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2 Research paper

Fabrication and characterization of silk fibroin-coated liposomes for ocular drug delivery

Yixuan Dong [a,b], Pin Dong [a], Di Huang [a], Liling Mei [a], Yaowen Xia [a], Zhouhua Wang [a], Xin Pan [a,c,*], Ge Li [d], Chuanbin Wu [a,b,c,*]

[a] School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, China
[b] Research and Development Center of Pharmaceutical Engineering, Sun Yat-sen University, Guangzhou, China
[c] Guangdong Research Center for Drug Delivery Systems, Guangzhou, China
[d] Guangzhou Neworld Pharm. Co. Ltd., Guangzhou, China

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A B S T R A C T

The unique structure and protective mechanisms of the eye result in low bioavailability of ocular drugs. Using a mucoadhesive material is an efficient solution to improve ocular drug therapeutic efficacy. This study was designed to prepare a liposomal formulation coated by a novel adhesive excipient, silk fibroin (SF), for topical ocular drug delivery. The regenerated silk fibroins (SFs) with different dissolving time were coated onto the ibuprofen-loaded liposomes. The morphology, drug encapsulation efficiency, *in vitro* release and *in vitro* corneal permeation of SF-coated liposomes (SLs) were investigated in comparison with the conventional liposome. Cellular adhesion and cytotoxicity assay of SF and SLs were tested using human corneal epithelial cells (HCEC). SLs showed sustained drug release and *in vitro* corneal permeation of ibuprofen as compared to drug solution and conventional liposome. The cellular fluorescence appeared after 7 min of exposure to SF, and the intensity increased sustainedly up to 12 h with no detectable cytotoxicity. Higher fluorescence intensity of Nile red in SLs was observed in a short period of 15 min showing a rapid uptake. These favorable properties make SF-coated liposomes be a promising ocular drug delivery system.

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1. Introduction

Ocular drug delivery is one of the most challenging pharmaceutical tasks, because of the critical and unique environment in the eye. For instance, most of the topically applied drugs will be washed off quickly from the surface of the eyes by various mechanisms, such as lacrimation, tear dilution and tear turnover. In addition, corneal and conjunctival epithelia, along with the tear film, construct a compact barrier preventing the drug absorption into the intraocular area [1]. Given its convenience of administration and clinical compliance of the patients, liquid eye drop is the most desirable dosage form. However, because of the low ocular bioavailability, frequent instillation of eye drop is often required to get the expected therapeutic effect, resulting in inconvenience, toxic side effects, and cellular damage at the ocular surface [2,3].

Researchers have focused on two ways to achieve more efficient ocular delivery: increasing the drug transport through the ocular barriers or extending the residence time of drug on the eye surface [4]. Several drug delivery systems, such as micro- or nano-emulsions [5], micro- or nano-particles [6], hydrogels [7], liposomes [8], have been demonstrated to improve the ocular drug transport. Liposomes in particular, which offer advantages including enhanced drug absorption, high biocompatibility, and biodegradability, have been proved as promising formulations for ocular drug delivery in the last decade [9]. In addition, the phospholipid bilayers and aqueous cavities impart liposomes the ability to encapsulate both hydrophilic and hydrophobic compounds. Various drug molecules, such as ganciclovir [10], ciprofloxacin [11] and tropicamide [12], have been exploited in liposomal formulations for ocular delivery, resulting in low tear-driven dilution in the conjunctival sac and high transcorneal permeability.

Abbreviations: SF, silk fibroin; SLs, SF-coated liposomes; HCEC, human corneal epithelial cells; SA, stearylamine.

* Corresponding authors at: School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, China. Tel.: +86 020 39943427; fax: +86 020 39943115 (X. Pan). Tel.: +86 020 39943120; fax: +86 020 39943117 (C. Wu).
E-mail addresses: pxin_1385@163.com (X. Pan), chuanbin_wu@126.com (C. Wu).

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However, the topical administration of conventional liposomes showed a similar *in vitro* drug release profile and precorneal retention time compared with drug solution [8]. For a controlled drug delivery and prolonged retention time on the corneal surface, it is necessary to utilize mucoadhesive polymers as viscosity enhancers to prolong the contact time between the formulations and the corneal/conjunctival epithelium [13]. Swan et al. firstly proposed using methyl cellulose to prolong the contact time with the eyes [14]. The interactions between polymers and the mucus layers of the eye tissues resulted in an increase of residence time in the precorneal area for the preparations with suitable polymers, which included chitosan, hyaluronan, polysaccharides, and cellulose derivatives [15]. Furthermore, polymer-coated liposomes also displayed prolonged *in vitro* drug release profiles due to additional diffusion barriers for drug molecules [8,15,16].

Silk fibroin (SF), a natural protein polymer obtained from mulberry silkworms of *Bombyx mori*, is composed of a heavy chain (350 kDa) and a light chain (25 kDa) which bound together by a sericin coating [17]. SF can be degummed to remove the highly immunogenic sericin coating, resulting in a liquid form. The liquid silk protein can be further processed into various carrier such as regenerated fibers, particles, membranes, films, gels and porous matrix for different applications [18]. These processes usually contain a structural transformation from random coil and α-helix (water soluble state) to β-sheet (water insoluble state) upon exposure to heat or organic solvents, resulting in excellent mechanical properties and modified drug delivery behaviors. SF properties, such as nontoxicity, non-immunogenicity, proteolytic degradability, and mechanical superiority, have been extensively investigated [19–22]. It was reported that SF related formulations showed increased cell adhesion and uptake behaviors [23,24], which were expected as the excellent properties of novel ocular drug delivery systems. However, to the best of our knowledge, no studies about the application and advantages of SF based nanoparticulate systems for ocular drug delivery have been reported yet.

