Studies on colloidal carriers for drug delivering to the ocular posterior segment in topical administration

薬物後眼部送達を目的としたコロイド粒子点眼製剤の設計と評価に関する研究

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Studies on colloidal carriers for drug delivering to the ocular posterior segment in topical administration

by

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Abstract

At present, the prevalence of diseases concerning with the posterior segment is growing rapidly. These diseases include age-related macular degeneration (AMD), diabetic retinopathy, and proliferative vitreoretinopathy. Currently, drug delivery to the back of the eyes is performed mainly through intravitreal injection in clinical practice, while frequent injection requirement may cause some potential threat and concomitant diseases to the patients. Traditional eye drop administration is taken as the most acceptable way for patients to use. However, it has been believed that eye drop administration can hardly deliver the drug to posterior segment of the eyes owning to the barriers in ocular structures. Recently, it was reported that colloidal drug carrier system liposomal particles in eye drops may deliver drugs to the posterior segment of the eye. In this study, lipid emulsions and polymeric micelles were prepared and evaluated as candidates of colloidal drug delivery system for posterior segment diseases of the eye. The properties of drug carriers were investigated to enhance the delivery efficiency via topical administration and also to understand the transit mechanism.

Particle design of lipid emulsion for ocular posterior segment delivery

In order to analyze the delivery efficiency of colloidal carriers visually, fluorescence labeled lipid emulsion was prepared by formulating coumarin 6 (C-6). The formulation and preparing process were optimized to obtain fine particle size of dispersed droplets of 100 nm. In vivo study with mice, apparently fluorescence was observed on the retinal part of the eye after ophthalmic application of the lipid emulsion. It indicated that the lipid emulsion can work well as well as liposomal systems as a drug carrier for delivering the drug to the posterior segment of eye. The drug delivery efficiency of lipid emulsion was clarified to be relevant with the surface properties of carrier than the formulation properties. Moreover, internalization of lipid emulsion particles containing C-6 with the eye tissue was assumed as an essential phenomenon in delivering C-6 to the posterior part of eye, because stability of lipid emulsions were confirmed by leakage of little amount of C-6.

Polymeric micelles for enhancing drug delivery to posterior segment

To evaluate the feasibility of polymeric micelles for drug posterior segment delivery, several formulations of Vitamin E TPGS (TPGS) and DSPE-PEG2000 (DSPE-PEG) were examined. Among these formulations, the molar ratio of 3:6 and 0:9 (TPGS:DSPE-PEG) made the micelles fine particle size and spherical morphology. The

two types of micelles (3:6 and 0:9) showed almost the same level in delivery efficiency in vivo study, while cellular uptake was improved in vitro test when TPGS was incorporated in the micelle formulation. Combined with the endocytosis inhibition study, the drug delivery efficiency to posterior segment was concluded to be controlled by both internalization of colloidal carriers into ocular cells and the transportation of carriers in the ocular tissues.

In conclusion, lipid emulsions and polymeric micelles might be good candidates of colloidal drug delivery systems to ocular posterior segment as well as liposomal systems. In addition, the surface properties and bio-stability of colloidal carriers were found to be important in improving the drug delivery to posterior segment in treatment of ophthalmic diseases. These conclusions might contribute greatly to development of ocular colloidal drug delivery systems targeting to posterior segment of the eye.

博士論文要旨

高齢化社会が進む近年、糖尿病網膜症、加齢黄斑変性など後眼部の疾患の患者数は増えている。これらの疾患の治療には、後眼部に薬物を送達する必要があるが、医療現場では、主として硝子体への注射が行われている。眼に注射することは患者さんにとっては恐怖感があるばかりか、繰返しの注射は炎症などの副作用も心配される。眼科領域の最も一般的な製剤である点眼剤では、薬物は後眼部まで送達できないと考えられてきたが、近年、リポソームを用いて点眼によって後眼部に送達ができることが報告された。本研究では、リポソームとは異なる微粒子薬物キャリアであるリピッドエマルション、高分子ミセルがリポソーム同様な後眼部送達機能を有するか評価した。これらの粒子の特性を検討して、点眼後の後眼部送達効率を向上させる物理化学的性質などの影響について研究を行った。

リピッドエマルションの粒子設計と後眼部送達機能の評価

後眼部送達機能を視覚的に評価することを目的として、蛍光物質で標識したリピッドエマルションを調製した。その際、100nm 程度の分散滴の粒子径となるように、処方および調製方法に関して最適化をした。得られた粒子を用いて、マウスに点眼して後眼部送達機能を評価したところ、リポソーム同様な効果が確認された。また、後眼部送達機能の観点では、処方よりも粒子の表面特性の制御が重要であることが判った。さらに、後眼部送達は内封された物質がリリースされて送達されるのではなく、封入されたまま、粒子の眼組織との親和性により達成されると推定された。

高分子ミセルによる後眼部送達

高分子ミセルによる後眼部送達の可能性を検討するため、TPGS (Vitamin E TPGS) および DSPE-PEG (DSPE-PEG2000) の構成成分からなるミセル粒子の調製を試みた。DSPE-PEG だけでも、微小なミセル粒子が形成されることが明らかとなった。さらに、このミセル粒子に適度な割合の TPGS を組み込むことが可能であった。DSPE-PEG のみの処方および TPGS が細胞毒性を示さない 3:6 (TPGS:DSPE-PEG) の処方比においては、目的とする微小な粒子径を有す球形度の高い粒子が得られることが明らかとなった。また、TPGS の処方によって細胞親和性は高くなることが示されたが、実際の後眼部送達機能には大きな影響を及ぼさなかった。細胞取り込みの阻害物質を用いた実験結果も考慮すると、後眼部送達機能には、組織内の移行性も関与し、細胞取り込み実験の結果だけでは判断できないという結論に至った。

以上の結果から、リピッドエマルション、高分子ミセルは、リポソームと同様に、薬物を後眼部に送達するキャリアであることが明らかとなった。また、後眼部への送達機能は、単に粒子のサイズだけではなく、粒子の表面特性、あるいは、生体内での安定性などの要因があることが示された。本研究の結果は、後眼部送達用点眼剤の開発に新たな知見を提供するものと考える。

Chapter 1:

General introduction

For the last decade, visual impairment from infectious diseases has decreased in virtue of overall socioeconomic development. However, in most countries, ocular posterior segment diseases have emerged as potential threats to the status of sight of the people. At present, it is assumed that more than 50% of the most enfeebling ocular diseases originated from the posterior part of the eyes [1]. Diabetic retinopathy has been added to the first by the increasing of diabetes among many population groups, while glaucoma, an eye disease known for centuries, remains on the public health agenda due to difficulties in its early diagnosis and frequent necessity of life-long treatment. Age-related macular degeneration (AMD) ranks next among the global causes of visual impairment with a blindness prevalence of 8~7%, which is the primary cause of visual deficiency in industrialized countries [2]. In addition, the world population is rapidly ageing. As reported, between 2000 and 2050, the proportion of the world's population over 60 years will double from about 11% to 22% [3]. With the aging population increasing, it could be expected that the more and more people will realize the threat of ocular posterior diseases by aging, which might cause irreversible vision loss if they were without any effective treatment.

Drug delivery to the posterior segment of the eye, namely, retina, choroid, vitreous humor and optic nerve, is very important for treating these disorders. It has been shown that vascular endothelial growth factor (VEGF) is responsible for many ocular pathologies involving retina neovascularization. To inhibit this target, lots of new drugs were listed as candidates; some of them have been already commercially available for intraocular use, such as Pegaptanib (Macugen®), Ranibizumab (Lucentis®), Bevacizumab (Avastin®) [4]. Moreover, increasing new agents for various therapeutic targets found would become potential liberators for patients who suffer from ocular posterior segment disease. Consequently, the problems facing to therapy of ocular posterior delivery can be assumed not to be lack of therapeutic agents. Developments of effective delivery methods of these agents to the objective site are strongly required.

Currently, the intravitreal route is widely used to deliver therapeutic entities to the retina in clinical practice. However, frequent administration requirements via this route would lead to retinal detachment, endophthalmitis, retinal hemorrhage, increased intraocular

pressure, and eye pain [5]. To minimize some of these complications, various controlled delivery systems have been developed in the form of biodegradable or non-biodegradable implants. Even so, there are still several other aspects of this treatment need to be addressed, including the long term stability of the implants and the possibility of retinal neuron response caused by long time stimulation [6]. In addition, drug delivery through periocular route seems to enable the diffusion of drug molecules directly to the external surface of the sclera bypass the elimination of drugs through the conjunctival lymphatics or even episcleral veins. Therefore, the periocular route is considered to be the least painful and efficient route of drug delivery to the posterior eye, because the route could minimize the risk of retina damage associated with intravitreal route of administration [7]. However, owing to the tight conjunctions of retinal pigment epithelium (RPE) and a network of blood vessels located between the sclera and retina, only few compounds with adequate permeability and the concentration gradient could flux across these barriers and reach to the retina [8,9].

Besides, the systemic route is also severely limited in effectively delivering drugs to the back of the eye. As reported, only estimated 1-2% of compounds could delivery via this route successfully cross the blood aqueous barrier (BAB) and blood retinal barrier (BRB) to reach and accumulate in the retinal tissues [10]. Thus, the higher dose of drug for administration may be required to reach the therapeutic concentration within the retinal tissues. However, it will increase the potential side effects of systemically exposure the body under high concentration of drugs, which makes systemic administration an undesirable route of delivery for posterior segment therapies. In general, these methods are invasive ones, which associated with requirements of medical facilities, low compliances and higher adverse effects.

Therefore it is important to develop a simple and less invasive way for delivering the drugs effectively to targeted tissues in the posterior segment of the eye. A topical eye drop represents the least invasive method for ocular delivery. However, the existence of anatomical and physiological barriers make eye drop administration difficult to accomplish effective drugs to targeted anterior tissues not to mention the posterior segment, such as cornea/conjunctiva barriers, the challenge of drug distribution, metabolism and clearance [13].

Recent developments in colloidal carriers for ocular drug delivery provided new potentials for posterior segment delivery via topical ophthalmic administration. In 2009,

Hironaka and co-workers first described drug delivery to the posterior segment of the eye using submicron-sized (100 nm) liposomes as the carriers [11]. In addition, nano-structured lipid carriers, were highlighted for their ability to deliver drugs to the retina after topical application [19]. The particle size, composition, and surface properties of colloidal carriers were reported to be important for the posterior segment delivery of drugs in previous researches [12,16,17]. It is also conceivable that endocytosis seem to be the main uptake mechanism for the drug loaded carriers on the surface of the eyes in delivering drugs to the posterior segment. Considering to these drug delivery beneficial properties, typical colloidal carrier lipid emulsions and polymeric micelles composited with biocompatibility materials seem to be potential candidates for ocular drug delivery as well as liposomal systems.

The purpose of this study was to apply the lipid emulsions and Vitamin E TPGS/DSPE-PEG2000 micelles for ocular delivery and to illuminate their feasibility for topical drug posterior segment delivery. In addition, to understand the transit mechanism, a variety details were collected and analyzed to reveal the possible transit routes of colloidal drug delivery systems to posterior segment of the eye.

Chapter 2:

Lipid emulsion for ocular posterior segment delivery

2.1 Introduction

Over the past several decades, emulsions, as one of typical dosage forms, have been explored for resolving a variety of drug delivery challenges. An emulsion system can be normally taken as a mixture of two immiscible phases (normally, water and oil) with emulsifier to stabilize one dispersed in the other (Fig.2-1).

