EE. This suggests that the decomposition of liposome was inhibited at low temperature. LCHL2/3 had a more stable particle size than Non-L, indicating the stabilizing effect of LCH coating layer.

3.6. In vivo precorneal retention

Precorneal retention can be used to evaluate the bioadhesion of ophthalmic formulation, and it may provide useful information for prediction of bioavailability in intraocular section. Fig. 2 and Table 5

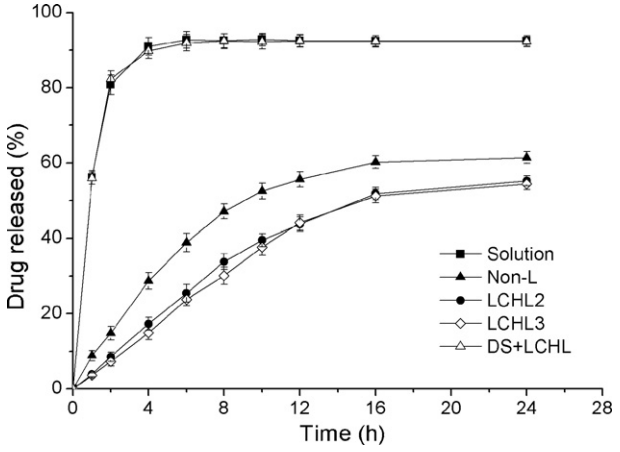

Fig. 1. In vitro release profiles of DS in different vehicles, each value represents the mean ± S.E. (n = 3).

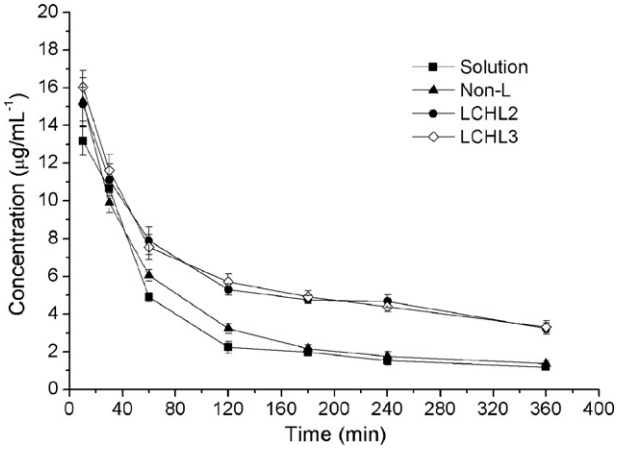

Fig. 2. DS concentrations in precorneal regions following topical instillation, each value represents the mean ± S.E. (n = 6).

Table 5
Pharmacokinetics parameters of DS in precorneal regions of rabbits after topical instillation, each value represents the mean ± S.E. ($n = 6$).

Sample	$AUC_{0-\infty}$ (μg mL^{-1} min)	MRT (min)	K_e ($\times 10^3$ min^{-1})
Solution	1566.2 ± 132.8	311.1 ± 31.6	3.37 ± 0.32
Non-L	1898.3 ± 176.7*	360.6 ± 56.9	3.29 ± 0.70
LCHL2	3565.5 ± 373.0**	436.8 ± 55.7	2.47 ± 0.30
LCHL3	3641.7 ± 386.5**,***	445.2 ± 42.3	2.37 ± 0.26

* $P > 0.05$, comparison with solution.
** $P < 0.001$, comparison with solution or Non-L.
*** $P > 0.05$, comparison with LCHL2.

show the drug elimination profile of LCHL in rabbit precorneal regions. Comparing to drug solution, Non-L only had a limited effect on the precorneal retention of the drug ($P > 0.05$). In contrast, LCHL achieved significantly higher AUC (area under the curve of concentration) and longer MRT (mean residence time) than either Non-L or solution ($P < 0.001$), while the difference between LCHL2 and 3 was not significant ($P > 0.05$). This suggests that the LCH coating was essential to prolong the retention of liposome encapsulated drug, but the difference of the amount of free LCH in the solution was not a prominent factor affecting the precorneal retention.

The surface of cornea and conjunctiva is covered by a thin fluid layer called mucus film (Ludwig, 2005). The primary component of mucus is mucin, a high molecular mass glycoprotein which is negatively charged at physiological pH. Thus, the positively charged LCH coating layer can provide a binding force to the eye surface. Nevertheless, the bioadhesion of chitosan is not exclusively determined by the positive charge. It could also be promoted by the presence of free amine and hydroxyl groups of chitosan molecules which form hydrogen bonds to the eye surface. This might explain that the LCHL could achieve a prolonged retention though its positive charge was limited at neutral pH.

