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Design and evaluation of a liposomal delivery system targeting the posterior segment of the eye

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ABSTRACT

The purpose of this study was to evaluate the potential of submicron-sized liposomes (ssLips) as a novel system for delivering ocular drugs to the eye's posterior segment. Fluorescence emission of coumarin-6 formulated into ssLip was obvious in that segment in mice after eyedrop administration of the liposomal suspension. Such fluorescence was not observed after administration of either multilamellar vesicles or dimethyl sulfoxide (DMSO) solution containing the same amount of coumarin-6. The highest fluorescence of ssLip occurred 30 min after eyedrop administration, and all fluorescence disappeared after 180 min. The ssLip based on L-α-distearoyl phosphatidylcholine (DSPC ssLip) showed higher fluorescence emission in the retina than that based on egg phosphatidylcholine (EPC ssLip). These results confirmed that the magnitude of fluorescence in the retina was closely related to both liposome rigidity and particle size. Images of the entire eye showed that ssLip was delivered via the non-corneal pathway after administration. The liposomes tested in ocular cells showed little cytotoxicity. These results suggest that ssLip can be used to deliver drugs to the posterior segment of the eye.

1. Introduction

The leading causes of vision impairment and blindness are posterior segment-related diseases including age-related macular degeneration, diabetic macular edema and endophthalmitis. Recently, pharmaceutical approaches to these diseases have used steroids and oligonucleotides [1,2]. These drugs are generally administered via invasive methods, such as intravitreal injections and subtenon injections, because noninvasive delivery of drugs is not available. However, repeated injections are associated with potential risks of complications, such as cataracts, vitreous hemorrhages and retinal detachment [3]. Moreover, patients may not comply with such regimens. Thus, there is a pressing need for noninvasive delivery systems targeting the posterior segment of the eye.

Systemic administration is one possible way to obviate intravitreal or subtenon injection. One of the disadvantages of systemic administration to deliver drugs to the retina is its efficiency. A very small fraction of the systemically administered dose reaches the ocular tissues because the blood-retinal barrier can prevent the drug from entering the retina [4]. Moreover, the doses that are needed in order to have a therapeutic effect via this route can lead to considerable side effects [5]. Topical administration with eyedrops is an alternative way to minimize side effects. However, corneal and conjunctival epithelia, along with tear film, serve as biological barriers to protect the eye from potentially harmful substances and drugs. Therefore, conventional eyedrop formulations usually cannot effectively overcome these barriers. The use of colloidal drug delivery systems, such as nanoparticles, nanoemulsions and liposomes, has received much attention as a way to enhance the bioavailability of drugs administered both systemically and topically. We have recently demonstrated that submicron-sized liposomes (ssLips) penetrated mucosal cells in rat intestine [6]. This finding encouraged us to try to use ssLip in an eyedrop formulation in this study.

In ophthalmic therapy, many researchers have investigated the use of liposomes extensively. Liposomes reportedly can come into intimate contact with the ocular surfaces, thus working as barriers, and they can be used to protect drug molecules from metabolic enzymes present at the tear/corneal epithelium interface [7]. Hathout et al. reported that multilamellar liposomes containing acetazolamide were more efficient than acetazolamide solution in lowering intraocular pressure [8]. Shen and Tu reported that the ocular bioavailability of liposomal ganciclovir in liposomes in rabbits was 1.7-fold higher than that of a

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ganciclovir solution [9]. However, there have been no reports on drug delivery to the retina via eyedrops formulating a colloidal drug carrier such as liposomes.

The purpose of this study was to evaluate the potential of ssLip as a novel system for delivering ocular drugs to the posterior segment of the eye, including the retina. The behavior of liposomes labeled with coumarin-6 as a fluorescence reagent was investigated after it was topically administered to mice via eyedrops. The cytotoxicity of the liposomes was also tested *in vitro* using ocular cells.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC) and L-α-distearoyl phosphatidylcholine (DSPC) were purchased from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). Dicetyl phosphate (DCP) and cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Coumarin-6 as a lipid marker and benzalkonium chloride were purchased from MP Biomedicals LLC (Illkirch, France). 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) was purchased from Nakalai Tesque (Kyoto, Japan). Hank's balanced salt solution (HBSS) was purchased from GIBCO BRL (Grand Island, NY, USA). Mica was purchased from Veeco Co., Ltd. (Tokyo, Japan). All other chemicals were commercial products of reagent grade.

