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Nanostructured lipid carrier (NLC) coated with Chitosan Oligosaccharides and its potential use in ocular drug delivery system

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ABSTRACT

The objective of the present investigation was to explore the potential of the Chitosan Oligosaccharides (COS)-coated NLC (nanostructured lipid carrier) for ocular drug delivery. NLC loaded with flurbiprofen was prepared by melt-ultrasonic method and then coated with COS with a molecular weight of 3000–6000 kDa. After coating, the particles reflected spherical morphology with smooth surface under transmission electron microscope (TEM) analysis and a changed zeta potential from −0.446 mV to +20.7 mV. The ocular bioadhesion property was evaluated by Gamma scintigraphic technique, revealing that the clearance of the formulations labeled with radioactive ^{99m}Tc-DTPA was significantly delayed in the presence of COS, and the AUC of the COS-coated formulation had a 7.7-fold increase comparing with non-coated ones. Additionally, enhanced transcorneal penetration was achieved by using the COS coating with a corresponding apparent permeability coefficients (P_{app}) which had a 2.4-fold increase comparing with the reference. Consequently, COS coating modified the properties of NLCs and presented a series of notable advantages in ophthalmic application.

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1. Introduction

Pharmaceutical preparations are applied topically to eyes to treat surface or intraocular conditions. Since the capacity of the eye can only retain a limited volume, excessive liquids, both normally produced and externally delivered, rapidly drain from the eye. Therefore, topical drug administration is restricted in small amount. On the other hand, due to the dynamics of the lacrimal system, the retention time of an ophthalmic solution on the eye surface is short, and the amount of drug absorbed is usually only a small fraction of the quantity administered. In addition, corneal and conjunctival epithelia of human eye, along with the tear film, construct a compact barrier preventing the drug absorption into the intraocular area (Li et al., 2009). This mechanism removes the exogenous substances involving entraps debris, microorganisms, and even drugs from the ocular surface, assisted by frequent blinking. These are the dominating reasons that many drugs have the difficulty in penetrating the ocular barrier and reaching the target tissues. Therefore, in clinical use of eye drops, frequent instillations are often required to get the expected therapeutic efficacy, which may lead to inconvenience and other system side effects through nasolacrimal absorption as well.

In recent years, studies on novel ocular drug delivery systems have been reported, such as in situ gel, microemulsion, microspheres, liposomes and solid lipid nanoparticles (SLN) (Qi et al., 2007; Chan et al., 2007; Gavini et al., 2004; Cavalli et al., 2002) all of which aim to prolong the pre-ocular retention and promote the absorption of the drug. As the second generation deriving from SLN (Müller et al., 2002), nanostructured lipid carriers (NLC) based on mixture of solid lipids with spatially incompatible liquid lipids (Müller et al., 2002) combines many features for application of pharmaceutics, i.e. controlled release of actives, drug targeting, and increasing the amount of drug penetrating into mucosa. On account of the physiological and/or biodegradable lipids, this carrier system also exhibits an excellent tolerability. Nevertheless, efforts are still needed to improve the drug delivery efficiency, especially, aiming to prolong the retention time of drug on the corneal surface to some extent.

Chitosan Oligosaccharides (COS), obtained through the decomposition of Chitosan, is a cationic polymer of low molecular weight. Unlike Polysaccharides Chitin and Chitosan, which are not water-soluble and therefore have some limitation in the use of pharmaceutics, high water solubility of COS makes it suitable for pharmaceutical application which is correlated to its structure and unique biological activities including favorable biocompatibility and mucoadhesiveness, in addition, its special property of

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Table 1
Composition of 0.03% flurbiprofen NLC dispersion.

Ingredient	Quantity (mg)
Oil phase	
Flurbiprofen	6
Compritol ATO 888	250
Miglyol 812	100
Gelucire 44/14	100
Water phase	175
Solutol HS 15	
Tween 80	75
Glycerol	400
Water ad	20

antimicrobial (Xia et al., 2010) is also taken into consideration in ophthalmic application.