In our study, SF, the novel mucoadhesive polymer, was utilized as the coating of liposomes for ocular drug delivery, and methanol was used to induce the structural transformation of SF. The molecular weight and the secondary structure of SF were studied. The *in vitro* release, isolated corneal permeation and cellular uptake behaviors of the formulations were also investigated. Sustained drug release and corneal permeation, rapid and sustained interactions with corneal epithelial cells were observed.

2. Materials and methods

Cocoons of *B. mori* were purchased from Guangxi Silk Group Co., Ltd. (Guangxi, China). Purified soybean lecithin (PC S100, ⩾94% phosphatidylcholine, approximately 70% linoleic acid, 8% lineolic acid, 5% oleic acid, 13% palmitic acid and 4% stearic acid residues) and stearylamine (SA) were obtained from Lipoid GmbH (Germany) and Sigma–Aldrich (USA), respectively. Ibuprofen was purchased from Shandong Xinhua Pharm. Co., Ltd., (Shandong, China). Albino New Zealand rabbits were obtained from Laboratory Animal Center of Sun Yat-sen University (Guangdong, China). Human corneal epithelial cells (HCEC) were kindly provided by Zhongshan Ophthalmic Center (Guangdong, China). Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Hyclone®, Thermo Scientific, USA) and fetal bovine serum (FBS, Hangzhou Sijiqing Bio. Eng. Materials Co., Ltd., China) were used for cell culture. All other commercially available chemicals were used at analytical grade.

2.1. Preparation of regenerated silk fibroin

SF was prepared from cocoons of *B. mori* as previously described [19,25,26]. Briefly, cocoons were boiled twice in a solution of 0.02 M Na_2CO_3 for 20 min and thoroughly rinsed with deionized water. After completely dying at 40 °C, the degummed silk fiber was dissolved in a ternary system containing $CaCl_2$–ethanol–H_2O (mole ratio = 1:2:8) under stirring at 78 °C for 2, 4, 6 and 8 h. The resulting fibroin solution was centrifuged at 4500 rpm for 15 min, and the supernatant was dialyzed in a cellulose tube (Viskase, USA; MWCO 8000–14,000 Da) against deionized water for 3 days to remove $CaCl_2$, smaller molecules and other impurities. The fibroin solution was then collected and freeze-dried for 48 h (Christ, Germany), and finally cotton-like SF solid was obtained and stored in an airtight container at room temperature till use.

2.2. Characterization of silk fibroin

2.2.1. Molecular weight

Molecular weights of SFs with different dissolving time were measured using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) [27]. Two polyacrylamide gels prepared by using 6% and 15% bisacrylamide were used for gel electrophoresis. After electrophoresis, proteins were stained with 0.25% Coomassie Blue, and the gels were destained in acetic acid.

2.2.2. Secondary structure

The secondary structures of SF with different dissolving time and methanol treatment were determined by X-ray diffraction (XRD) and Fourier transform infrared spectroscopy (FT-IR) [28,29]. Lyophilized SFs were incubated with different concentrations of methanol (0, 20, 40, 60, 80, 100%, v/v) for 10 min. Then the structural transformations of methanol-treated SFs were detected by XRD (D-MAX 2000 VPC, Rigaku, Japan) at a voltage of 10 kV and the glancing angle of 4–50°. Regenerated SF and SF treated with 50% methanol were tested by a FT-IR spectrophotometer (Bruker Tensor 37, Germany). FT-IR spectra in transmission mode were obtained in the region of 1000–1800 cm^{-1} by accumulation of 16 scans with a resolution of 4 cm^{-1}.

2.2.3. Cell culture

HCEC were cultured in DMEM/F12, supplemented with 10% FBS, 100 μg/mL penicillin, and streptomycin in 37 °C atmosphere containing 5% CO_2. The culture medium was changed every 2 days throughout the experimental operation. HCEC at population numbers of 2–3 were used in the experiments.

2.2.4. In vitro cytotoxicity

In vitro cytotoxicity of SF was determined using MTT assay, which is based on the mitochondrial conversion of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). HCEC were seeded in 96-well plates at 5000 cells per well overnight. Various concentrations of SFs (0.25, 0.5, 1.0 and 2.0%, w/v) with different dissolving time (2, 4 and 8 h) in DMEM/F12 which contained 0.4% FBS were added to the cells (200 μL per well). After 24 h of exposure, the culture medium containing SF was removed, and then 180 μL of fresh medium and 20 μL of MTT (5 mg/mL) were added to each well and incubated for 4 h at 37 °C. At the end of the assay, the blue formazan reaction product was dissolved by adding 150 μL of dimethyl sulfoxide, and the absorbance was measured by using a microplate reader (BIORAD, model 550, USA) at wavelength of 490 nm. Five replicates were read for each sample.

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