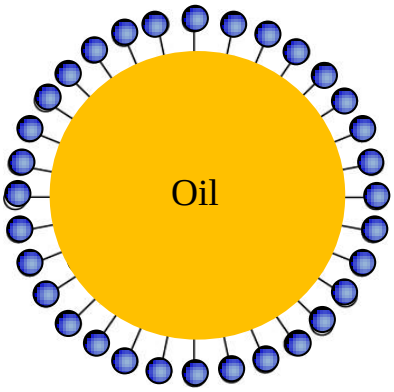

Fig.2-1 Scheme of emulsion

As concern the side effect of surfactants, the most common used emulsifier in emulsion formulations is lecithin (phosphatidylcholine) normally derived from egg yolk or soybean. The emulsion, in which oily materials are dispersed in an aqueous phase with lecithin was called lipid emulsions (LEs). LEs dispersed as submicron-sized droplets has been accepted for their good biocompatibility and well known as a parenteral nutrition. The parenteral nutritional LEs have been applied to patients for supplying high-caloric nutrition in many countries. As LEs can incorporate lipophilic drugs, it has been applied to design parenteral drug delivery systems also in aims of reducing side effects, increasing the bioavailability, and prolonging the pharmacological effects [16]. A variety of poorly water soluble drugs loaded emulsion formulations for intravenous injection have been marketed in a lot of countries for solubilizing and improving delivery also [17,18].

In the field of ocular dose forms, lipid emulsion systems have also been used to provide a better balance between ocular bioavailability improvement and patient comfort via

instillation [19]. Some of them have already been commercialized in the markets. An anionic LEs containing 0.05% cyclosporine A (Restasis®, Allergan, Irvine, USA), was first approved by FDA for anterior segment disease (chronic dry eye). Subsequently, a number of cationic emulsion formulations, such as Cationorm® and Novasorb®, were proved to be efficient drug delivery for dry eye syndrome and cure anterior segment diseases. As could be noticed previously, there has been few researches focused on targeting to the ocular posterior segment.

In the present study, we hypothesized that submicron-sized LEs would be suitable for drug delivery to the posterior segment via eye drops administration. As previously reported, liposome could behave as a drug carrier for delivering drugs to posterior segment of the eye in virtue of endocytosis [11]. In view of the same surface structure of LEs with liposome covered with the phospholipid, LEs was assumed to be a potential candidate for drug posterior segment delivery.

In order to testify the hypothesis, submicron-sized (100 nm) LEs were prepared and examined their feasibility in applying to topical ocular posterior segment delivery test. Subsequently, the formulation effects on retinal drug delivery efficiency were tested by different emulsion formulations with different types of core oils and emulsifiers. Finally, to improve the drug delivery efficiency, the surface of submicron-sized LEs was modified with functional polymers, chitosan (CS) and poloxamer 407 (F127).

2.2 Preparation and characterization of lipid emulsion

The formation of LEs relies on the emulsifiers to stabilize the emulsion by reducing interfacial tension between the oil and the water phases. In the current study, submicron-sized LEs were prepared using typical high-pressure homogenizer method and by means of emulsion phase inversion (Method A in Fig. 2-2) [20].

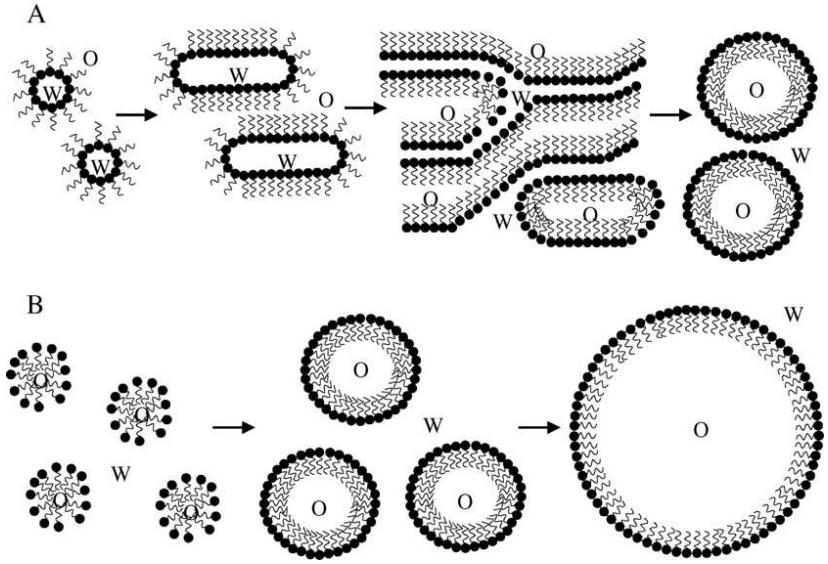

Fig.2-2 Scheme of the emulsification procedure (W: water phase; O: oil phase). Method A: first water-in-oil droplets are produced; those inverted structures merge together to give elongated and bicontinuous or lamellar structures that finally decompose into submicron-sized oil droplets. Method B: small oil droplets are immediately produced and grow in size upon oil and surfactant addition. Their size is a result of the mechanical process only. (Reference to [20])

By the slow addition of water phase into the oil phase, with increasing the ratio of the water phase, the formulation experienced a phase transition from water in oil emulsion to oil in water emulsion, which was believed to have ability to formulate homogeneous small-sized droplets and stabilize the formulation [20]. Preliminary investigations revealed that the particle size of oily droplets in formulations decreased dramatically by using high pressure homogenizer under the conditions at 150MPa for 5 min. Under these conditions, stable and uniform lipid emulsions in the size around 100 nm with low polydispersity at the final pH about 7.4 were obtained after pH adjustment. The optimal weight ratio between emulsifier and oil phase was also found to be 3:10 for the formulation and high entrapment efficiency (~100%) was confirmed. Owing to the needle like crystallization of coumarin-6 (C-6), the portion of un-entrapped C-6 in the LEs was removed by filtration through 0.8μm membrane filter. The amount of C-6 encapsulated in the oily droplet was determined by measuring C-6 concentration with and without filtration. C-6 assay was performed by using a fluorescent spectrophotometer; resultant calibration curve shown in Fig. 2-3 was used. The compositions and characteristics of different formulations of emulsion are presented in

Table 2-1.

As could be noticed in Table 2-1, further increasing in castor oil ratio resulted in the increase in particle sizes and broadening in particle distribution. Owing to the higher viscosity of castor oil, the particle size of dispersed oil droplet in LEs increased and the higher energy was required to achieve the same particle level than emulsion made by Medium-chain triglycerides (MCT) as oil phase. In contrast, the particle size of the emulsion containing MCT as the only oil (Formulation A) could achieve an ideal particle size of 100 nm with a relatively narrow particle distribution. Since the emulsifier is an important determinant to enhance the stability of oily droplets, two kinds of natural phospholipids, egg yolk phospholipid (EPC) and hydrogenated soybean phospholipids (HSPC), were tested in the formulation study. The results indicated that both of the phospholipids own the same emulsifying capacity by producing same level of particle size.

Table 2-1 Physical characterization of different lipid emulsions

Form.	Oil Phase MCT/Castor oil	Emulsifier (w/v)	Charge supplement	Isoosmotic adjustment	Particle Size(nm)	PI	ζ-potential (mV)
A	1:0	EPC 3%	DCP	Glycerin 2.5%	94.69	0.165	-55.4 ± 9.59
B	0.5:0.5	EPC 3%	DCP	Glycerin 2.5%	98.63	0.192	-38.1 ± 7.55
C	0:1	EPC 3%	DCP	Glycerin 2.5%	160.3	0.181	-47.6 ± 5.15
D	1:0	EPC 3%	-	Glycerin 2.5%	98.69	0.134	-15.7 ± 2.32
E	1:0	HSPC 3%	-	Glycerin 2.5%	100.37	0.152	-46.0 ± 5.14
F	1:0	HSPC 3%	DCP	Glycerin 2.5%	99.89	0.142	-64.1 ± 6.83
G	1:0	EPC 3%	SA	Glycerin 2.5%	75.95	0.145	51.9 ± 7.58

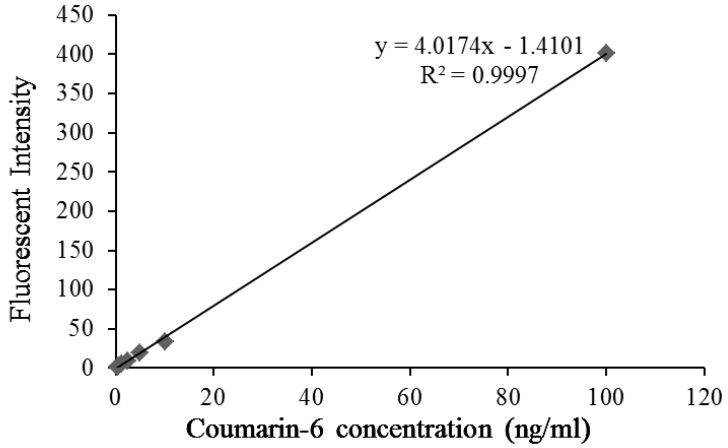

Fig.2-3 Calibration curve of coumarin-6 by Fluorescent Spectrometer

2.3 The delivery efficiency in vivo of lipid emulsion

It has been reported that liposomes could deliver drugs to the ocular posterior segment via topical administration [11]. Structurally, liposomes are vesicles in which an aqueous volume is entirely enclosed by a membranous lipid bilayer mainly composed of natural or synthetic phospholipids [21]. Similarly, LEs is also a dispersed system, where oily droplets are dispersed in a water phase with the help of emulsifiers. The oily droplet surface of the LEs is covered by the phospholipid emulsifier indicating that the surface structure of LEs is almost the same as that of liposomes. Therefore, submicron-sized lipid emulsion systems have been expected for drug delivery to the posterior segment via eye drop administration *in vivo*.

To evaluate the delivery efficiency of carriers, the fluorescence intensity on inner plexiform layer (IPL) of retina was quantified by Image J. The results were shown in Fig.2-4. Ganglion cell layer (GCL) is a layer of the retina that consists of retinal ganglion cells (RGC) and displaced amacrine cells. The death of RGC is reported to be a common feature of many ocular disorders such as glaucoma, optic neuropathies, and retinovascular diseases and so on [22]. In this study, the IPL was the region of interest and chosen as the target site because it is located close to the inner GCL of the retina, which is assumed to be the most suitable part to evaluate the delivery efficiency to the posterior segment. Compared to untreated eye, it was observed that significantly strong fluorescent intensity in the posterior ocular segment after one drop administration. However, this was not observed when MCT containing the same amount of C-6 was applied under the same conditions (Fig. 2-4). This result strongly suggested that MCT itself does not have delivery functions for the dissolving C-6. The very fine droplets of

LEs carriers are responsible for delivering C-6 to the posterior part of eye after topical administration.

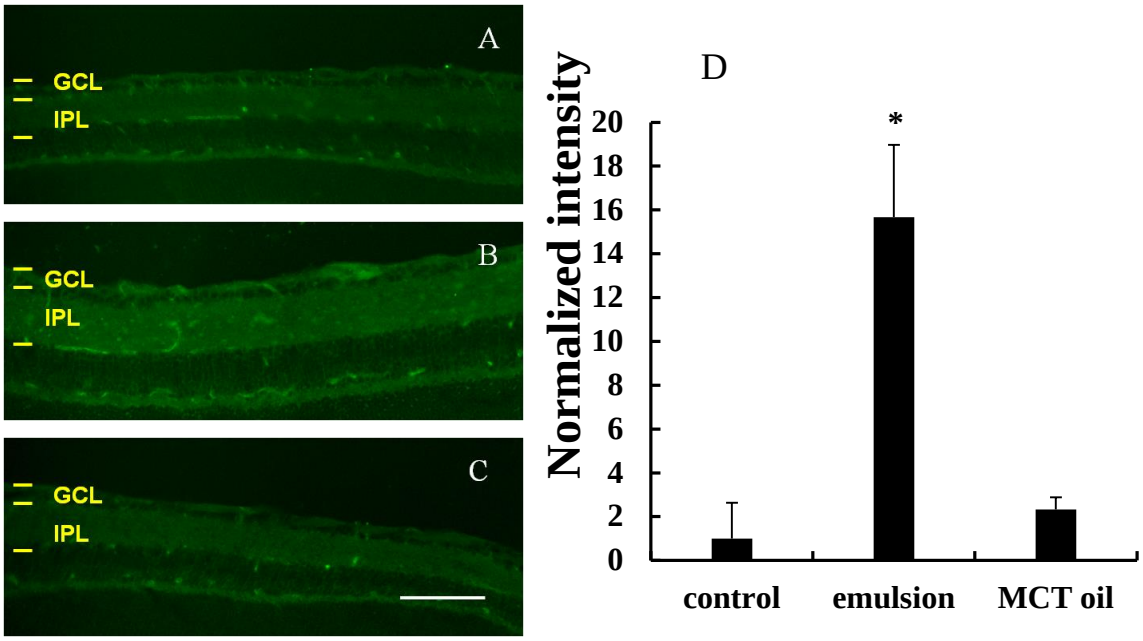

Fig.2-4 The effects of coumarin-6 loaded lipid emulsions on drug delivery to ocular posterior segment in mice. Representative epifluorescence microscopic images of the retina 30 min after eye drop administration. (A) Untreated (control); (B) coumarin-6-loaded lipid emulsion (Formulation A); (C) coumarin-6 dissolved in MCT; (D) the accumulated fluorescence intensity in the IPL after eye drop administration in mice. Data are shown as mean ± SEM. (n = 3). *$P < 0.05$ versus control. The scale bar represents 100 μm. GCL, ganglion cell layer; IPL, inner plexiform layer.