It is reported that cationic polymers were probably admirable mucoadhesive materials due to an ability to develop molecular attraction forces by electrostatic interactions with the negative charge of the mucus (Ludwig, 2005). Taking this into account, the polycationic COS has been investigated as an ophthalmic vehicle and its superiority was extensively studied in drug delivery. The linking of COS which has a molecular weight of 3000–6000 kDa with the surface of the nanoparticles would presumably modify the action of NLC and ameliorate its efficiency in ocular drug delivery.

2. Materials and methods

2.1. Materials

Compritol 888 ATO (glyceryl behenate) and Gelucire 44/14 (polyoxylglycerides) were obtained as a gift from Gattefosse (France), Solutol HS-15 (polyoxyethylene esters of 12-hydroxystearic acid) was kindly supplied by BASF (Germany), Miglyol 812N (C8–C12 triglyceride) was given by Sasol (Germany). Flurbiprofen (FP) was supplied by Hangzhou Keben Chemical Co., Ltd. (Zhejiang, China). Chitosan Oligosaccharides (COS) was purchased by Tianjin Hiromi Biotechnology Development Co., Ltd. (Tianjin, China). All other chemicals and reagents used were of analytical grade.

2.2. Preparation of FP loaded NLC

The formulations of FP-NLCs, listed in Table 1, were prepared by melt-emulsification and ultrasonication technique. Briefly, appropriate amounts of FP, Compritol 888 ATO, Miglyol 812N and Gelucire 44/14 were blended and melted at 85 °C to form a uniform and clear oil phase. Meanwhile, the aqueous phase consisting of dispersing surfactant Solutol HS-15 and Tween 80 in double distilled water was added dropwise to the oil phase at the same temperature by the aid of agitation at 600 rpm for 10 min. The coarse emulsion was then treated by probe-ultrasonic (JY-92-II, Xinzhi, China) cell disruptor for 5 min (active every 3 s for a 3 s duration, 400 W). Subsequently the dispersion was cooled to room temperature to solidify nanoparticles and stored at 4 °C.

2.3. COS coating of NLC

For COS-coated NLCs, appropriate amounts of the polymer was dissolved in water in order to form a series of various concentrations (0.1%, 0.3%, 0.5%, 1%, w/v), then mixed with FP loaded NLC dispersions. In each case, an aliquot of NLC was mingled with an equal volume of COS liquor by adding it dropwise to the polymer solution under continuous agitation at room temperature (20 °C) for a 30-min incubation.

2.4. Drug encapsulation efficiency (EE)

Free FP (non-incorporated in the FP-NLC) was separated by ultrafiltration centrifugation technique (Zhuang et al., 2010). Briefly, 1 mL of FP-NLC colloidal solution was placed in the upper chamber of a centrifuge tube matched with an ultrafilter (Amicon ultra, Millipore Co., USA, MWCO 10 kDa) and centrifuged for 15 min at 4000 rpm. The total drug content in FP-NLC was determined as follows: aliquots of 1 mL FP–NLC dispersion were diluted appropriately by ethanol to dissolve the lipid ingredient and then the obtained suspension was filtrated through 0.45 μm membrane filters. The resulting solution was analyzed by HPLC. 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 (73:22:5). The flow rate was 1.0 mL min^{-1} and the column temperature was 35 °C. The drug loading content was the ratio of incorporated drug to lipid (w/w). The encapsulation efficiency (EE) and drug loading could be calculated by the following equations, respectively:

$$\text{EE} \quad (\%) = \frac{W_{\text{Total}} - W_{\text{Free}}}{W_{\text{Total}}} \times 100$$

$$\text{Drug} \quad \text{loading} \quad (\%) = \frac{W_{\text{Total}} - W_{\text{Free}}}{W_{\text{Lipid}}} \times 100$$

where W_{Total}, W_{Free}, W_{Lipid} were the weight of total drug in NLC, the weight of unentrapped drug in ultrafiltrate and the weight of lipid added in system, respectively.

