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Thiolated nanostructured lipid carriers as a potential ocular drug delivery system for cyclosporine A: Improving *in vivo* ocular distribution

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ABSTRACT

Ophthalmic drug delivery with long pre-corneal retention time and high penetration into aqueous humor and intraocular tissues is the key-limiting factor for the treatment of ocular diseases and disorders. Within this study, the conjugate of cysteine-polyethylene glycol monostearate (Cys-PEG-SA) was synthesized and was used to compose the thiolated nanostructured lipid carrier (Cys-NLC) as a potential nanocarrier for the topical ocular administration of cyclosporine A (CyA). The rapid cross-linking process of Cys-PEG-SA *in vitro* was found in simulated physiological environment. The *in vitro* CyA release from Cys-NLC was slower than that of non-thiolated nanostructured lipid carriers (NLC) due to the cross-linking of thiomers on the surface of nanocarriers. After topical ocular administration in rabbits, the *in vivo* ocular distribution of CyA was investigated in comparison of Cys-NLC with non-thiolated NLCs and oil solution. The results showed that CyA concentration in systemic blood was very low and close to the detection limit. The area-under-the-curve ($AUC_{0-24\,h}$) and mean retention time ($MRT_{0-24\,h}$) of Cys-NLC group in aqueous humor, tear and eye tissues were significantly higher than that of oil solution, non-thiolated NLCs ($p < 0.05$). These results demonstrated that the thiolated NLC could deliver high level of CyA into intraocular tissues due to its bioadhesive property and sustained release characteristics.

1. Introduction

Cyclosporine A (CyA) is a potent immunosuppressive agent, which could specifically inhibit the activation of T lymphocytes (Nussenblatt and Palestine, 1986). Over the past years, numerous studies have showed the potential applications of CyA in ophthalmology (Hingorani et al., 1999; Murphy et al., 2005; Tang-Liu and Acheampong, 2005). Systemic administration of CyA is an effective therapy for the corneal graft rejection and some eye disorders. However, the long term systemic administration of a relatively high dose of CyA may lead to the serious systemic adverse effects, such as hypertension, nephrotoxicity and hepatotoxicity (Algros et al., 2002; Rumelt et al., 2002). Topical ocular administration of CyA provides a good alternative to the systemic delivery. It has been reported that topical CyA could be used in the treatment of a variety of immune-mediated ocular surface disorders like vernal conjunctivitis (Gupta and Sahu, 2001), dry eye syndrome (Kunert et al., 2002), and the prevention of corneal allograft rejection (Perry et al., 2002).

Due to its high hydrophobicity ($\log p = 3.0$) and low aqueous solubility (0.04 mg/ml, 25 °C) (el Tayar et al., 1993; Prasad et al., 2007), CyA cannot be formulated into the commonly aqueous formulations. Meanwhile, the intraocular penetration of CyA is very poor. To overcome these obstacles, various attempts have been made to develop ophthalmic formulations of CyA, including vegetable oils (BenEzra and Maftzir, 1990), colloidal carriers (liposomes, nanoparticles, emulsions, micelles, and solid lipid nanoparticles) (Calvo et al., 1996; Gokce et al., 2009; Kuwano et al., 2002; Milani et al., 1993; Stevenson et al., 2000), hydrogel (Kapoor and Chauhan, 2008), and collagen shields (Pleyer et al., 1994). Generally, the most frequent reference vehicle, CyA oil solution, is considered to be poorly tolerated and provide a low ocular bioavailability. RESTASIS® (0.05% CyA ophthalmic emulsion), the first and only therapy for patients with keratoconjunctivitis sicca, cannot achieve therapeutic efficiency in preventing rejection after corneal allograft (Price and Price, 2006). To treat intraocular diseases like uveitis and other intraocular inflammation, high concentration of CyA should be delivered to the aqueous humor and the iris/ciliary body (Lallemand et al., 2003). Although progress has been made, none of the topical ocular delivery systems mentioned above could really succeed in delivering therapeutic amount of CyA into the target intraocular tissues with low toxicity.

Abbreviations: Cys-PEG-SA, cysteine-polyethylene glycol monostearate; PEG-SA, polyethylene glycol monostearate; NLC, nanostructured lipid carrier; Cys-NLC, Cys-PEG-SA modified NLC; PEG-NLC, PEG-SA modified NLC; STF, simulated tear fluid.