2.4 The formulations effect on ocular drug delivery

To compare the effect of formulations on drug delivery efficiency to the back of the eye, the normalized fluorescent intensity of C-6 on the IPL of the retina was calculated from the fluorescence images. As reported, particle size was considered to be one of important factors for intraocular drug delivery using liposomal drug delivery systems; 100 nm was the best size for drug delivery to the posterior segment when comparing the delivery efficiency for the different particle sizes of liposome from 100 to 800 nm [15]. Therefore, we attempted to prepare LEs with particle sizes of approximately 100 nm with different formulations to clarify the formulations effect on drug ocular posterior segment delivery. Moreover, other formulation effects of LEs such as the types of inner

oil, phospholipids, on the delivery efficiency were evaluated.

2.4.1 The effect of inner oil types

Two kinds of core oils in the formulations were administrated based on their different physical and viscosity properties. In virtue of its less viscous, MCT was reported to be 100 times more soluble in water than long chain triglycerides and had been well used in a lot of LEs formulations [14]. Castor oil is one of the most viscous oils reported to be commonly used in preparation of ophthalmic LEs [23]. Due to its high viscosity, it was difficult to prepare LEs of 100 nm with castor oil as the single oil phase even when increasing the working pressure of homogenizer. Therefore, a mixture of MCT and castor oil with ratios of 0.5:0.5 and 1:0 were used *in vivo* on comparing inner oil formulations effects on drug delivery to the ocular posterior segment. Negligible differences were observed on intraocular delivery efficiency between the different oil formulations (formulation A and B in Table 2-1), which suggested that the oil composition has little influence on drug delivery to the ocular posterior segment (Fig.2-5).

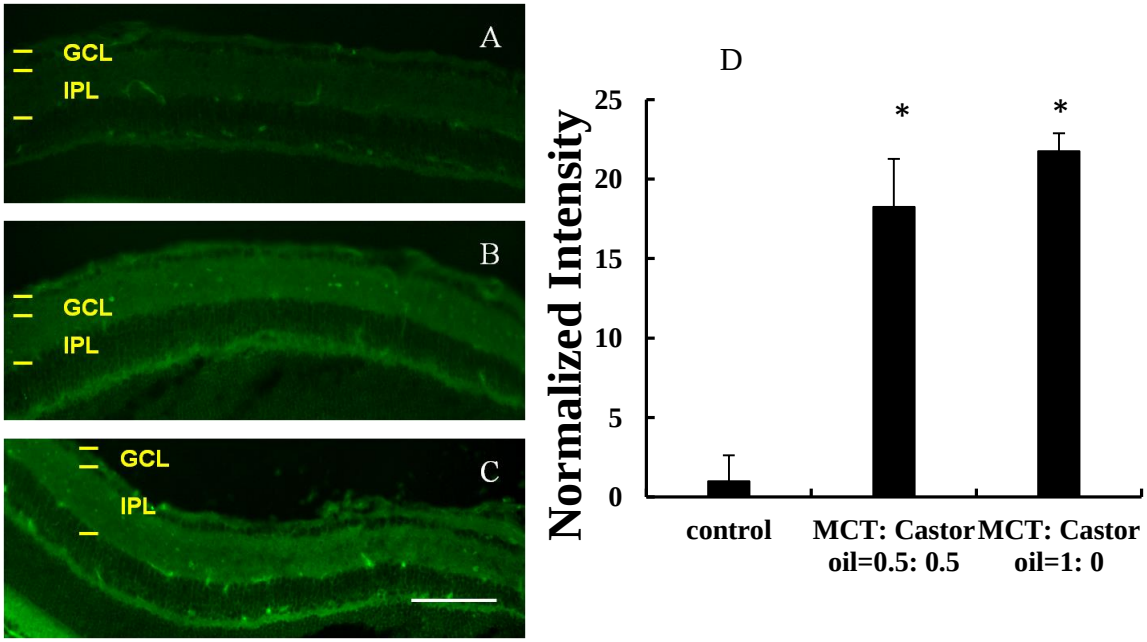

Fig.2-5 Effects of core oil formulations on the efficiency of drug delivery to the retina. Representative epifluorescence microscopic images of the retina 30 min after eye drop administration. (A) Untreated (control); (B) MCT:Castor oil = 0.5:0.5 (Formulation B); (C) MCT:Castor oil = 1:0 (Formulation A); (D) the accumulated fluorescence intensity

in the IPL after eye drop administration in mice. Data are shown as mean ± SEM (n = 4). *P < 0.05 versus control. The scale bar represents 100 μm. GCL, ganglion cell layer; IPL, inner plexiform layer.

2.4.2 The effect of phospholipid types

The emulsifier effects on drug delivery to ocular posterior segments were determined by two types of eye drop emulsions prepared with EPC and HSPC as emulsifiers, respectively. They were selected in this study because both of EPC and HSPC have been commonly used in the formulation of emulsion and have significantly different phase transition temperatures (EPC, −15 to −17°C, HSPC 50 to 56°C) and unsaturated/saturated properties, respectively. Since both of the formulations (A and F in Table 2-1) revealed no apparently difference in delivery efficiency of C-6 to the IPL of the retina in mouse (Fig. 2-6), it was concluded that the properties of phospholipids in the formulations do not influence drug delivery efficiency to the ocular posterior segment in the same manner as the core oil composition.

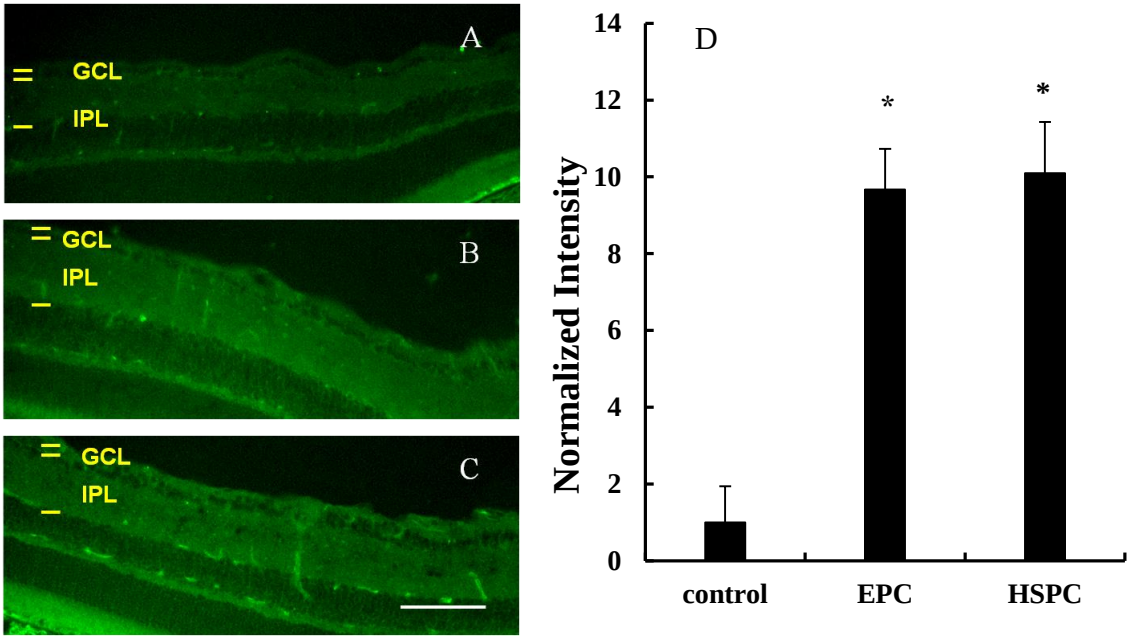

Fig.2-6 The effect of emulsifiers in lipid emulsion on the efficiency of drug delivery to the retina. Representative epifluorescence microscopic images of the retina 30 min after eye drop administration. (A) Untreated (control); (B) EPC emulsion (Formulation A); (C) HSPC emulsion (Formulation F); (D) the accumulated fluorescence intensity in the IPL after eye drop administration in mice. Data are shown as mean ± SEM (n = 4). *P <

0.05 versus control. The scale bar represents 100 μm. GCL, ganglion cell layer; IPL, inner plexiform layer.

2.5 Surface modification for enhancing the trans-ocular posterior delivery

2.5.1 The effect of surface charge of lipid emulsion

Surface properties of LEs are important factors for the interaction of emulsion droplets with ocular cells and tissues. Negatively charged and positively charged emulsions were prepared using dicetylphosphate (DCP) and stearylamine (SA) as charge inducers, respectively (Formulation A and G in Table 2-1). The positively charged emulsion showed significantly higher delivery efficiency to the retina than the negatively charged in the ocular drug delivery study (Fig.2-7). It was considered that the bio-adhesive properties of cationic emulsion contributed to ocular drug delivery on account of the cell membrane naturally negatively charged [24]. EI-Shabouri has reported the similar results using positively charged nanoparticles to improve drug oral bioavailability [25]. These evidences suggest that the electrostatic interactions between the positively charge of the emulsion and the negatively charged cell membranes on the ocular surface enhanced the association, thereby increasing the interaction of the droplet with eye tissues. It was confirmed that the surface property of LEs seems to have much more influences on delivery efficiency to ocular posterior segment than the inner structure or the inner properties of LEs.

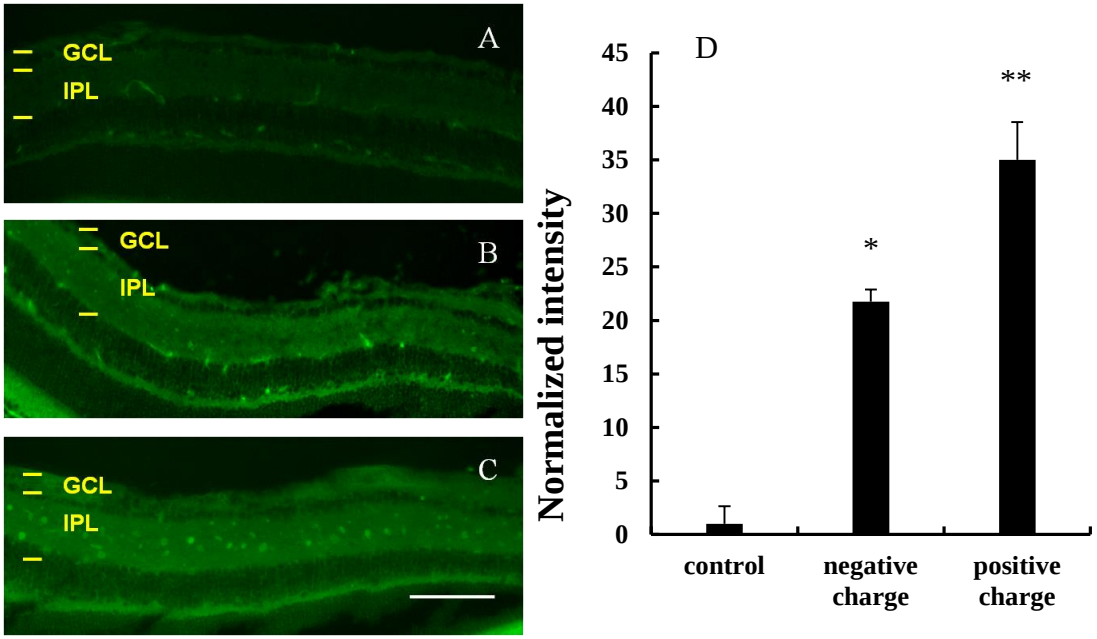

Fig.2-7 The effect of surface charge in the lipid emulsion on the efficiency of drug delivery to the retina. Representative epifluorescence microscopic images of the retina 30 min after eye drop administration. (A) Untreated (control); (B) DCP-modified emulsion (Formulation A); (C) SA-modified emulsion (Formulation G); (D) the accumulated fluorescence intensity in the IPL after eye drop administration in mice. Data are shown as mean ± SEM (n = 4). *P < 0.05 verses control. **P < 0.05 verses negative charge emulsion. The scale bar represents 100 μm. GCL, ganglion cell layer; IPL, inner plexiform layer.