2.5. Particle size and zeta potential measurement

The average particle size and polydispersity index (PI) of FP–NLCs were determined by Laser Particle Size Analyzer (Coulter LS-230, Beckman Coulter Co. Ltd., USA). The zeta potential was analyzed by a Nano-ZS zeta sizer (Malvern Instruments, Malvern, UK) at 25 °C after appropriate dilution with ultra-purified water. Each measurement was made in triplicate.

2.6. TEM analysis

The morphological observation of COS-coated NLC was performed by transmission electron microscopy (JEM-1200EX, JEOL). The sample demanded was prepared by placing a drop of formulation which was diluted 50-fold with double-distilled water onto a 400-mesh copper grid coated with carbon film and followed by negative staining with 1% phosphotungstic acid.

2.7. Corneal penetration study

The penetration-enhancing effect of COS-coated NLCs was evaluated on isolated rabbit corneas (available areas 0.70 cm^2) using a perfusion apparatus (Camber, 1985). Albino New Zealand albino rabbits (male, weighing 2.5–3.0 kg) were used. Resumptively, 1 mL sample and 7.8 mL glutathione bicarbonate Ringer (GBR) buffer (O'Brien and Edelhauser, 1977) were applied into the epithelial (donor) side and endothelial (receptor) side of the cornea, respectively. The apparatus were maintained at 35 °C. At 40-min intervals, 1.0 mL sample was withdrawn from the receiving compartment, and was immediately replaced with an equal volume of preheated GBR buffer. Each experiment was continued for 4 h in triplicate. The osmolality of all perfusion solutions was adjusted with glycerol to 292–298 mOsm/kg, evaluated by freeze 256 drying point measurements on a Fiske osmometer.

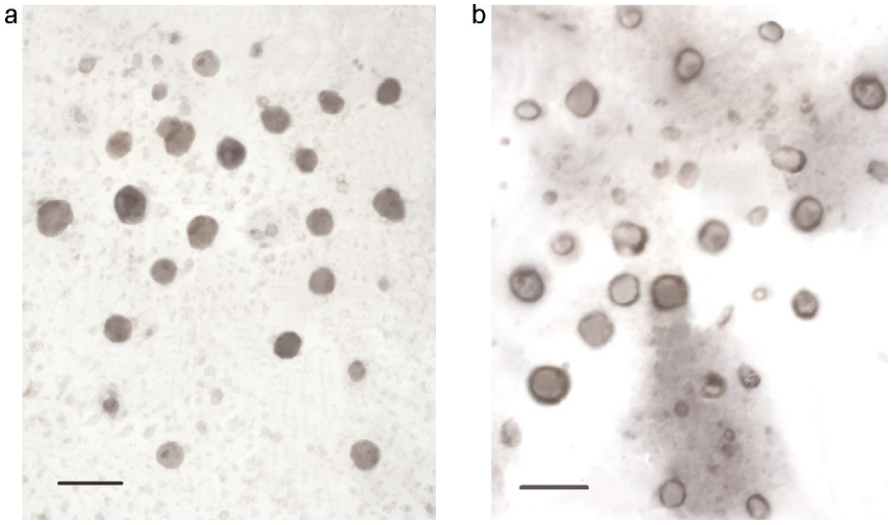

Fig. 1. TEM morphology of (a) NLC and (b) COS-coated NLC bar = 200 nm.

In this study, the cumulative penetration quantity at different intervals (Q_n, $\mu g\,cm^{-2}$) 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); 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}, $cm\,min^{-1}$) and J_{ss} as follows (Schoenwald and Huang, 1983), respectively:

$$P_{app} = \frac{\Delta Q}{\Delta t\, C_0\, A\, 60} \qquad J_{ss} = C_0 P_{app}$$

where $\Delta Q_n/\Delta t$ is the slope rate of the straight line portion on Q_n–t plot; A is the area of the penetrating region (0.70 cm^2); C_0 is the initial drug concentration in the epithelial compartment (1.0 mg/mL).