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The specific anatomy and physiology of eye limit the ocular absorption of most bioactive compounds (Dey and Mitra, 2005; Koevary, 2003). Attempts to improve the ocular bioavailability of drugs have been focused on overcoming pre-corneal constraints through improving corneal penetration and prolonging pre-corneal retention (Kaur and Smitha, 2002). Novel colloidal carrier, nanostructured lipid carriers (NLCs) are known to good biocompatibility, feasibility of large scale production by high pressure homogenization, high drug loading capacity and controlled drug release compared with other colloidal carriers. Recent reports indicated that NLC could increase the ocular bioavailability of lipophilic drug, ibuprofen (Li et al., 2008). Our previous research showed that NLC could improve the penetration of bioactive compounds into ocular tissues with a good ocular tolerance (Shen et al., 2010). Moreover, we demonstrated that NLC with thiolated modification could effectively prolong pre-corneal retention time *in vivo* (Shen et al., 2009).

The aim of this study was to investigate whether the thiolated NLC could really deliver the encapsulated CyA into intraocular tissues, thus could be an effective therapeutic strategy to the treatment of intraocular diseases, such as uveitis.

2. Materials and methods

2.1. Materials

4-Nitrophenyl chloroformate was obtained from Suzhou Time-Chem Technologies Co., Ltd., China. Polyethylene glycol monostearate (PEG-SA, the polymerization degree of ethylene glycol is 40) was kindly donated by Nanjing WELL Chemical Co., Ltd. China. L-Cysteine and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Bio Basic Inc., Canada. Glyceryl palmitostearate (Precifac ATO® 5) was obtained from Gattefosseĭ (France). Propylene glycol dicaprylate/dicaprate (Miglyol® 840) was obtained from Sasol (Germany). Tween 80 (analytical reagent) was purchased from Shanghai Chemical Reagent Co., Ltd., China. Cyclosporine A (CyA) was purchased from Galena (Czech Republic). Methanol and acetonitrile were HPLC grade. Dichloromethane, triethylamine, and other chemicals were all analytical reagent grade.

2.2. Stability of Cys-PEG-SA

Thiolated polyethylene glycol monostearate (Cys-PEG-SA) conjugates were synthesized and stored at 4 °C as previously reported (Shen et al., 2009). The storage stability of Cys-PEG-SA conjugates at 4 °C was investigated in a period of 3 months. At intervals aliquots of samples were withdrawn for the free thiol group amount measurement.

To investigate the cross-linking process of thiolated agent in different aqueous solutions, the decrease of thiol group was quantified as a time function. Cys-PEG-SA was hydrated in acetic acid buffered saline (ABS, pH 5.0) and freshly prepared simulated tear fluid (STF, composition: NaCl 0.67 g, $NaHCO_3$ 0.20 g, $CaCl_2{\cdot}2H_2O$ 0.008 g and distilled, deionized water to 100.0 g) (Lin and Sung, 2000) in a final concentration of 0.2% (m/v), respectively. The samples were incubated at 4, 25 and 34 °C with water bath shaker. At predetermined time points, aliquots were withdrawn and the amount of remaining free thiol group was determined. The experiment was carried out in triplicate.

2.3. Preparation of NLC formulations

The NLC formulations were prepared by the melt-emulsification method. Briefly, 500 mg lipid matrix (consisted of 70% Precifac ATO® 5 and 30% Miglyol® 840) and 20 wt.% CyA relative to total lipid were mixed and melted at 80 °C. The melted mixture was then dispersed in aqueous solution (heated up to 80 °C) containing 2 wt.% Tween 80 and 2 wt.% PEG-SA using a mechanical stirrer to form a coarse emulsion. The warm emulsion was further treated 90 times (work 2 s and stand 3 s) by a probe sonicator (JY92-II, Ningbo Scientz Biotechnology Co., Ltd., China) at 400 W to form homogenous nanoemulsion. Then, the nanoemulsion was incubated with the same volume of aqueous solution, 2% Cys-PEG-SA solution and 2% PEG-SA solution at 80 °C for 5 min, respectively. The resultant nanoemulsion was rapidly cooled in an ice bath (0 °C) to solidify the lipid matrix and form NLC, Cys-NLC and PEG-NLC.