2.5.2 The effect of surface modification with polymers

Several surface modification techniques for colloidal drug carriers have been developed by using several polymers for the purpose of improving drug delivery [26,27]. Biodegradable cationic polymers such as chitosan (CS) have often been used to improve the mucoadhesive properties and cell association of colloidal drug carriers [28,29]. In addition, it has been reported that surface modification by a nonionic surfactant could increase the cellular association of nanoparticles [30]. Thus, two kinds of surface modifiers, CS and Pluronic® F-127 (F127) were selected to modify the surface of LEs for enhancing the drugs delivery efficiency to the posterior part of eyes.

The particle size and zeta potential of the CS-modified emulsion changed from 89.25 nm to 94.49 nm and −34.6 mV to −14.9 mV, respectively. The slight changes in particle size were supposed to result from surface modification with CS. The increasing in zeta potential confirmed the electrostatic interactions by the adsorption of positively charged CS on the surface of negatively charged emulsion particles. CS is a well-known biodegradable polymer that has been demonstrated excellent ocular compatibility with positive charge [31]. DCP was firstly used to induce a negative charge on the LEs. Therefore, the amino groups attached to the chain of CS molecule could interact with the phosphate groups of DCP incorporated in the emulsion particles electrostatically. The fluorescence intensity of C-6 in the retina increased by CS-modified emulsions compared with unmodified ones (Fig. 2-8). It suggested that CS modification could apparently enhance the ocular delivery efficiency of LEs to the posterior segment. The function of CS to open the tight conjunction of cells may be partly responsible for increasing drug delivery [32]. In addition, the residual amino acid groups, which do not interact with the negative phosphate group of DCP on the emulsion surface, may electrostatically interact with negatively charged mucus of eye surface tissue to prolong the retention time of emulsions.

In the case of F127 modification, the particle size and zeta potential of LEs were changed from 89.25 nm to 110.6 nm and −34.6 mV to −42.7 mV, respectively. Polyoxyethylated nonionic surfactants have been reported to increase drug permeability through corneal epithelial cell membranes [33]. F127, as a type of nonionic surfactants, is a thermosensitive amphiphilic polymer. With increasing the temperature, it could form gel in a certain concentration [34]. Furthermore, it was reported 5% (w/v) of F127 indicated excellent ocular tolerance in eye irritation test [35]. With consideration of the irritation and cytotoxicity, 5% (w/v) was used in the study, although the thermosensitive property could not appear under this concentration. As shown in Fig. 2-8, it was indicated that F127 modified LEs significantly enhanced the drug delivery efficiency to the retina. The reason for delivery improvement by F127 was taken to be different from that of CS. One possibility is that F127 in the emulsion systems may increase the fluidity of cell membrane on the eye surface, leading to increase C-6 permeability. The other possibility is that the presence of F127 helped stabilize the LEs by increasing the viscosity in the system, thereby prolonging its retention time on the surface of the eye [34]. Above all, our data suggested that the positive surface properties of LEs have large impacts than their inner formulation properties on drug delivery to ocular posterior segment.

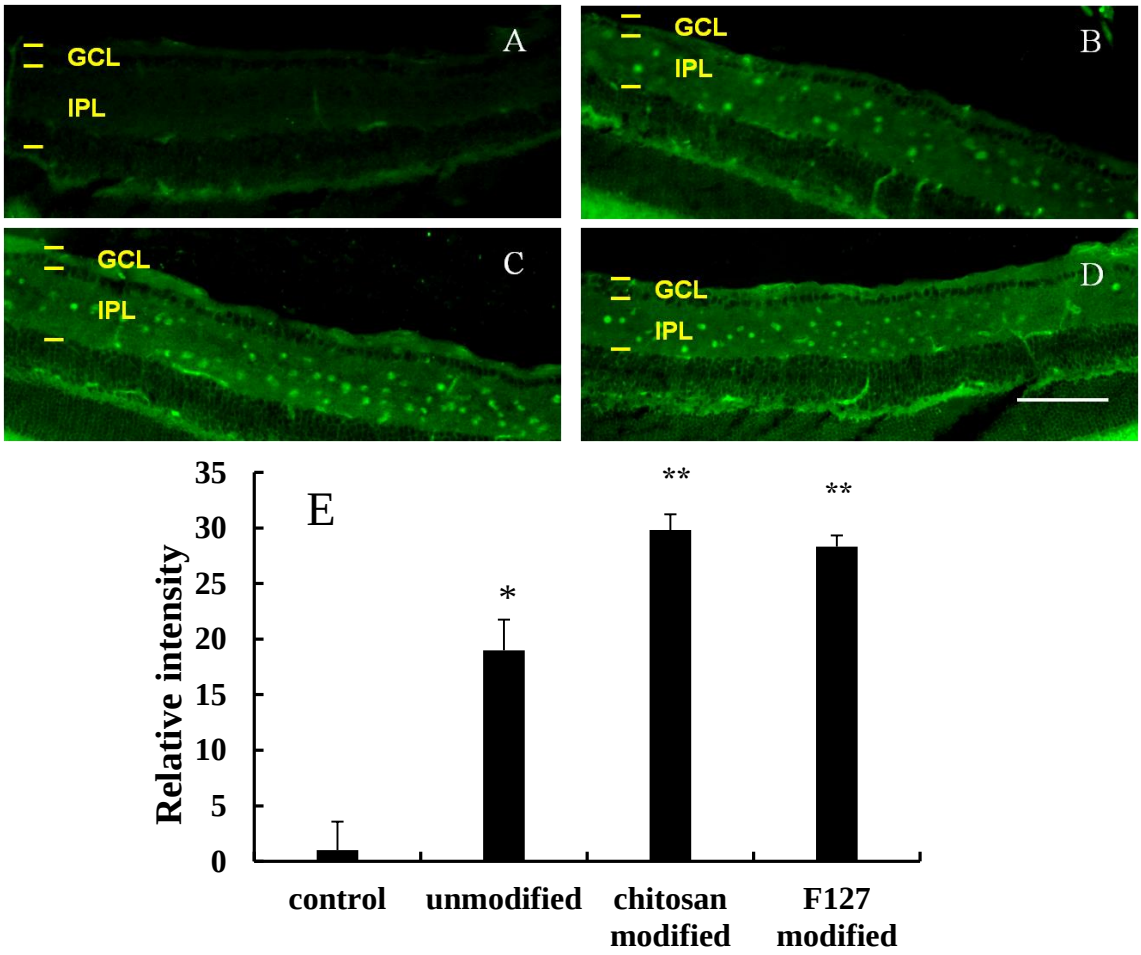

Fig.2-8 The effect of CS and P407 surface modification on delivery efficiency in mice retina. Representative epifluorescence microscopic images of the retina 30 min after eye drop administration. (A) Untreated (control); (B) unmodified lipid emulsion; (C) CS-modified lipid emulsion; (D) F127-modified lipid emulsion; (E) The accumulated fluorescence intensity in the IPL after eye drop administration in mice. Data are shown as mean ± SEM (n = 3). *P < 0.05 versus control. **P < 0.05 versus unmodified. The scale bar represents 100 μm. GCL, ganglion cell layer; IPL, inner plexiform layer.

2.6 In vitro release study

In order to clarify delivery mechanism of C-6 entrapped in LEs on the surface of the eye, in vitro release behaviors were examined by dialysis method. Rarely drug release was observed even after 12 h (720 min) incubation in the release test. It further proofed that C-6 internalized into the ocular cells was not in a release manner on the surface of the eye.

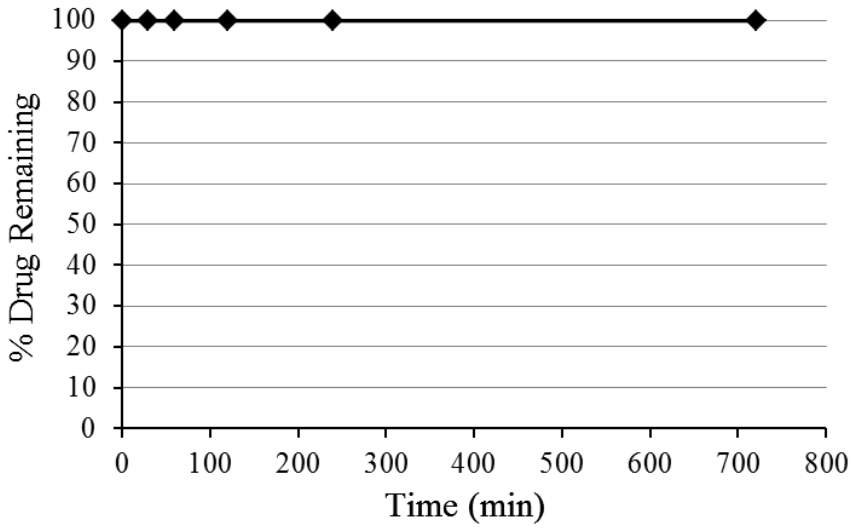

Fig. 2-9 In vitro release study of coumarin-6 loaded lipid emulsion in simulated tears (pH 7.4).

2.7 Transit routes illumination from surface to posterior segment of the eye

Present understanding suggests there are three potential routes of drug penetration from the corneal/conjunctiva surface to the retina for local drug delivery (Fig. 2-10) [36]: (1)trans-vitreous, which trans-corneal diffuse into aqueous humor then entry into vitreous subsequent distribution into the target posterior part; (2)periocular paths, permeation through the conjunctiva followed by diffusion across the sclera, choroid and retina; (3)uvea-sclera, drugs firstly trans-corneal pass into anterior chamber, and then reach to uvea-sclera tissues subsequent diffusion into posterior tissues. In order to elucidate the transit routes of the colloidal carriers to the posterior segment of the eye, the observation of the change of C-6 as a marker of LEs on entire of the eyes was carried out.

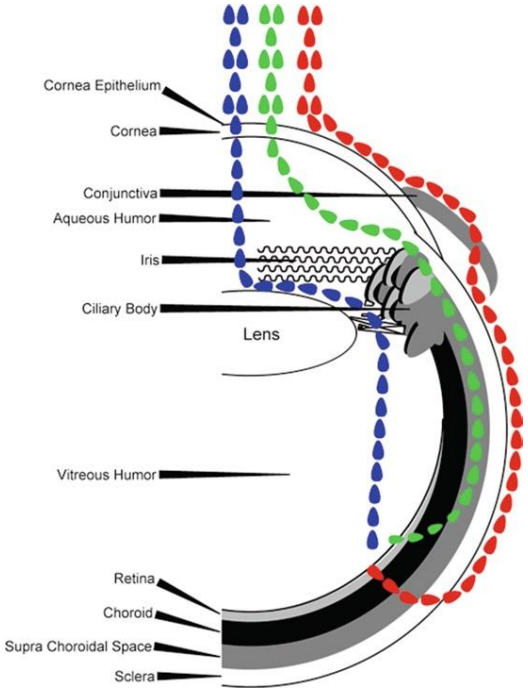

Fig. 2-10 Three potential routes for the penetration of topically applied ophthalmic drugs to the posterior segment. (1) The trans-vitreous route: trans-corneal diffusion followed by entry into vitreous and subsequent distribution to ocular tissues (blue line). (2) Periocular route: diffusion around the sclera followed by trans-scleral absorption (red line). (3) Uvea-scleral route: trans-corneal diffusion followed by progression through the uvea-sclera (green line). From Reference [36]

The fluorescent micrographic picture was taken 30 min after topically administrating with LEs (Fig. 2-11). The photograph confirmed that LEs administration achieved a strong fluorescence level on the cornea, and relatively weaker level on the aqueous side on lens, while negligible fluorescence was observed on the vitreous side of lens. Observations of C-6 fluorescence on the cornea and aqueous side of lens indicated that the colloidal carriers containing C-6 access to ocular tissues by way of corneal penetration into aqueous humor of anterior chamber. However, the undetectable level on the vitreous side of lens represented that drugs were restricted to continue distribution. This drug distribution pattern may exclude trans-vitreous as mainly transit into the posterior segment of the eye for colloidal drug delivery systems by the existence of lens block the distribution of drugs to the retina as discussed in Fig. 2-10 route (1).