2.8. In vivo pre-corneal retention

Albino rabbits ($n = 9$) were used in the study and each rabbit was restrained and administrated in front of the Gamma scintigraphic camera. The flurbiprofen formulation was labeled by adding a specified amount of radioactive substance ^{99m}Tc in the water phase and then processed via the same method as for preparation of FP-NLC and COS-coated NLCs. Exactly 25 μL (Meseguer et al., 1993) of test formulation containing radioactive materials were instilled directly into the lower fornix of the conjunctival sac of the right eye. Recording was started 5 s after instillation and frames were photographed by a Gamma camera to detect the radiation of ^{99m}Tc (140 keV) over a period of 10 min using a 128 × 128 pixel matrix.

2.9. Ocular irritation test

Three male albino New Zealand rabbits weighing 2.0–2.5 kg were used to evaluated ocular tolerance of COS coating, by instilling 50 μL samples in the right eye, while the contralateral eye was served as control. For irritation test, the rabbits received a single instillation 5 times a day in a 7-day period, and were examined at the end of the application. According to the scoring system of guidelines for ocular irritation testing (Diebold et al., 2007), symptoms and signs were examined by slit-lamp involving cornea (opacity), iris (inflammation degree), and conjunctiva (congestion, swelling and discharge) to evaluate the irritation value. After the examination, 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 and characterization of NLC coated COS

3.1.1. Morphological shape

We designed a lipid formulation coated with COS aiming that the COS molecules would adhere to the NLC surfaces by means of hydrogen bonding and hydrophobic interaction between COS and neutral lipid. Fig. 1 shows the ultrastructure of FP-NLC and COS-coated NLC determined by TEM. The micrograph of FP-NLC illustrated a spherical structure which was ascribed to the nano-droplets (Fig. 1, left). The COS-coated NLC dispersion demonstrated a quite different appearance of micrographic photo (Fig. 1, right): the spherical-shaped particle with an obvious double-layer structure was observed. The existence of COS polymer uniformly surrounded on the surface of NLC particle leading to a slightly lager vehicle, which was also consistent to the experimental results of the particle size analysis.

Table 2
Physicochemical properties of samples coated with different COS concentrations (mean ± SD, $n = 3$).

Sample	COS concentration (%)	Average size (nm)	Zeta potential (mV)	EE (%)
NLC	0	55.4 ± 2.3	−0.446 ± 0.2	98.3 ± 0.4
NLC-COS1	0.3	68.3 ± 3.7	+18.0 ± 0.9	98.7 ± 0.2
NLC-COS2	0.5	77.9 ± 2.4	+19.2 ± 0.4	99.1 ± 0.2
NLC-COS3	0.8	169.9 ± 8.8	+20.7 ± 1.3	97.9 ± 0.7

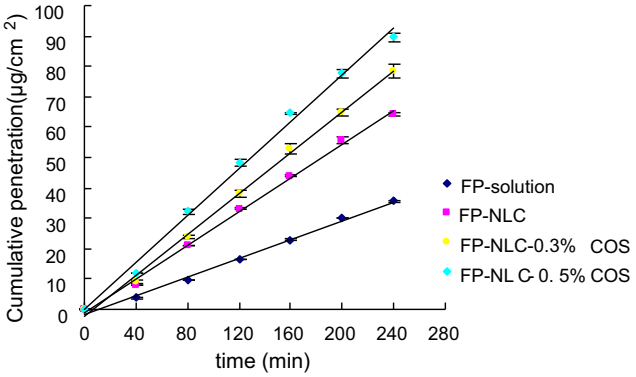

Fig. 2. Corneal penetration profiles of FP in different vehicles (mean ± SD, $n = 4$).