2.2. Preparation of liposomes

Multilamellar vesicles (MLV), composed of phospholipid (EPC or DSPC), DCP, and cholesterol at a molar ratio of 8:2:1, were prepared using the hydration method. The lipid mixture containing coumarin-6 was dissolved in a small amount of chloroform in a round-bottom flask and dried in a rotary evaporator under reduced pressure at 40 °C to form a thin lipid film. The film was dried in a vacuum oven overnight to ensure complete removal of the solvent. After addition of HBSS–Hepes buffer, lipid film was incubated in water bath at 70 °C for 30 s, and then vortexed for about 30 s. This cycle was repeated 5 times. The obtained MLV were incubated at 10 °C for 30 min. The ssLip was prepared using an extruder (LipoFast™-Pneumatic; Avestin, Inc., Ottawa, Canada) with a size-controlled polycarbonate membrane (0.1 μm membrane filter pore size; Whatman Japan KK, Tokyo, Japan). Extrusion was performed 41 times under nitrogen pressure (200 psi). The final phospholipid and coumarin-6 concentrations in the resultant liposomal suspension were 20.4 μmol/ml and 0.143 μmol/ml, respectively. The coumarin-6 entrapment efficiency into ssLip was calculated as follows. The ssLip was obtained by ultrasonication of the MLV followed by filtering the ssLip through a cellulose acetate filter (0.8 μm). The filtered suspensions (0.5 ml) were dissolved in chloroform/methanol (1/1, 3 ml), and the coumarin concentration was measured with fluorometry (F3010, Hitachi, Tokyo, Japan) at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The entrapment efficiency percentage of ssLip was calculated by the following equation: % entrapment efficiency $= A_{flt} / A_{int} \times 100$, where A_{flt} is the filtered amount of coumarin-6 in ssLip and A_{int} is the initial amount of coumarin-6 in ssLip. The ssLip particle size was measured with an aliquot of the particulate suspension diluted with a large amount of distilled water by the dynamic light scattering (DLS) method (Zetasizer, Malvern, Worcestershire, UK). The MLV particle size was measured by a laser diffraction size analyzer (LDSA-2400A; Tonichi Computer applications, Tokyo, Japan). The zeta potential of liposomes was measured using a laser Doppler method (Zetasizer, Malvern). Each batch was analyzed in triplicate. Both EPC and DSPC confirmed that more than 98% of coumarin-6 was entrapped into ssLip. As shown in Table 1, there were no differences in particle size, zeta potential or entrapment efficiency of coumarin-6 between the two types of phospholipids.

Table 1
Characterization of liposomes.

	Mean particle size (nm)	Zeta potential (mV)
EPC MLV	5750	−97.5
DSPC MLV	6450	−101.1
EPC ssLip	125.3	−62.9
DSPC ssLip	105.4	−66.2

2.3. Animal studies

Un-anesthetized male adult ddY mice (Japan SLC, Hamamatsu, Japan) weighing 30–35 g were used. The mice were fed a regular diet. All experiments were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University. A single dose of 3 μl of the liposomal formulation was dropped onto the surface of the left eye. The contralateral eye was used as the control and received no treatment. The mice were then sacrificed 5, 10, 30, 60 or 180 min after administration of the liposomal formulation. Both eyes were enucleated immediately and washed with excess amount of saline, and then fixed overnight in 4% paraformaldehyde at 4 °C. Fixed eyes were immersed in 20% sucrose for 24 h at 4 °C and embedded in optimal cutting temperature compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan). The samples were then sliced with a cryostat (CM1850, Leica Instrument GmbH, Nussloch, Germany) into sections 10 μm thick and placed onto slides under a cover slip. The retinal images, taken at distances between 375 and 625 μm from the optic disc of frozen sections, were observed using epifluorescence microscopy (BX50; Olympus, Tokyo, Japan) with an attached CCD camera (DP30VW, Olympus) and fluorescence filters for coumarin-6 (U-WNIBA, Olympus). In the inner plexiform layer (IPL) at a distance between 475 and 525 μm (50 μm × 50 μm) from the optic disc, the fluorescence intensity of coumarin-6 was evaluated with 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 fluorescence intensity of coumarin-6 was measured in the range of 0–255 as the mean density, using Image J at the constant area (50 μm × 50 μm). The relative intensity indicates the value of a treated sample when the fluorescence intensity of an untreated sample is estimated as 1. A frozen section of eyeball was prepared, and the entire eye was scanned with fluorescence microscopy (BZ-9000, Keyence, Osaka, Japan) in order to obtain a schematic representation of the entire eye.

2.4. Evaluation of the rigidity of ssLip with atomic force microscopy (AFM)

We used a commercial AFM apparatus (Nanoscope IIIa system controller, Digital Instruments Inc., Santa Barbara, CA, USA) with an E-scanner possessing a maximum range of 10 μm × 10 μm × 2.5 μm. All images were captured in distilled water at room temperature with a silicon nitride probe (DNP-S20, Veeco Co., Ltd, nominal spring constant: 0.32 N/m). The scanning speed was optimized between 1.0 and 2.5 Hz depending on the scan size. All images were recorded by both height and amplitude modes, and they were analyzed in the height image mode. Surface-modified mica was used as the substrate for AFM observation. The mica was kept in a vacuum oven and was later prepared for surface modification using 3-aminopropyltriethoxysilane (AP) and N,N-diisopropylethylamine (DI) as reported by Thomson et al. [10]. Positively charged mica was used for adsorption between the mica and negatively charged liposomes. Freshly cleaved mica was incubated in a petri dish with AP (2 μl) and DI (1 μl) containing the top layer from the microcentrifuge tubes for 2 h.

The liposomal suspension was diluted with distilled water, and the phospholipid concentration of liposomes was 20.4 μM. The surface-modified mica and a quartz glass cell were set for the fluid tapping mode. Then, a suspension of ssLip was adsorbed onto the substrate surface, which was then washed three times with distilled water to