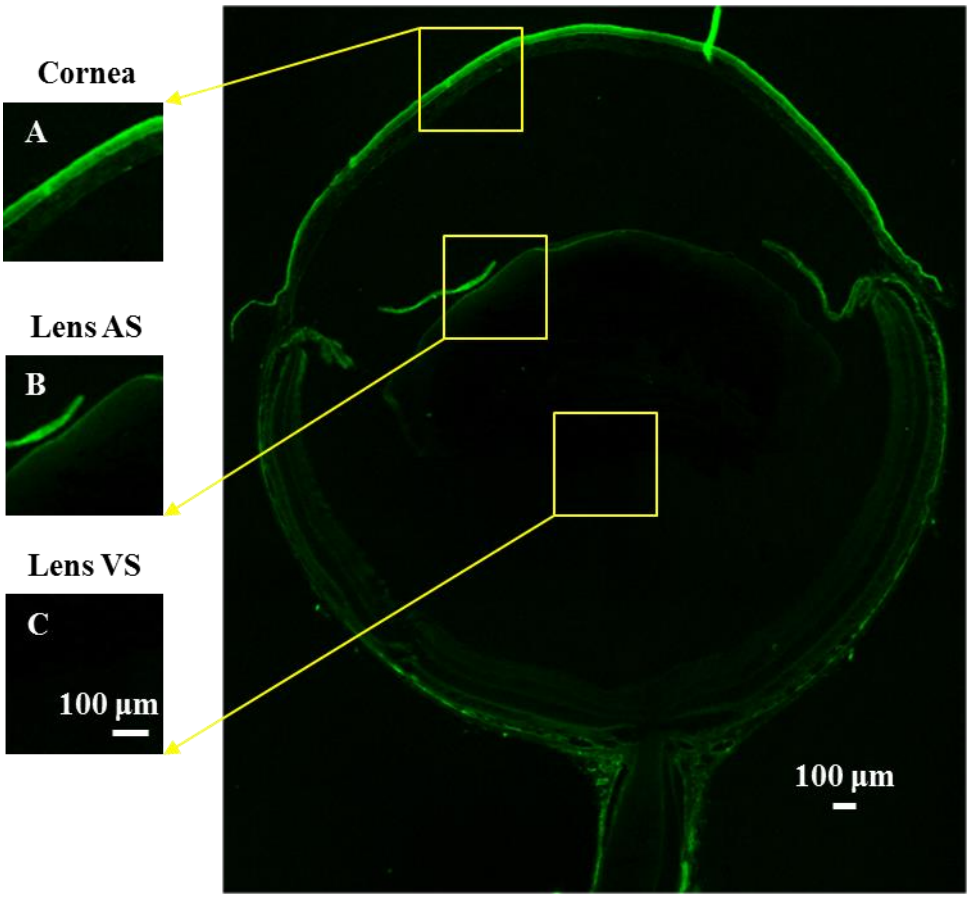

Fig. 2-11 A schematic representation of the entire eye 30 min after administration of lipid emulsion. (A) Cornea, (B) Lens aqueous side, (C) Lens vitreous side.

To confirm the further drug distribution behavior on the posterior tissues such as retina, two images of ocular posterior parts and their fluorescent quantification were taken (Fig. 2-12). As could notice, the fluorescent intensity of IPL of retina was shown to gradually decrease with regular directions. The diffusion of coumarin-6 moved from ocular cornea side (anterior side) across to optical nerve side (posterior side) in combine with distribution from sclera side to retina side. These data strongly suggested that the formulated colloidal carriers help drugs reach the posterior segment of the eye via the periocular trans-sclera route and combination of the uvea-scera route but not trans-vitreous route as shown in Fig. 2-10 routes (2) and (3).

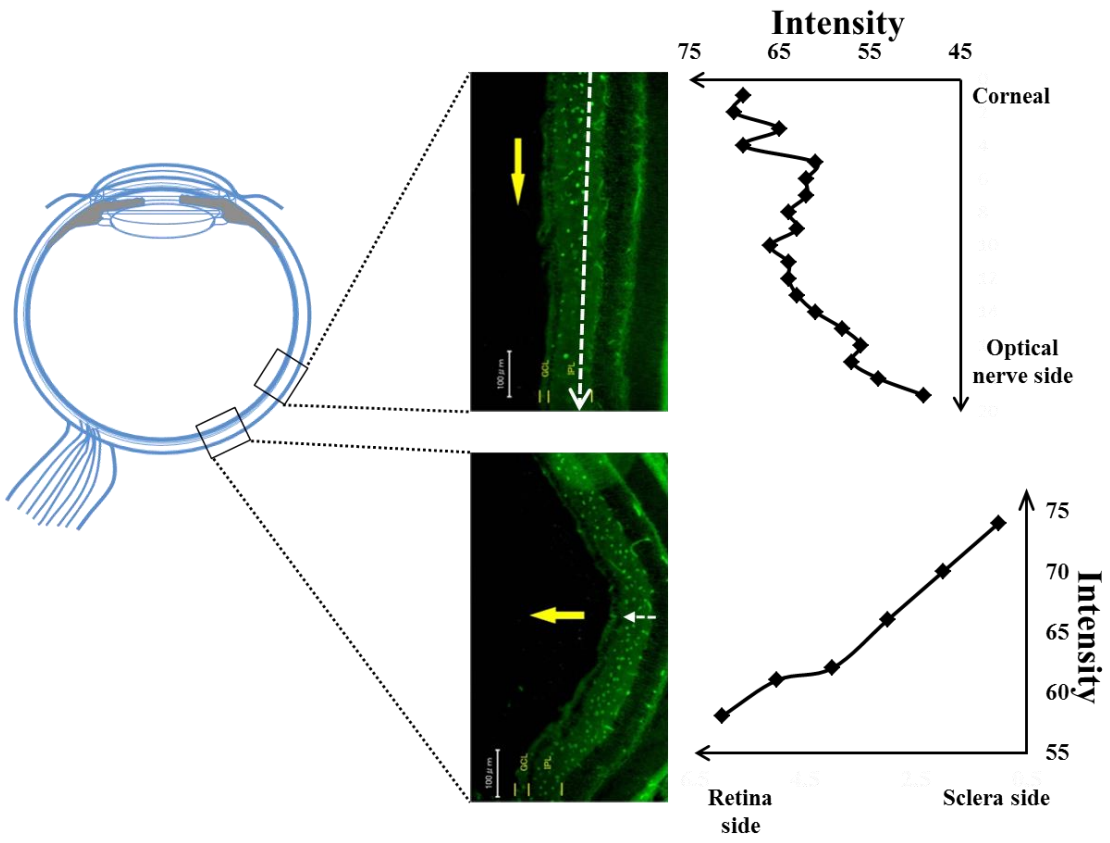

Fig. 2-12 A schematic representation of the ocular posterior segment parts 30 min after administration of lipid emulsion.

2.8 Conclusion

The main objective in this chapter was to investigate the feasibility of LEs in ocular posterior delivery of drugs via topical administration. The formulation effects on the drug delivery process and potential transit routes were discussed also. The conclusions were listed as follow:

(1) Very fine particle sized LEs (ca.100 nm) were achieved by using high pressure homogenizer. The formulations and operation conditions would affect the final physicochemical characteristics of LEs.

(2) It was confirmed that LEs could enhance the hydrophobic drug posterior segment delivery *in vivo*. However, it would not achieve by the oil droplet loaded with the same level of C-6. Moreover, the lipid emulsion showed very slow released behavior in vitro. All these data suggested that the drug posterior segment delivery

was contributed by the colloidal structure but not in a released manner on the surface of the eye.

(3) The positively charged and mucoadhesive functional polymer modified on the surface of LEs were important in improving the ocular drug posterior segment delivery. Nevertheless, the inner properties of the formulations showed little effect on the drug delivery.

(4) The mechanisms underlying topical administration for drug posterior segment delivery was supposed that drug distribution was not trans-vitreous route uptake into the posterior tissues but via periocular tans-scleral route and accompanied by the uvea-sclera route.

Chapter 3:

Polymeric micelles for enhancing trans-ocular absorption of hydrophobic drugs

3.1 Introduction

Nano sized spherical polymeric micelles, composed of an inner core and outer corona, are frequently considered to be promising drug delivery carriers. They are formed by amphiphilic block copolymers with the size between 10 nm to 100 nm at low critical micelle concentrations compared with those micelles made by low-molecular-weight ionic surfactants. Polymeric micelles have attracted increasing interests for their potential application to deliver various therapeutic compounds, such as hydrophobic drugs and gene-based therapy medicine. A number of studies have been reported in this field. Abdelbary and Tadros [37] discussed the application of block copolymer nano-micellar carriers as nose-to-brain targeted delivery system. Kataoka and co-workers described the progress of synthetic polymeric micelles for the intravenous delivery of chemotherapy drugs [38–40]. The polymeric micelles also have been well reported to be used as topical ocular delivery vehicle due to their good biocompatibility [41,42]. Although previous studies have demonstrated that polymeric micelles had a great potential to be employed as ocular drug delivery system, rarely researches focused on ocular posterior segment delivery.

D-α-tocopheryl polyethylene glycol succinate (Vitamin E TPGS, or simply TPGS), an amphiphilic block copolymer, has been used to overcome multidrug resistance (MDR) and promote the drug delivery efficiency as an inhibitor of P-glycoprotein (P-gp). Owing to its relatively low critical micelle concentration (CMC) of 0.02% w/w, TPGS was considered to be an ideal biomaterial in developing various drug delivery systems [43,44], especially in combination with PEG-DSPE [45,46]. PEG-DSPE is a biocompatible, micelle-forming pegylated lipid, with different level of PEG modified. Micelles formed by N- (Carbonyl- methoxy polyethylene glycol 2000)-1,2-distearoyl-sn-glycero-3-phosphorethanolamine, sodium salt (DSPE-PEG) exhibit very low critical micelle concentration (CMC) of approximately 1×10^{-6} M. This is attributed to the strong hydrophobic driving force for self-assembly originating from two saturated 18-carbon acyl chains, which makes DSPE-PEG2000 an ideal material to construct micelles for drug delivery [47–49].

In applying the polymeric micelles composed of these PEG-modified block copolymers as drug delivery systems, the outer corona of the micelles is condensed layer of PEG which can act as an excellent repellent for biomolecules. Such pegylated micelles are assumed to be poorly immunogenic and antigenic [50]. Considering their low CMC, the formed micelles should be sufficiently stable to withstand dilution after instillation.

In this study, we attempted to design and evaluate TPGS/DSPE-PEG polymeric micelles to reveal the potential for ocular posterior drug delivery and the effect of their formulations on delivery process via topical administration.

3.2 Preparation and characterization of polymeric micelles

In this study, micelles with TPGS and DSPE-PEG were processed using film casting method as described in the experimental section. The formation of polymeric micelles was prepared using various concentration and weight ratios of TPGS and DSPE-PEG. Four types of formulations were tested to characterized formation of the micelles of TPGS and DSPE-PEG. C-6 was added as a fluorescence marker in the formulations to detect their drug delivery *in vivo*.

The obtained micelles containing C-6 were clear and transparent with the color of fluorescence green. As shown in Table 3-1, the mean particle size of the four formulations was approximately 10 nm and all formulations displayed a similar neutral zeta potential. The size and zeta-potential values were negligibly affected by the ratio of TPGS and DSPE-PEG. For orange needle like crystallization of C-6, the entrapment was carried out with filtration method. As shown in Table 3-1, almost 100% of C-6 was encapsulated into the polymeric micelles prepared with molar ratio of 9:0, 6:3, and 3:6 (TPGS:DSPE-PEG). In the case of DSPE-PEG2000 micelles (0:9 of TPGS:DSPE-PEG), entrapment efficiency was decreased as low as ca. 50%.