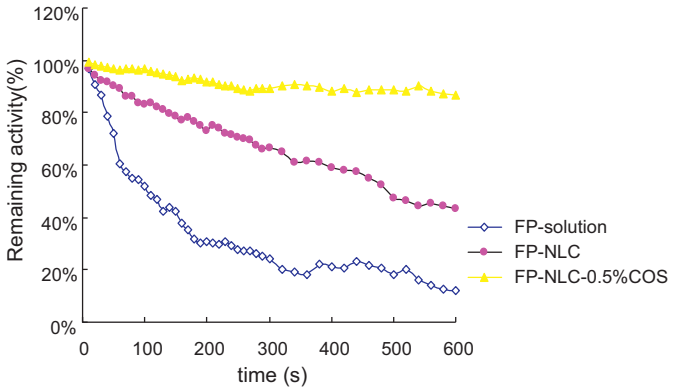

Fig. 3. Pre-corneal drainage of ^{99m}Tc-DTPA labeled formulations after topical instillation.

3.1.2. Particle size and zeta potential

Table 2 depicts physicochemical properties of NLCs coated with different COS concentrations. FP-NLC was coated with different concentrations of COS, and its effects on NLC physicochemical properties were evaluated, as shown in Table 1. In the case of NLC, particle size of which was 55.4 nm, possessed the zeta potential of nearly zero (−0.446 mV). With the increasing amount of COS coating from 0% to 0.8% (v/w), zeta potential values varied from −0.446 to +20.2 mV, owing to the interaction between the COS and the NLC surface. Meanwhile, particle size rises slightly along with the augment of COS, representing 68.3 nm and 77.9 nm with the concentration of 0.3% and 0.5% COS, respectively, compared with non-coated NLC with an average particle size of 55.4 nm. As the COS amount was up to 0.8%, a significant difference was implied in respect of particle size, however, the zeta potential came to a relatively constant value. It can be concluded that the coating layer had reached a saturated status in the presence of 0.5% COS, and excessive amount of COS in the solution would not change the coating strength and zeta potential of NLC, instead, they lead to a sharp rise with regard to particle size.

Due to the highly positive charge that COS carried, the adsorption of COS increased the density of positive electron cloud that resulted in the positive electricity of integral particle as well. This increment in the surface charge was ascribed to the formation of complexes with the coating mechanism involving hydrogen bonding between the polysaccharide and the glyceride head groups (Grant and Allen, 2006). The result of zeta potential and scanning electron micrograph supported the conclusion that COS adsorption occurred.

3.2. In vitro corneal permeation study

The corneal penetration study was carried out in order to evaluate the effect of COS on the drug transcorneal transportation. Fig. 2 illustrates the corneal penetration profiles of three different dosage forms, FP-phosphate eye drop (0.03% FP in phosphate buffer saline, pH 7.4), FP-NLCs and COS-coated FP-NLCs. A straight line was obtained for all preparations in the time range from 0 to 240 min, while the corresponding apparent permeability coefficients (P_{app}) and R^2 (correlation coefficient) are displayed in Table 3. The penetration profiles were linear in all cases ($R^2 > 0.994$), which indicated that the cornea integrity was maintained throughout the experimental conditions and the penetration rate was constant. Compared with FP-phosphate eye drop, FP-NLCs showed a slightly higher P_{app}, which would be attributed to the biocompatibility of the lipid matrix with the corneal epithelial cells. However, while NLCs were coated with COS, it produced a significantly higher P_{app}, which exhibited a 2.4-fold increase more than that of the non-coated ones. This would be explained that the COS could improve the permeability of cornea by opening the tight junctions among corneal epithelial cells or by intracellular routes, which was similar to the case of Chitosan (Colo et al., 2004). Furthermore, the polycationic COS layer could strengthen the adherence of NLCs to the corneal surface by both electrostatic force and hydrogen bonds, and consequently facilitated the drug absorption into the cornea.

In comparing the influence of COS amount to the transcorneal penetration, NLCs coated with two different concentrations of COS in terms of 0.3% and 0.5% were investigated as well. The curve graph obtained demonstrated that with the increasing of the COS amount in the formulations from 0.3% to 0.5%, P_{app} of the samples ascended steadily from 18.39 to 20.26 ($cm\,s^{-1} \times 10^4$), while the cumulative penetration amount of 0.5% COS coating remained higher than that of 0.3% COS coating during the entire period.