2.4. Characterization of NLC formulations

The diluted NLC formulations were dissolved in methanol and the total drug amount was measured by HPLC. The drug entrapment efficiency (Ee) was determined as reported previously (Shen et al., 2010). The mean particle sizes of the NLC formulations were determined by Zetasizer (3000HS, Malvern Instruments, UK) after being diluted 20 times with distilled water. The pH of the NLC formulations was determined at 25 °C using pH-meter (Shanghai Precision & Scientific Instrument Co., Ltd., China), which was calibrated at pHs 4.0 and 7.0 with standard solution. Osmotic pressure was determined by the freezing-point method using Osmomat 030-D (Gonotec, Germany) on a 0.1 ml aliquot of the sample. The osmometer was calibrated using standard solution (0–0.3 osmol/kg).

2.5. Determination of thiol groups content

The amount of free thiol groups on the Cys-PEG-SA was determined via Ellman's reagent (DTNB) as described previously (Hornof et al., 2003). The quantity of free thiol groups was calculated from a standard curve obtained by the unmodified PEG-SA solutions mixing with L-cysteine in a series of concentration (2–150 μg/ml). The actual amount of thiol groups modified onto NLC was determined as reported previously (Shen et al., 2009).

2.6. In vitro CyA release study

The *in vitro* drug release profiles of CyA-loaded NLC formulations in STF were investigated by dialysis method. The fluid contained 0.1% sodium dodecyl sulfate to assess sink conditions during *in vitro* release studies. To start the release, 0.5 ml of NLC formulations were transferred into dialysis bag with a membrane MWCO of 100 kDa, which were then put into 50 ml of release medium stirred at 100 rpm at 34 ± 0.5 °C. The dialysis bag was rapidly moved into fresh release medium (50 ml) constantly in the scheduled time intervals, and the CyA content was determined by HPLC after filtration through 150 nm filters. The experiment was carried out in triplicate.

2.7. In vivo ocular distribution investigation of CyA

Male New Zealand albino rabbits (weighing 2–2.5 kg), housed on standard laboratory diet at an ambient temperature and humidity in air-conditioned chambers were used for the present studies. All animal experiments were conducted in full compliance with local, national, ethical and regulatory principles for animal care. Reference formulation for *in vivo* studies was CyA castor oil solution. Animals were randomly divided into 4 groups, and each received 25 μl of an oily control solution or the same volume of NLC, PEG-NLC and Cys-NLC containing 500 μg of CyA in two instillations at 90 s intervals using a micropipette without actually touching the eyes and irritating the corneal surface. At 0.5, 1, 2, 4, 8 and 24 h following the administration, the ocular tear samples (2 μl) were collected into eppendorf tubes using a capillary. The blood samples were immediately taken before sacrificing the animals with a sodium pentobarbital overdose. Then, the ocular surface was rinsed

with normal saline and dried with filter paper to remove any drug remaining in the tear fluid. The aqueous humor, the conjunctiva, the cornea and the iris-ciliary body were collected from each eye and placed into tubes which were later weighed. All samples were stored at −80 °C until analysis.

2.8. Sample extraction

Tear samples were directly diluted with 100 μl of methanol and vortexed for 5 min, and then centrifuged at 12,000 rpm for 10 min. Aliquots of the supernatants were analyzed by the HPLC method. The blood samples, aqueous humor samples and ocular tissue homogenates were extracted by vortexing with 2.4 ml of *n*-hexane/ethyl ether (1/1, v/v) for 5 min and centrifuged at 4000 rpm for 5 min. Then, 1.8 ml of the upper organic layers were transferred to a glass vial and evaporated to dryness in a freeze dryer. The residues were dissolved in 30 μl of methanol. Aliquots of the supernatants were analyzed by the HPLC method.

2.9. Cyclosporine A quantification

CyA concentrations were determined by the HPLC method. The HPLC system employed in the study consisted of an Agilent G1100 HPLC series system (Agilent Technologies, Palo Alto, CA) with an Agilent G1310A pump equipped with an Agilent G1314A variable wavelength UV–VIS photo diode array detector (DAD), set at 210 nm. An Inersil®-C18 ODS column (250 mm × 4.6 mm, 5 μm) was operated at 65 °C due to the existence of various conformers of CyA. The mobile phase was consisted of acetonitrile–water (9/1, v/v). The flow rate was 0.8 ml/min. A calibration curve was established on each running day. These curves showed good linearity, with correlation coefficients of >0.99. The limit of quantification (LOQ) and detection (LOD) were 75 and 25 ng/ml, respectively.