To confirm the formation of polymeric micelles, transmission electron microscopic (TEM) analyses with molar ratios of 3:6 and 0:9 are illustrated in Fig. 3-1. In both formulations, the resultant TEM photographs indicated formation of monodisperse spherical micelles particles, whose size was about 10 nm as measured by dynamic light scattering (DLS) (Table 3-1). In comparing the two formulations, the 3:6 micelles appeared to be somewhat irregular shape compared with that of 0:9 micelles (Fig. 3-1). Moreover, as seen in Fig. 3-1, the particles of 3:6 micelles showed better dispersibility

than that of 0:9 micelles, on account of 0:9 micelles regularly arranged in a nice pattern compared to 3:6 micelles. It was considered to be attributable to the interposition of TPGS (with shorter PEG segments and single hydrophobic chain) into the core of mixed micelles, which disturbed the regular arrangement of DSPE-PEG micelles and also changed the repulsive force among particles. From this viewpoint, the TEM study also confirmed the interposition of TPGS in each micelles but not TPGS and DSPE-PEG forming micelles separately.

In addition, 0:9 polymeric micelles displayed aggregation in 24 h at ambient temperature. However, the other three formulations provided better stability of over one week in the refrigerator. These data suggested that the incorporation of TPGS in 3:6 micelles formulation may help to disperse the micellar system and achieve better stability.

Table 3-1 Summary of formulations, Particle Size, and Zeta potential of micelles

The ratio of TPGS/DSPE-PEG2000	**TPGS (mM)**	**DSPE-PEG (mM)**	**Size*** **(nm)**	**Zeta-potential*** **(mV)**	**Entrapment Efficiency %**
9:0	6.6	0	18.1	-3.62	~100
6:3	4.4	2.2	10.1	1.05	~100
3:6	2.2	4.4	8.7	-1.35	~100
0:9	0	6.6	11.7	-3.01	46

*Determined by DLS.

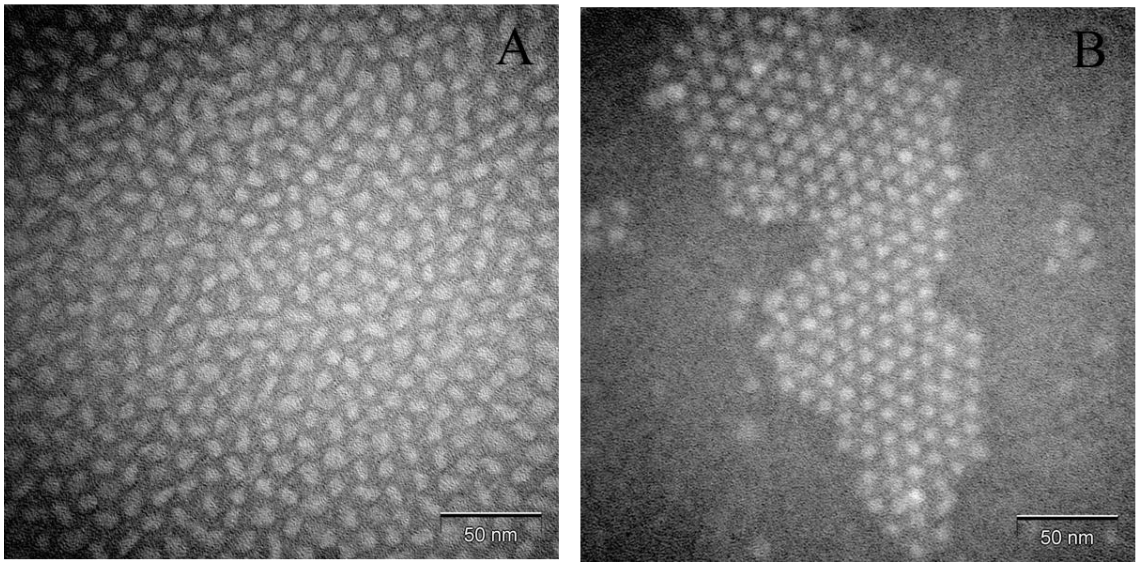

Fig. 3-1 Transmission electron microscopic image of coumarin-6-loaded TPGS/DSPE-PEG micelles with molar ratios of 3:6 (A) and 0:9 (B).

3.3 Cytotoxicity study

A human conjunctiva cell line was employed to investigate the cytotoxicity of blank and C-6 loaded polymeric micelles with four formulations shown in Table 3-1. TPGS has been well reported to use as a permeation enhancer, stabilizer, or emulsifier in drug delivery systems in several studies [43,51]. As shown in Fig. 3-2, the cellular viability decreased with increasing amount of TPGS in polymeric micelle formulations. However, DSPE-PEG single micelles showed great ocular tolerability. This indicated that the cytotoxicity of micelles exhibited a highly TPGS concentration dependent manner. In addition, encapsulated C-6 did not induce further cytotoxicity in contrast to that without C-6 encapsulated formulations. According to these data, the polymeric micelles prepared with molar ratio of 3:6 and 0:9 with good cellular viability were selected to use for further researches in the following sections.

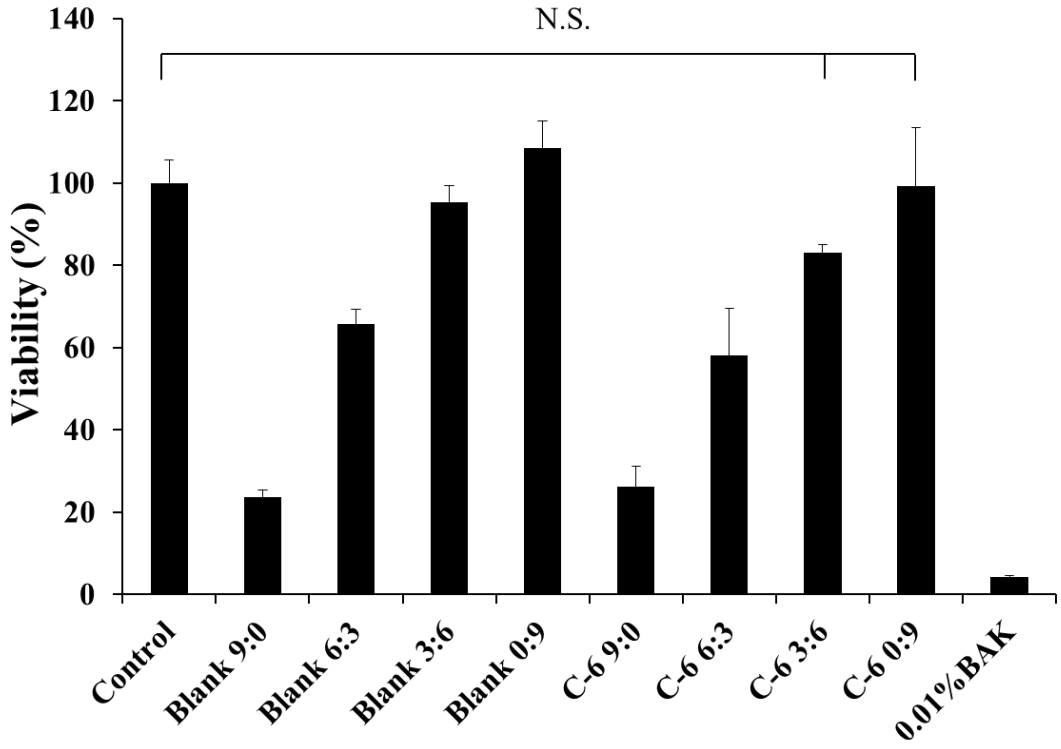

Fig. 3-2 Cytotoxicity of TPGS/DSPE-PEG2000 mixed micelles with and without loaded C-6 in the conjunctiva cells. The concentration of the mixed micellar solution was 6.6 mM in all experiments irrespective of the molar ratios of the components. Data are presented as the mean ± SD (n = 4-8). N. S. means no significant difference was observed.

3.4 In vitro release study

In order to understand the release behavior of micelles, the in vitro release of C-6, from 3:6 and 0:9 micelles were evaluated in an aqueous medium containing tween 80. As shown in Fig. 3-3, sustained drug release profiles were seen in both formulations, while 3:6 micelles showed slightly faster release than 0:9 micelles. Moreover, very small amount of the drugs were released in the first 30 min from both formulations. Considering the short residence time (<5 min) of topically applied drugs and neglecting the influence of enzyme, it may suggest that during the retention time on the surface of the eye, only very little amount of drug was released after instillation. In addition, in the study 1% Tween 80 was added to the medium to create a sink condition, establishing a more ideal environment than present on the surface of the eye, which may suggest the micelles would be more stable after installation than observed in the vitro release study. All evidences indicated that both of 3:6 and 0:9 micelles are relatively stable resistant to

the incubation *in vitro*.

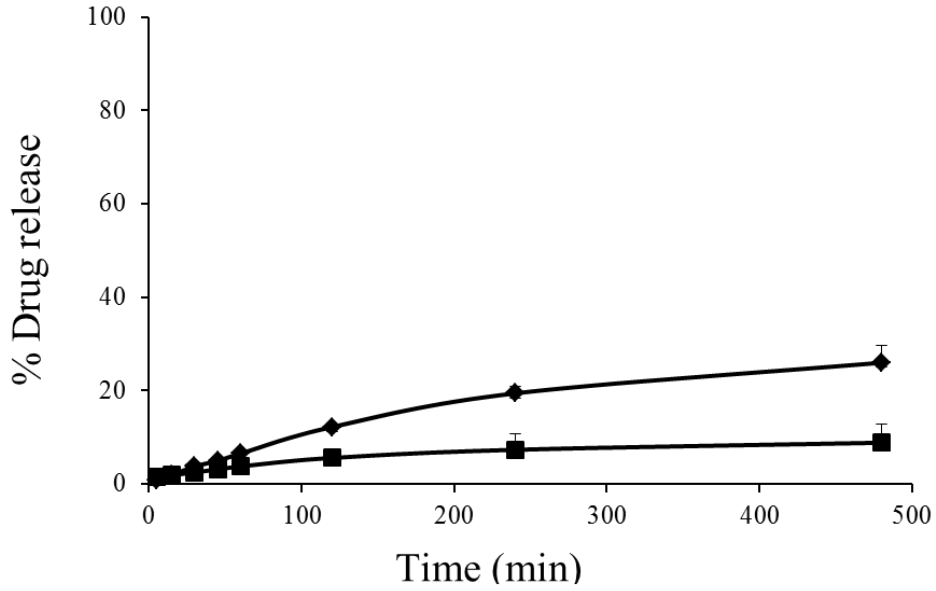

Fig. 3-3 *In vitro* release profile of coumarin-6-loaded TPGS/DSPE-PEG2000 3:6 (♦) and 0:9 (■) micelles (mean ± SD, n = 3).

3.5 Cellular uptake of polymeric micelles

In order to elucidate the uptake behavior of the two polymeric micellar formulations, a preliminary internalization delivery study was conducted using confocal laser scanning microscope (CLSM) against conjunctiva cells. The results showed that TPGS incorporation was effective for model drug uptake into conjunctiva cells. In another word, 3:6 micelles indicated stronger cellular association than 0:9 micelles (Fig. 3-4).

Moreover, both formulations showed great stability in the first 30 min incubation in the test. However, drug crystals were discerned after 1 h incubation of 3:6 micelles. This may resulted from the relatively high mobility of 3:6 micelles with faster drug release as well as the partial destruction of 3:6 micelles under ocular circumstance, while 0:9 was not. With increasing incubation time, crystallization increased and more amount of drugs were liberated from the micelles, as shown in Figs. 3-4(B-C). These results could prove that the interposition of single chain of TPGS into the core of 3:6 micelles, may cause a loosen structure, irregular shape and a better dispersion but with the loss of bio-resistant property. In the case of 0:9 micelles, aggregated fluorescent spots were observed instead of crystals after 2 h incubation. Based on these observations, it appeared that 0:9 micelles had better bio-stability on the surface of the ocular cells, and

C-6 had a strong association with the hydrophobic chains of DSPE-PEG, which was not relived from the structures of micelles. This may explain the slower in vitro drug release rate and the lack of crystal precipitation for the 0:9 micelles.