Along with the development of ophthalmic pharmacy, ocular penetration enhancers have been widely studied 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 as well (Chetoni et al., 2003). Whereas, COS, along with its biocompatible and biodegradable properties, was proved to be a non-toxic penetration enhancer, especially for ophthalmic application with its pronounced penetration-enhancing efficacy.

3.3. In vivo pre-corneal retention

Gamma scintigraphy is a well-established technique for *in vivo* evaluation of pre-corneal retention time (Wei et al., 2002), which could be used to evaluate the bioadhesion of ophthalmic formulation and played a key role to provide useful information for prediction of bioavailability in intraocular section as well.

Table 3
Permeation parameters of FP through the excised corneas (mean ± SD, $n = 3$).

Sample	P_{app} ($cm\,s^{-1} \times 10^4$)	J_{ss} ($\mu g\,s^{-1}\,cm \times 10^4$)	R^2
Phosphate solution	8.958 ± 0.11	25.68 ± 0.33	0.9951 ± 0.0004
NLC	15.56 ± 0.19	46.15 ± 0.58	0.9961 ± 0.0002
NLC–0.3%COS	19.07 ± 0.45	55.82 ± 1.36	0.9972 ± 0.0034
NLC–0.5%COS	20.26 ± 0.43	64.48 ± 1.34	0.9947 ± 0.0017