2.10. Statistical data analysis

Statistical data analysis was performed using the student *t*-test with $p < 0.05$ as the minimal level of significance.

3. Results and discussion

3.1. Stability of Cys-PEG-SA

The free thiol groups of Cys-PEG-SA conjugates were 368.43 ± 15.57 μmol per gram conjugate. There was no significant decrease in free thiol group amount of Cys-PEG-SA during 3-months storage at 4 °C, which suggested that Cys-PEG-SA conjugates could be stored at low temperature. The influence of pH and temperature on the percent remaining of the free thiol group on the thiomer solution was shown in Fig. 1. A significant decrease in the free thiol group content of the polymers was observed at 34 °C in STF, indicating the rapid formation of disulfide formation in the simulant physiological environment. At 4 °C in ABS, the cross-linking process of polymer proceeded rather slowly. The observed cross-linking rate constants $K_{STF,\ 34\,^\circ C}$ and $K_{ABS,\ 4\,^\circ C}$ were estimated to be 0.510 and 0.005 h^{-1}, respectively.

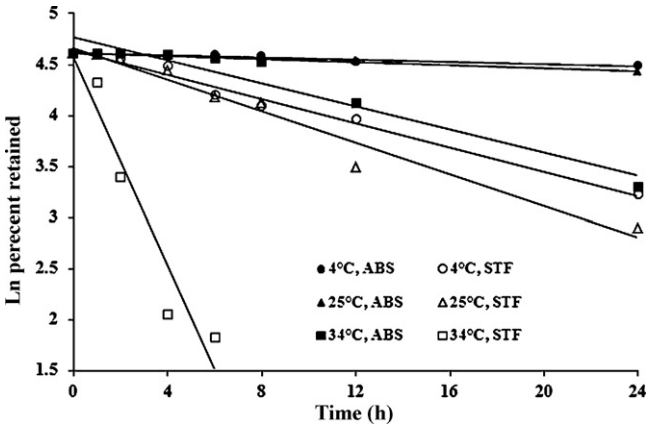

Fig. 1. Apparent first-order plots of disulfide bonds formation at various temperatures and pHs.

It has been reported that the improved mucoadhesive properties of thiomers were based on their in situ cross-linking properties, and the disulfide bonds within the thiomer itself led to additional anchor via chaining up with the mucus gel layer (Bernkop-Schnurch, 2005). The rapid cross-linking process of Cys-PEG-SA at physiological condition (STF, 34 °C) could facilitate the bonding between Cys-PEG-SA and ocular mucus gel layer, thus, prolong the pre-corneal retention time of Cys-PEG-SA and its modified nanocarriers. Furthermore, the thiolated polymer in aqueous solution should be stored in the environment with low pH-value (<5) and low temperature since there is a direct correlation between the content of free thiol groups and the mucoadhesive properties of thiolated polymers.

3.2. Preparation and characterization of NLC formulations

The components and properties of NLC formulations are listed in Table 1. The modification of Cys-PEG-SA or PEG-SA resulted in the slight increase of the particle size comparing with NLC. There were no significant differences in CyA content among NLC, PEG-NLC and Cys-NLC ($p > 0.05$). Modification by PEG-SA or Cys-PEG-SA would not have an influence on the Ee of NLC in this study (approximately 97%) due to the high lipophilicity of CyA. The osmotic pressures of the NLC formulations were very close to the physiological values of lachrymal liquid (0.3 osmol/kg). Furthermore, the pH-values of all NLC formulations were >4. Therefore, the physiochemical parameters of the NLC formations studied should not induce a feeling of discomfort and irritation, which was in accord with the *in vivo* tolerance investigation results in our previous study (Shen et al., 2009). The relatively low pH of Cys-NLC formulation could effectively retain the activity of the free thiol group modified on the surface of Cys-NLC.

3.3. In vitro CyA release study

The CyA release profiles from the NLC formulations were shown in Fig. 2. The drug release of all NLC formulations was relatively fast

Table 1
The components and properties of NLC formulations.