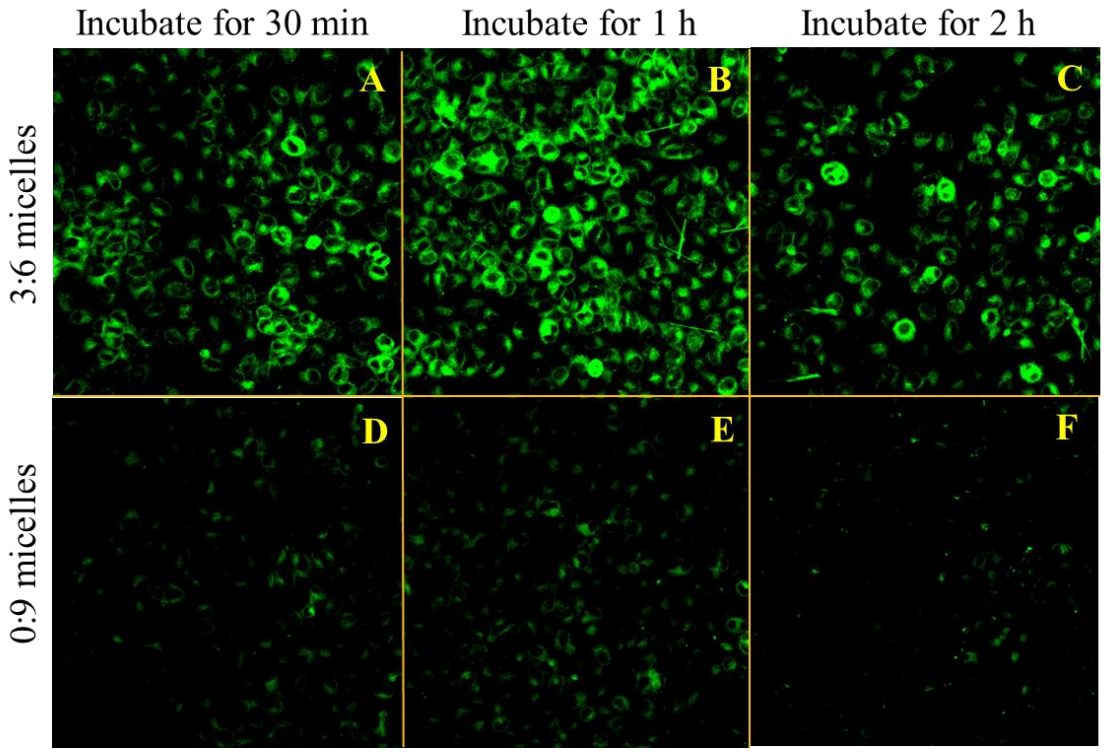

Fig. 3-4 Evaluation on the association of micelles with the conjunctiva cells via confocal laser scanning microscopy. D-α-tocopherol polyethylene glycol 1000 succinate/DSPE-PEG2000 3:6 and 0:9 micelles were incubated for 30 min (A), (D), 1 h (B), (E) or 2 h (C), (F).

3.6 Endocytosis inhibition study

The main objective in this section was to study the uptake mechanism of polymeric micelles by endocytosis inhibition study for 3:6 polymeric micelles. As shown in Fig. 3-5, the uptake of 3:6 polymeric micelles incorporating C-6 was significantly inhibited by both clathrin-dependent endocytosis inhibitor and caveolae-dependent endocytosis inhibitor. These results suggested that clathrin- and caveolae-dependent endocytosis were involved in conjunctiva cellular uptake of polymeric micelles.

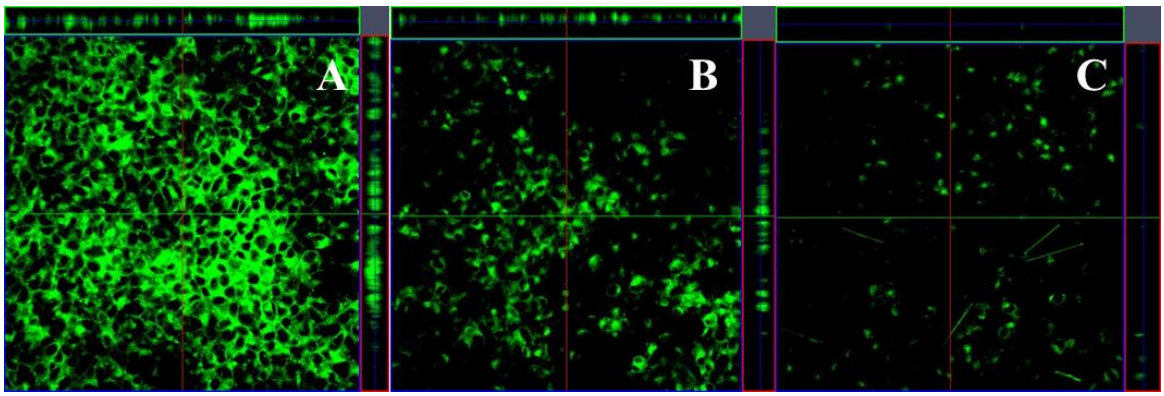

Fig. 3-5 Uptake mechanism study of 3:6 polymeric micelles by conjunctiva cells. Confocal microscopy images of 3:6 polymeric micelles when conjunctiva cells were pretreated without (A) or with 10μg/mL chlorpromazine for 1 h to inhibit clathrin (B) and 1μg/mL filipin III for 1 h to inhibit caveolae endocytosis (C) and then treated with 200 μL 3:6 micelles for 1 h.

3.7 In vivo animal study

The drug delivery efficiency of entrapped C-6 was assessed after topical eye drop of 3:6 and 0:9 polymeric micelles. Measurement of fluorescent level on IPL was performed to estimate the drug posterior segment delivery efficiency. Data in Fig. 3-6 revealed the absence of any significance difference between 3:6 and 0:9 micelles, although both micelles exhibited promising drug delivery efficiency to the posterior segment of the eye compared to the untreated one. These results confirmed that both micellar systems were potential carriers to be used in ocular drug posterior delivery. However, it was slightly inconsistent with the data in CLSM uptake study. In view of topical administration, eye drop formulations experienced rapid elimination due to tear turnover, nasolacrimal drainage, non-productive adsorption, and other factors. The residence time of topically applied formulation is normally less than 5 min, and with tear dilution, only 1%–5% of applied drugs can reach the intraocular tissues [41]. The drug delivery to the ocular posterior may result from two possibilities, the first one may be attributed to the released drugs on the surface of the eye, and the other one may be caused the internalization of drug carriers for delivering to the ocular posterior segment. Therefore it can be hypothesized that if the drug ocular delivery was mainly because of drugs released from carriers to the surface of the eye, the results of 3:6 micelles *in vivo* should appear to higher drug level delivery than 0:9 micelles. However, this was not the case here. As previously reported, the ocular posterior delivery of topically administrated

drugs was caused mainly by the endocytosis of hydrophobic drug carriers but not in a released manner [11,12]. This was in agreement with our results in the study, which indicated that ocular posterior segment delivery was not mainly caused by drugs released from carriers. In agreement with these researches, with considering the endocytosis inhibition results, we identified the endocytosis of micellar drug carriers as the main mechanism for drug ocular posterior delivery after topical administration [52]. Therefore, the retina delivery of the model drug was believed to be mainly affected by the penetration of intact micelles loaded with the drug on the surface of the eye.

As discussed above, the interposition of TPGS contributed to the mobility of micelles and produced a positive effect on drug release in the case of 3:6 polymeric micelles. Furthermore, the incorporation of TPGS in 3:6 micelles significantly increased the ocular cell association and internalization compared with the case of 0:9 micelles. These results suggested that drug delivery to the posterior segment was not only related with the internalization of colloidal carriers in ocular cells on the surface of the eye after eye drop, but also well associated with the transitions of carriers in the ocular tissues, which may be relevant to the bio-stability of colloidal carriers during the delivery process.

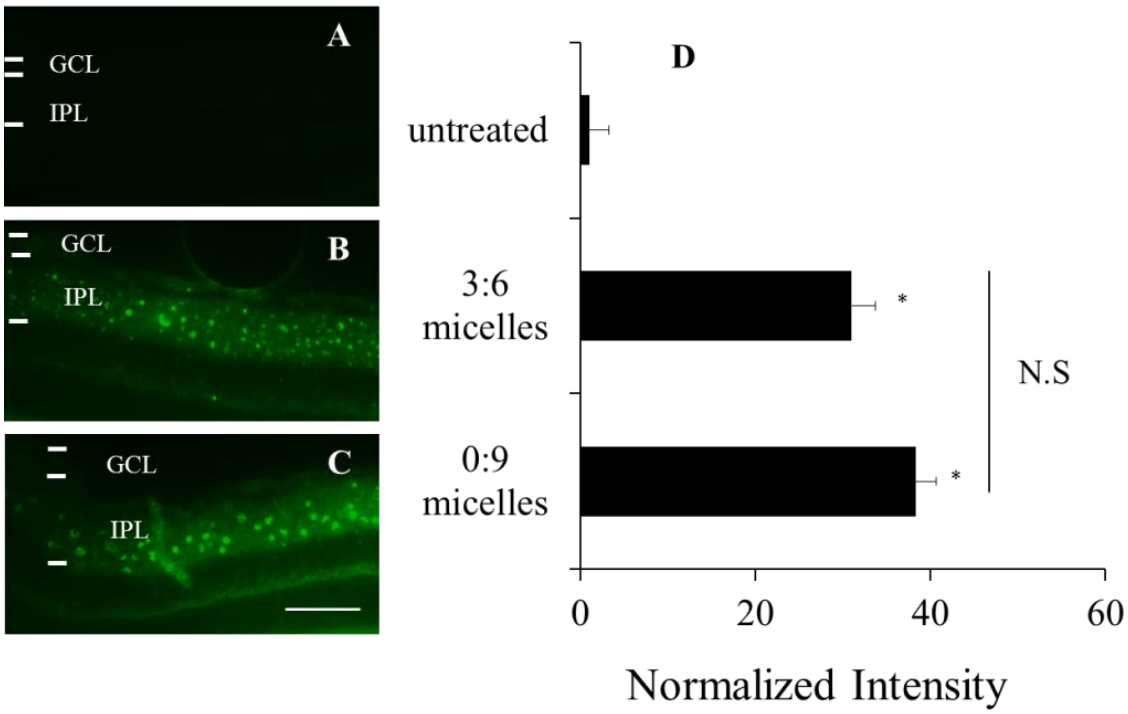

Fig. 3-6 Epifluorescence microscopic images of the posterior segment 30 min after the topical administration of TPGS/DSPE-PEG2000 micelles with molar ratios of 3:6 (B), 0:9 (C) and untreated (A). Changes in the measured median fluorescence intensity in the IPL after the administration of 3 μl of micelles were measured (D). Data are presented as the mean ± SEM. (n = 8–10). *P < 0.05 versus untreated. The scale bar represents 100 μm. GCL, ganglion cell layer; IPL, inner plexiform layer.

3.8 Conclusion

The feasibility of TPGS/DSPE-PEG micelles for enhancing the trans-ocular posterior segment delivery of hydrophobic drugs was investigated in the chapter. Different molar ratio of formulations were prepared and evaluated. The conclusions were listed as follow:

(1) The uniform and spherical micelles formulated by TPGS/DSPE-PEG with molar ratios of 3:6 and 0:9 showed good biocompatibility and were indicated to have good potential as drug carriers for topical ocular posterior segment delivery.

(2) Although uptake study of the two formulations showed apparently difference in conjunctiva cells, the drug posterior delivery was indicated almost the same level. This result suggested that drug ocular posterior segment delivery was not only relative with the internalization of colloidal carriers on the surface of the eye but also well associated with the transitions of carriers in the ocular tissues, which could not determinate one-sided data.