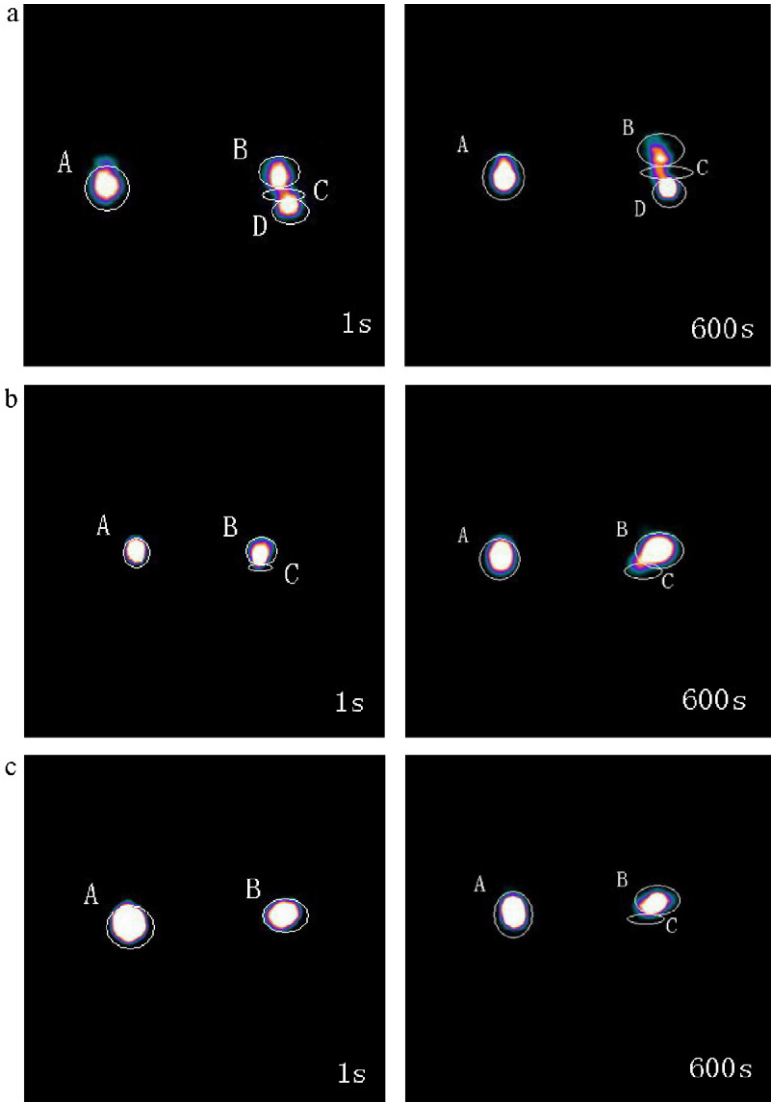

Fig. 4. Gamma scintigraphic images showing the migration of the formulations labeled with ^{99m}Tc: (a) FP-phosphate solution, (b) FP-NLC, (c) FP-NLC-COS in 1 s and 600 s dynamic image, regions of interests (ROIs) were divided into four parts according to the results of the residual radioactive activity, A: position reference, B: cornea/conjuctiva surface, C: inner canthus, D: naso-lacrymal duct.

Localization of ^{99m}Tc-solution in rabbits' cornea over time, as determined by Gamma camera imaging, is displayed in Fig. 3. Regions of interests (ROIs) were divided into four parts according to the results of the residual radioactive activity, A: position reference, B: cornea/conjuctiva surface, C: inner canthus, D: naso-lacrymal duct. The observation of the acquired photos exhibited a good spreading over the entire pre-corneal area for COS based formulations immediately after administration (Fig. 3, Group C), as compared to the control sample of FP phosphate solution. To be specific, the reference formulation (Fig. 3, Group A) illustrated that no adhesive properties existed, thus the activity in the corneal region moved constantly forward from the pre-ocular regions to naso-lacrymal duct with a rapid elimination. Up to 10 min, only 16.3% radioactive ^{99m}Tc remaining on the corneal surface. When instilled with COS-coated FP-NLC, a stable intensity of radioactivity was observed mainly in the cornea/conjuctiva surface rather than other regions as well as inner canthus and naso-lacrymal duct, which indicated the good bioadhesion and spreadability of COS. At 10 min, a relatively strong intensity of 86.9% still existed in the corneal surface, which was almost 5.33 and 2.02 times more than that of FP-solution group and FP-NLC group, respectively. This suggests that the COS coating may serve to prolong the retention of NLC encapsulated drug. From the results obtained, a possible explanation could be obtained that cationic polymers such as Chitosan and its Oligosaccharides probably provide superior mucoadhesive properties on account of either secondary chemical bonds such as hydrogen bonds or ionic interactions between the positively charged amino groups of chitosan and the negatively charged sialic acid residues of mucins (Lehr et al., 1992; Henriksen et al., 1996). Moreover, this *in vivo* performance was proved to be significantly higher at neutral or slightly alkaline pH as in the tear film (Rossi et al., 2001).

The curves of the remaining activity on the corneal surface as a function of time (600 s dynamic imaging) are plotted in Fig. 4, and the parameters describing the pre-corneal clearance are summarized in Table 4. Statistical analysis of these data have demonstrated that comparing with drug PBS solution and FP-NLC, the addictive of COS led to significantly higher $AUC_{0-10min}$ with a 7.7-fold and a 2.8-fold improvement on the cornea, respectively. Meanwhile, the clearance of COS coated FP-NLC in the initial phase was much slower, as the clearance rate (k) was obviously lower than that of the other two formulations. In terms of $t_{1/2}$, PBS solution displayed a rapid half-value period of 2.821 min, which was far below the COS-coated formulation. This remarkable rise also confirmed

Table 4
Pre-corneal clearance parameters (mean ± SD, $n = 3$).