Formulation	PEG-SA (wt.%)	Cys-PEG-SA (wt.%)	CyA content (mg/ml)	Mean particles size (nm)	Polydispersion index	Free thiol groups on the NLC (mM)	Osmotic pressure (osmol/kg)	pH
NLC	–	–	10.16 ± 0.41	59.4 ± 1.5	0.307 ± 0.045	0.00 ± 0.01	0.266 ± 0.32	5.42 ± 0.62
PEG-NLC	2	–	10.78 ± 0.33	67.9 ± 1.6	0.303 ± 0.021	0.01 ± 0.01	0.296 ± 0.23	5.15 ± 0.23
Cys-NLC	–	2	10.52 ± 0.78	66.9 ± 0.4	0.284 ± 0.028	2.65 ± 0.34	0.330 ± 0.19	4.71 ± 0.22

Data are represented with mean ± S.D. ($n = 3$).

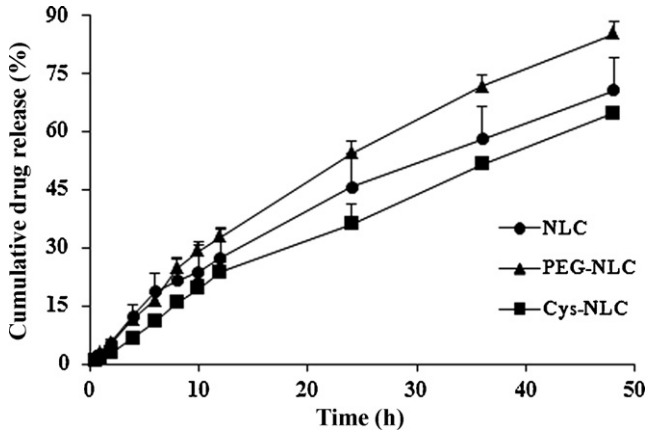

Fig. 2. *In vitro* release profiles of CyA from NLC formulations (NLC, PEG-NLC and Cys-NLC). Each value represents the mean ± S.D. ($n = 3$).

in the initial 12 h, and followed by a sustained release profiles. The cumulative drug release rate of PEG-NLC within 48 h was significantly higher than that of NLC and Cys-NLC ($p < 0.05$). Both the diffusion and erosion mechanism would be the limited factors of drug release from the NLC formulations. It has been reported that the incorporation of amphiphilic PEG-SA may facilitate the particle surface to be wet easily and the swollen state of the particle may result in the faster drug release (Yuan et al., 2007). Therefore, the faster drug release of PEG-NLC could be contributed to the accelerated corrosion of NLC with the PEG-SA addition. As we have discussed above, the free thiol group modified on the surface of the Cys-NLC would form disulfide bonds at physiological condition (STF, 34 °C) very quickly. Therefore, these cross-linked thiolated agents may act as a shield surrounding Cys-NLC and result in the slower release of the incorporated drugs, compared with that of PEG-NLC and NLC.

3.4. Distribution of CyA in ocular tissues

According to previous study, the NLC formulations exhibited a good ocular tolerance *in vivo*. No signs of red swelling, inflammation or vision effect were found during the study period (Shen et al., 2010). The biodistribution of CyA in different rabit eye tissues was shown in Fig. 3. The amount of CyA distributed in blood was close to the limit of detection (data not shown) and the CyA in eye