(3) The clathrin- and caveolae-dependent endocytosis was suggested to be involved in the conjunctiva cellular uptake of micelles in this study.

(4) The involvement of permeation enhancer (TPGS) in the formulation may be beneficial to drug encapsulated, formulation stability and internalization on the surface of the eye, but with sacrificing the bio-stability of micellar carrier. This may help the internalization of colloidal drug carriers into surface of the eye, but in vivo data suggested the drug posterior segment delivery was also well associated with the integrity of the drug carriers during transition in the ocular tissue.

Experimental Methods

Chapter 2

2.1 Materials

Egg phosphatidylcholine (EPC) and hydrogenated Soy L-α-phosphatidylcholine (HSPC) were purchased from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). Dicetylphosphate (DCP) and Stearylamine (SA) were supplied by Sigma Chemical Co. (St. Louis, MO, USA) and Tokyo chemical Industry Co., Ltd. (Tokyo, Japan), respectively. Medium chain triglyceride (MCT, Triester F-810) was supplied by Nikko Chemical (Tokyo, Japan). Castor oil was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Coumarin-6 was purchased from MP Biomedicals LLC (Illkirch, France). Oligo CS (Oligo Chitosan MW around 1000) was supplied by Katakura Chikkarin Co., Ltd. (Tokyo, Japan). P407 (Poloxamer 407) was supplied by BASF (Ludwigshafen, Germany). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Nakalai Tesque (Kyoto, Japan).

2.2 Preparation of lipid emulsion

Lipid emulsions were prepared using a high pressure homogenization system (Nanomizer, Yoshida Kikai Co., Ltd., Nagoya, Japan). In brief, purified phospholipids with DCP or SA and coumarin-6 were dissolved in MCT with gentle heating. Glycerol was dissolved in deionized water as the aqueous phase. To achieve a coarse emulsion, the two phases were heated separately to 70°C and then were emulsified using a high-shear mixer (Polytron homogenizer, Kinematica, Lucerne, Switzerland) at the speed of 12000 rpm for 5 min. This emulsion was then cooled to room temperature, the pH was adjusted to 8.0, and the desired volume was made up with purified water. Finally, the emulsion was homogenized into a fine mono-dispersed emulsion with the high pressure homogenizer under 150 MPa for 10 min.

2.3 Surface modification of lipid emulsions with CS and P407

The prepared lipid emulsions (Formulation A, Table 2-1) were then mixed with an appropriate amount of 0.4% w/v and 10% w/v solution of CS and P407 in HEPES buffer respectively to produce a final concentration of 0.2% and 5% w/v. The samples were then incubated 1 h at room temperature after gently stirring for 30 min.

2.4 Determination of particle size and zeta potential

The diameter of the dispersed oil droplets in the emulsions and the zeta potential were

analyzed using Zetasizer Nano-ZS (Malvern Instrument Ltd., Worcestershire, UK) at 500 times dilution with deionized water at room temperature.

2.5 Determination of entrapment efficiency

In consideration of the crystallization properties of coumarin-6, the un-entrapped coumarin-6 will not dissolve in the water phase of emulsion. Therefore, the prepared lipid emulsion was passed through 0.8μm filter. Then the fluorescent intensity of coumarin-6 in the formulations was quantified by Fluorospectro Photometer. The entrapment efficiency of lipid emulsion loaded with coumarin-6 was calculated as followed formula:

Entrapment efficiency % = Fluorescent intensity of lipid emulsion after filtration/Fluorescent intensity of lipid emulsion before filtration ×100

2.6 Animal study

2.6.1 Animal

Male ddY mice (Japan SLC, Hamamatsu, Japan), 4 weeks old (30g-35g), were used as the animal model. All protocols were approved by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University, and the experiments were monitored as they were being carried out.

2.6.2 In vivo protocol

Ophthalmic lipid emulsion (3 μl) was instilled directly onto the left eye of unanesthetized mice. The contralateral eye was used as a control and received no treatment. After 30 min of instillation, the mice were sacrificed by cervical dislocation, and the eyes were enucleated immediately with excess saline washing. The eye balls were fixed overnight in 4% paraformaldehyde (Wako, Osaka, Japan) at 4°C and immersed into 20% sucrose at 4°C for 48 h. The eye balls were frozen using liquid nitrogen and rapidly embedded in optimal cutting temperature (OCT) compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan). The frozen eye balls were kept in a −20°C refrigerator prior to slicing and observation. A cryostat (CM1850, Leica Instrument GmbH, Nussloch, Germany) was used to slice the embedded eye balls into 10 μm sections. The sliced frozen sections were placed on glass slides, Fluoromount^{TM} (DBS, Pleasanton, CA, USA) was added, and they were covered with a glass cover slip.

2.6.3 Observation and evaluation on the drug posterior segment delivery efficiency

The retinal image was observed by fluorescence microscopy (model BZ-9000; Keyence,

Osaka, Japan) with a 20 × objective lens or a 4× objective lens for the entire eye observation. Coumarin-6 was visualized using a BZ filter GFP (excitation wavelength 480/30 nm; emission wavelength 510 nm). The fluorescence intensity of coumarin-6 in the inner plexiform layer (IPL), at a distance between 475 and 525 μm (50 μm × 50 μm) from the optic disc, was evaluated by appropriately calibrated computerized image analysis using median density as an analytic tool (Image Processing and Analysis in Java; Image J, National Institute of Mental Health, Bethesda, MD, USA). The IPL is a suitable target for evaluating retinal delivery because it is located very close to the ganglion cell layer (GCL). GCL contains retinal ganglion cells (RGCs) and amacrine cells. The death of RGCs is a common feature of many ophthalmic disorders, such as glaucoma, optic neuropathy, and retinal vein occlusion. The fluorescence intensity of coumarin-6 in the range of 0–255 was considered as the mean density in a constant area (50 μm × 50 μm) using Image J. The fluorescence intensity of treated samples was calculated relative to that of the untreated sample, which was adjusted to 1.

2.7 In vitro release study

The release profiles of C-6 from the lipid emulsion were performed in phosphate buffer solution (pH 7.4) using the dialysis method. The fresh prepared lipid emulsion was poured into cellulose ester dialysis bag (MWCO: 2000Da, Spectrum, USA) against 50 mL of simulated tears [53] with gentle stirring at 37°C. At pre-determined time intervals, aliquots (0.5 ml) were withdrawn and replaced with fresh release medium. The amount of C-6 released was determined using fluorescence spectrometry.

2.8 Statistical analysis

Statistical analyses were carried out using SPSS statistical software (IBM, SPSS statistics, Ver. 20.0). Multiple comparisons between different formulation groups and their significance were analyzed using ANOVA followed by independent sample t test. Data are shown as the mean ± SEM. A difference was considered to be statistically significant when the P value was less than 0.05.

Chapter 3

3.1 Materials

Vitamin E TPGS and DSPE-PEG2000 were obtained from BASF (Ludwigshafen, Germany) and NOF Corporation (Tokyo, Japan), respectively. Coumarin-6, as a model drug, was purchased from MP Biomedicals LLC (Illkirch, France). Hank's balanced salt solution (HBSS) was purchased from GIBCO BRL (Grand Island, NY, USA). All other

chemicals were commercial products of the reagent grade.

3.2 Preparation of TPGS/DSPE-PEG polymeric micelles

Two kinds of micelle-forming block copolymer, TPGS and DSPE-PEG, were used to prepare by the film casting method as described previously, with minor modifications. Briefly, both of micellar-forming materials (TPGS and DSPE-PEG with molar ratios of 9:0, 6:3, 3:6, and 0:9) in desired concentration (final 6.6 mM) with 0.05 mg/ml coumarin-6 were dissolved in 5 ml of methanol and 5 ml of chloroform in a round-bottom flask. The organic solvent was removed by evaporation under vacuum to obtain a thin film, and the film was then dried in a vacuum oven overnight to ensure complete removal of the organic solvent. The resulted film was hydrated with PBS pH 7.4 in a water bath at 40°C for 10 min. Un-entrapped coumarin-6 was removed by centrifugation at 5000 rpm for 10 min at 4°C. The blank micelles were prepared in the same way except that coumarin-6 was eliminated.

3.3 Determination of particle size and zeta potential

The diameter of TPGS/DSPE-PEG polymeric micelles and the zeta potential were analyzed using Zetasizer Nano-ZS (Malvern Instrument Ltd., Worcestershire, UK) without dilution at room temperature.

3.4 Morphology study

The morphology of micelles was observed via transmission electron microscopy (JEOL JEM-1400Plus, JEOL Ltd, Tokyo, Japan) following negative staining with 2% sodium phosphotungstate solution (pH 7.0).

3.5 Cytotoxicity study

The in vitro cytotoxicity of blank and C-6-loaded micelles against the human conjunctiva cells (Wong-Kilbourne derivative of Chang conjunctiva, clones 1 to 5c-41, CCL-20.2; American Type Culture Collection, Manassas, VA, USA) were tested by the MTS assay. The conjunctiva cells were seeded at a density of 9.6×10^3 cells/well in 96-well transparent plates and incubated for 24 h under standard cell culture conditions. After the attachment period, medium was removed by washing twice with HBSS solution. The medium was then changed to TPGS/DSPE-PEG micelles. After 2 h of incubation, the cells were rinsed with HBSS three times to wash out the sample. Then, the cells were incubated with 120 μl of MTS solution (317μg/ml in medium) at 37°C in a CO_2 incubator for another 2 h. Absorbance values were measured with a microplate

reader (MTP 120, Corona Electric, Tokyo, Japan) at a wavelength of 490 nm.

3.6 In vitro release study

The measurements of the degree of drug release are based on the increase of C-6 fluorescence intensity in mediums when C-6 is transferred from the dialysis bag to the outer mediums. Briefly, the sample (1 ml) was poured into cellulose ester dialysis membrane bags, with a cutoff molecular weight of 2000 Da, (Spectrum, USA) and it was dialyzed against 100 ml of water containing Tween 80 (1% w/v), with gentle stirring at 37°C. At pre-determined time intervals, aliquots (0.5 ml) were withdrawn and replaced with fresh release medium. The amount of C-6 released was determined using fluorescence spectrometry.

3.7 Cell uptake study

Cells were seeded 24 h prior to treatment in 8-well chamber slides, with an initial density of 1.05×10^5 cells/well. After 30 min, 1 h, and 2 h of exposure to 200 µl of 6.6 mM freshly prepared micelle solutions at 37°C in a CO_2 incubator, cells were rinsed twice with PBS. Then, fixed using 4% PFA and incubated for 30 min at room temperature. Cells were observed to identify the cellular association of micelles and their intracellular locations using CLSM.

3.8 Endocytosis inhibition study

As the method of 3.7, before exposure to the polymeric micelles, conjunctiva cells were pretreated with 10µg/mL chlorpromazine for 1 h to inhibit clathrin and 1µg/mL filipin III for 1 h to inhibit caveolae endocytosis. Then treat with 200 µL 3:6 micelles for 1 h. After these processes, the endocytosis inhibition study was carried out with the same method as 3.7.

3.9 Animal study

In this study animal study carried out with the same condition as 2.5.

3.10 Statistical analysis

Statistical analyses were performed using SPSS statistical software (IBM, SPSS statistics, Ver. 20.0). Multiple comparisons between different groups and their significance were analyzed using ANOVA followed by independent sample t-tests. Data are presented as the mean ± SEM. A difference was considered statistically significant when the P value was less than 0.05.

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Abbreviations

EPC	egg phosphatidylcholine
HSPC	hydrogenated soy L-α-phosphatidylcholine
DCP	dicetyl phosphate
SA	stearylamine
MCT	medium chain triglyceride
C-6	coumarin-6
GCL	ganglion cell layer
IPL	inner plexiform layer
LEs	lipid emulsions
TPGS	D-α-tocopheryl polyethylene glycol succinate, Vitamin E TPGS
DSPE-PEG	N-(Carbonyl-methoxypolyethyleneglycol2000)-1,2-distearoyl-sn-glycero-3-phosphorethanolamine, sodium salt
CLSM	confocal laser scanning microscopy
TEM	transmission electron microscope

List of Publications

Articles in peer-reviewd journals:

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