Sample	RA_{10} (%)	$AUC_{0-10\ min}$ (μg/mL min)	k (min^{-1})	$t_{1/2}$ (min)
Phosphate solution	16.3 ± 2.11	3293.1 ± 553.0	0.421 ± 0.043	2.821 ± 0.288
NLC	43.6 ± 6.28	9049.8 ± 3267.9	0.282 ± 0.021	15.519 ± 1.085
NLC–0.5%COS	86.9 ± 8.87	15,356.6 ± 4050.7	0.121 ± 0.017	164.834 ± 12.488

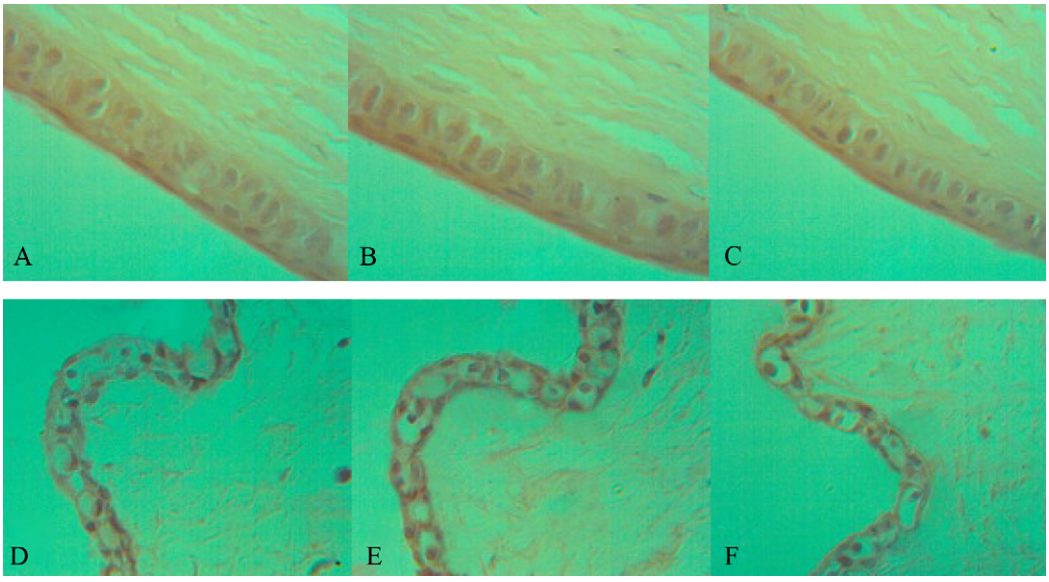

Fig. 5. Histopathology microscopy of the ocular tissues including cornea (a–c) and conjunctiva (d–f) after treated with different solutions for 7 days. (a and d) Non-treated; (b and e) NLC; (c and f) COS-coated NLC.

the relation between COS and bioadhesion that incurred a notable stagnation time in the cornea/conjuctiva region.

3.4. Ocular irritation test

Fig. 5 presents the histopathology of the tested rabbit eyeballs with various preparations to investigate their influence on cell structure and tissue integrity.

Pathology of rabbit eyeballs and lids confirmed the presence of normal ocular surface structures in both control and COS treated eyes. Corneal and conjunctival epithelial cells maintained normal morphology and integrated epithelium. The basal columnar cells of cornea can be recognized as single layer cell and were normally packed by tight junction complex (Fig. 5a–c). Similarly, normal levels of scattered polymorphonuclear cells were observed in the conjunctival stroma (Fig. 5d–f), indicating that there were no signs of inflammation and tissue edema. Treatments of corneas with COS-coated formulations are exemplified in Fig. 5c and f, and the histopathology confirmed that no ocular irritating effects were induced by COS compared with non-treated eyes. The combination of NLCs and COS, both of which showed a good corneal biocompatibility, demonstrated a preferable ocular tolerance.

4. Conclusion

From the results obtained it is concluded that after NLCs were coated with COS, they may have a series of predominance when considering their effectiveness as carriers throughout the ocular drug delivery. The particle was coated with the COS polymer surrounding the surface uniformly, which resulted in the positively charged of NLC dispersions, thus provided a longer retention time by interacting with the negative mucous. Eventually, an improved penetration rate was achieved with the presence of COS concerning its effective contribution to the corneal permeability. In addition, a most notable advantage that COS-coated NLCs, comparing with non-coated ones, was that they possess superior mucoadhesive properties which was beneficial to the ocular drug delivery system. Taking these findings into account, COS-coated NLC might represent a promising vesicle in the ophthalmic application.

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