In another in vivo study (Henriksen et al., 1996), it was reported that liposome coated with chitosan (high MW) did not significantly increase the precorneal retention of ^{125}I-BSA comparing to non-coated liposome. This might be explained that high MW chitosan is only soluble in acidic solution, and when transferred into physiological pH, the amine groups will be deprotonated, which will result in formation of a large amount of intramolecular hydrogen bonds along with change of molecule conformation. Consequently the hydrogen bonds between chitosan molecule and eye surface were largely diminished. Meanwhile, the increased pH also resulted in aggregation of chitosan coated liposome (Henriksen et al., 1997). Therefore, the bioadhesion of chitosan coated liposome would be interrupted in vivo. However in the case of LCHL, at physiological pH LCH remains its free amine and hydroxyl groups to interact with the eye surface and therefore significantly improves the precorneal retention.

3.7. Corneal penetration study

The corneal penetration study was carried out in order to evaluate the effect of LCH on the drug transcorneal transportation. The corneal penetration profile is shown in Fig. 3, and the corresponding P_{app} and R^2 (correlation coefficient) are shown in Table 6 (where the linear regression was carried out at the interval of 120–360 min). The penetration profiles were linear in all cases ($R^2 > 0.996$), which indicated that the cornea integrity was maintained under the experimental conditions and the penetration rate was constant. The P_{app} of Non-L showed no significant difference with the solution ($P > 0.05$), this is because that DS is a small molecule (MW = 318) and it can give a relatively high penetrating rate without the aid of liposome. Although the contact of drug with the corneal surface can be improved by liposome, it would be offset by the prolonged drug release from liposome and the P_{app} was virtually decreased.

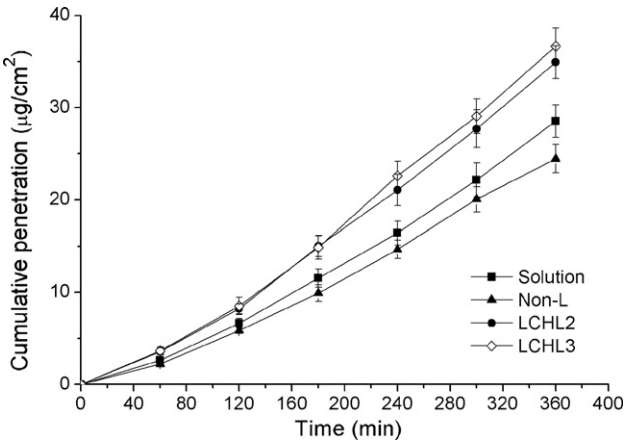

Fig. 3. Corneal penetration profiles of DS in different vehicles, each value represents the mean ± S.E. ($n = 4$).

However, LCHL produced a significantly higher P_{app} than Non-L ($P < 0.05$). This can be explained that the LCH layer could intensify the binding of liposome to the corneal surface by both electrostatic force and hydrogen bonds, which consequently facilitate the drug absorption into the cornea. On the other hand, the polycationic LCH could improve the permeability of cornea by opening the tight junctions among corneal epithelial cells, which was similar to the case of chitosan (Di Colo et al., 2004). Consequently, the drug penetration was significantly enhanced by the LCH coating. The difference between LCHL2 and 3 was not significant ($P > 0.05$), suggesting that the penetration enhancing effect of LCHL would not further increase with the excess amount of LCH.

Ocular penetration enhancers have been widely investigated in recent years (Liu et al., 2006; Saettone et al., 1996) such as benzalkonium chloride, non-ionic surfactants, bile salts and EDTA, and many of them were effective to increase the corneal penetration rate. However, their potential toxicity to ocular tissues should be considered (Chetoni et al., 2003). LCH is a biocompatible and biodegradable polymer, and LCH coating of liposome achieved a pronounced penetration enhancing effect comparing to non-coated liposome. Therefore, the potential use of LCH as a non-toxic penetration enhancer, especially for liposomal drugs, is notable.

3.8. Ocular irritation test

Both acute and long term ocular irritation of LCHL2 and 3 were studied. In all the experimental rabbits, no signs of discomfort appeared (grade 0) during either acute or long term test. LCHL treated eyes showed mild discharge (grade 1) after several instillations, and this might be due to the aggregation of lipid from liposome and the physiological secretion. For the symptoms of cornea, conjunctiva and eyelids, only grade 0 was recorded for both treated and control groups, in either acute or long term test.

Table 6
Effects of LCHL on apparent permeability coefficient (P_{app}) of DS through corneal, each value represents the mean ± S.E. ($n = 4$).