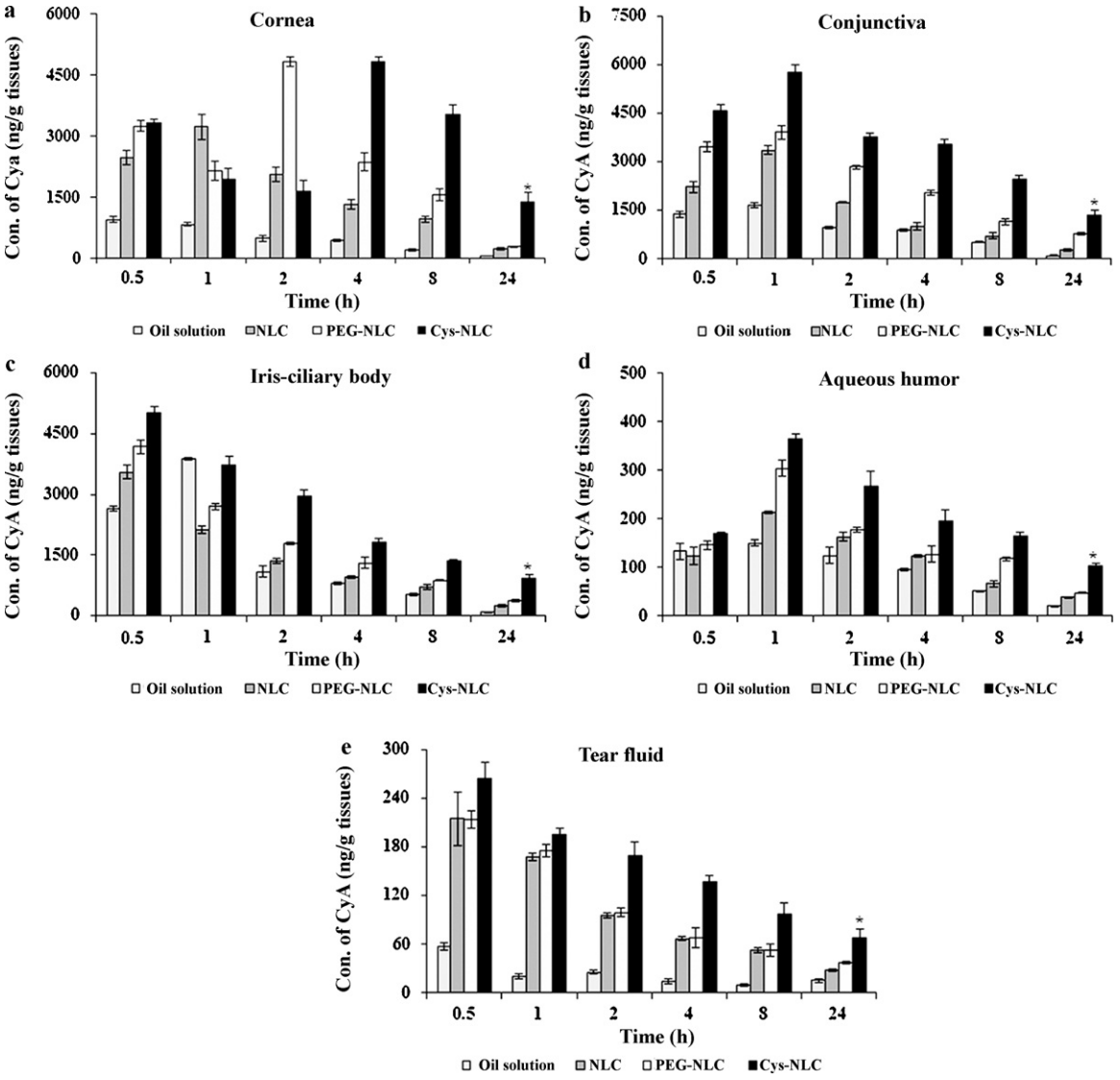

Fig. 3. Biodistribution of CyA in rabbit eyes after topical administration of CyA-loaded formulations (500 μg CyA): (a) cornea; (b) conjunctiva; (c) iris-ciliary body; (d) aqueous humor; (e) tear fluid ($n = 3$). * differs from PEG-NLC, NLC and oil solution, $p < 0.05$.

Table 2
Pharmacokinetic parameters of CyA in different eye tissues after topical administration ($n = 3$).

Samples	Pharmacokinetic parameters	Cornea	Conjunctiva	Iris-ciliary body	Aqueous humor	Tear fluid
Oil solution	AUC (ng h/ml)	5200.507	11934.03	13623	1293.191	328169.9
	MRT (h)	7.872	8.763	7.165	12.047	9.680
NLC	AUC (ng h/ml)	21448.19***	18269.83***	16605.61***	1775.628***	885624.6***
	MRT (h)	9.882***	14.064***	13.504***	18.730***	19.325***
PEG-NLC	AUC (ng h/ml)	22499.15	31902.13**	21618.46	2468.935	1364984**
	MRT (h)	12.272	22.355**	15.721	18.222	35.145**
Cys-NLC	AUC (ng h/ml)	65528.07*	57148.4*	35184.03*	3775.923*	2238796*
	MRT (h)	17.194*	27.104*	31.065*	31.344*	43.245*

* Differs from oil solution, NLC and PEG-NLC, $p < 0.05$.
** Differs from oil solution and NLC, $p < 0.05$.
*** Differs from oil solution, $p < 0.05$.