Sample	P_{app} (cm min^{-1} × 10^4)	R^2
Solution	0.906 ± 0.077	0.9962 ± 0.0012
Non-L	0.789 ± 0.069*	0.9981 ± 0.006
LCHL2	1.102 ± 0.067**	0.9992 ± 0.0003
LCHL3	1.174 ± 0.080**,***	0.9990 ± 0.0003

* $P > 0.05$, comparison with solution.
** $P < 0.05$, comparison with Non-L.
*** $P > 0.05$, comparison with LCHL2.

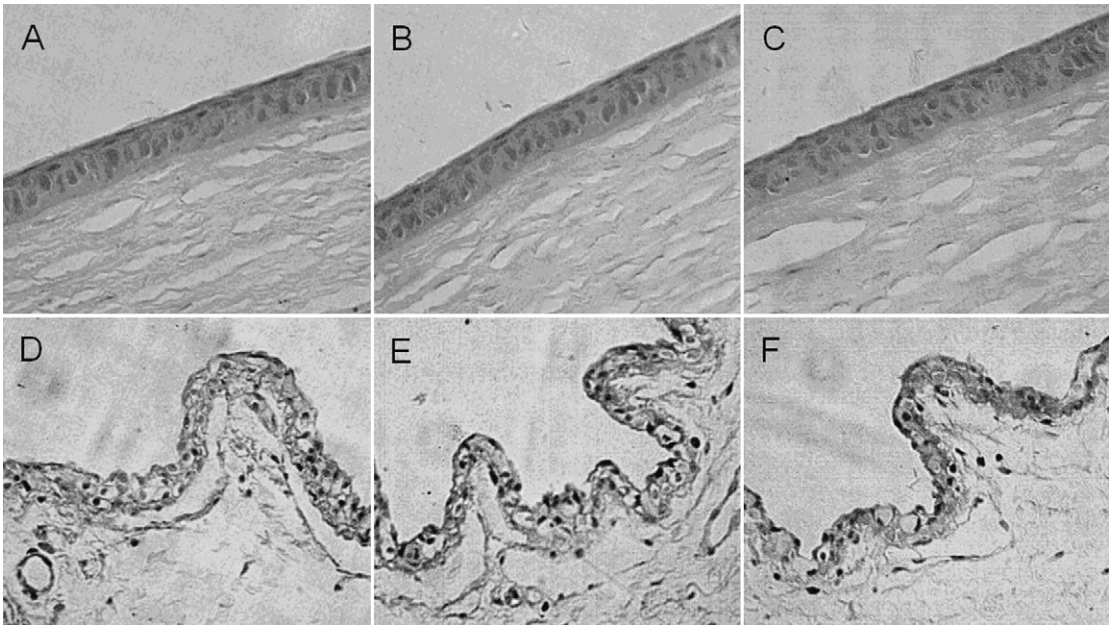

Fig. 4. Histopathology microscopy of the ocular tissues after treated with LCHL for 7 days. A–C: cornea of non-treated (A), treated with LCHL2 (B) and LCHL3 (C); D–E: conjunctiva of non-treated (D), treated with LCHL2 (E) and LCHL3 (F).

Fig. 4 shows the histopathology of the tested rabbit eyeballs after long term irritation test. Normal and healthy structures of ocular tissues were observed in all the tested eyes and there were no differences between LCHL treated groups and control group. Corneal and conjunctival epithelial cells maintained normal morphology and constructed integrated epithelium (Fig. 4A–E). The basal cells of cornea remained abundant and were normally packed by junction complex (Fig. 4A–C). Conjunctival lymphoid tissue was identified in all the conjunctivas without the abnormality of its size and location (Fig. 4D–F). Normal levels of polymorphonuclear cells were observed in the conjunctival stroma (Fig. 4D–F), indicating that there were no signs of inflammation. The histopathology confirmed that no ocular irritating effects were induced by LCHL compared with non-treated eyes. The combination of liposome and LCH, both of which are biocompatible, demonstrated a preferable ocular tolerance.

4. Conclusion

LCH coating of liposome has brought a significant modification on its ocular drug delivery behaviors. The LCH coating layer gave the liposome a positive surface charge as well as an excellent bioadhesive property, as shown in this study. The precorneal retention was significantly prolonged by LCHL, compared with either non-coated liposome or drug solution. LCHL also demonstrated an improved transcorneal drug penetration rate, which was attributed to the penetration enhancing effect of LCH. Meanwhile, LCHL displayed preferable physicochemical stability and pronounced in vivo ocular tolerance. These findings proposed a novel ocular drug delivery system, and its mechanism and application in delivery of other molecules, such as macromolecule drugs, will be further investigated in our undergoing studies.

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