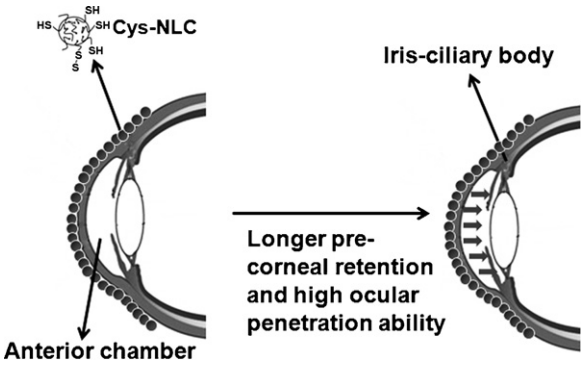

Fig. 4. Schematic representation of intraocular drug delivery of Cys-NLC.

tissues was relatively high, which could effectively avoid adverse effect caused by systemic absorption. CyA of Cys-NLC group was broadly distributed in eye tissues including iris-ciliary body. 24 h after topical ocular administration, CyA concentrations of Cys-NLC in different eye tissues and tear fluid were significantly higher than that of oil solution, NLC and PEG-NLC formulation ($p < 0.05$). The immune response and inflammation in the ocular tissues could be suppressed at a CyA concentration of 50–300 ng/g of tissue (Kaswan, 1988). CyA concentration of Cys-NLC in iris-ciliary body was 934.96 ng/g of tissue at 24 h after topical ocular administration, which was sufficient for the immunomodulation.

Pharmacokinetic parameters of CyA in different eye tissues are listed in Table 2. The values of the area-under-the-curve ($AUC_{0-24\,h}$) and mean retention time ($MRT_{0-24\,h}$) of NLC formulations in eye tissues and tear fluid were significantly higher than that of oil solution ($p < 0.05$). Considering its high drug loading ability, sustained drug release properties and good tolerance *in vivo*, these results demonstrated that NLC could be a promising system to deliver lipophilic drugs to eyes. With PEG-SA modification, the $AUC_{0-24\,h}$ and $MRT_{0-24\,h}$ of PEG-NLC in eye tissues and tear fluid were higher than that of the NLC. It has been reported that PEG chains on the surface of the particles could influence the mucoadhesion and drug absorption, due to its ability to enhance interpenetration with the mucus layer (De Campos et al., 2003; Smart, 2005). Therefore, NLC with PEG modification may significantly prolong the pre-corneal retention and improve the biodistribution of CyA in hydrophibic conjunctiva compared with that of NLC without PEG modification ($p < 0.05$). Among all formulations studied, the $AUC_{0-24\,h}$ and $MRT_{0-24\,h}$ of Cys-NLC was significantly higher than that of the others ($p < 0.05$). The pharmacokinetics of Cys-NLC showed high $AUC_{0-24\,h}$ and sustained drug retention. The PEG moiety and free thiol group around the particle surface of Cys-NLC could facilitate the interpenetration and in situ cross-linking between the Cys-NLC and the mucus gel layer, which resulted in the enhanced mucoadhesion of Cys-NLC to the eye surface. Meanwhile, the prolonged pre-corneal retention time of thiolated NLC would provide an intimate contact between drugs and ocular surface tissues, thus, result in the high penetration of drug into eye tissues and aqueous humor (Fig. 4). These results demonstrated that the thiolated NLC could really deliver CyA into eye tissues, even high distribution of CyA into the iris-ciliary body, and achieve the sustained drug delivery.

4. Conclusion

Within this study, the mucoadhesive nanostructured lipid carrier modified by thiolated agent was evaluated as a promising carrier for ocular drug delivery *in vitro* and *in vivo*. The results showed the thiolated PEG derivates rapidly cross-linked in the simulant physiological environment. Thiolated NLC would not induce a feeling of discomfort and irritation, and present a sustained drug release *in vitro*. The *in vivo* distribution investigation indicated that thiolated NLC could prolong pre-corneal residence time, and deliver high CyA level into eye tissues in ocular surface and anterior chamber. The results of this work have shown that thiolated NLC could be a promising strategy to the treatment of ocular surface diseases and anterior segment inflammatory diseases (e.g. uveitis).

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