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A method for enhancing the ocular penetration of eye drops using nanoparticles of hydrolyzable dye

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ABSTRACT

This report describes a method for enhancing the ocular penetration of eye drops using nanoparticles of hydrolyzable dye, which is similar to a prodrug approach. The entry of eye drops into the ocular globe is restricted predominantly by corneal barrier functions. The barrier functions are epithelial tight junctions as well as a physicochemical property consisting of the opposite characteristics of a lipophilic epithelium and a hydrophilic stroma. We found that using a formulation of nanoparticles of hydrolyzable dye (with particles of 200 nm in diameter on average) attained a greater than tenfold higher (about 50-fold) ocular penetration than that of micron-sized particles. The nanoparticles were prepared by a carrier-free technique; i.e., the reprecipitation method. Confocal laser fluorescence microscopy showed that dyes originating from the nanoparticles surmounted the corneal epithelium barrier, which has tight junctions, and achieved deeper penetration into the cornea. The high penetration rate of the dyes into the cornea was attributed to the size of particles (i.e., nanoparticles) and a transformation of dye polarity from lipophilic to hydrophilic in *in vivo* hydrolysis reactions. We concluded that utilizing *in vivo* hydrolysis reactions to alter the physicochemical nature of nanoparticles consisting of hydrolyzable compounds was an effective approach for enhancing the ocular penetration of eye drops.

1. Introduction

A drug passing through tissue-specific barriers (e.g., blood–brain barrier, epidermal barrier or corneal barrier) is expected to reach targeted diseased organs. Current studies on designing adequate formulations of drugs to penetrate such barriers are of interest in medicine, pharmacy, chemistry and nanotechnology [1–5]. Enhancement of ocular penetration of eye drops remains one of the most challenging tasks in ophthalmology [6–9]. The majority of ophthalmic formulations are administered as eye drops because of their ease of use, low invasiveness and the simplicity with which such drugs can be formulated [6,7]. However, the anatomy, physiology and biochemistry of the eye form a unique structure that restricts the entry of drug molecules at the required site of action [10–12]. Most of the drug is washed out from the ocular surface by various mechanisms (e.g., lacrimation, tear dilution and tear turnover) before they are able to penetrate into the desired tissue [11]. Furthermore, the specific barrier that exists in the cornea strictly restricts the entry of the drugs remaining on the ocular surface. Eventually, less than 5% of the administered drug penetrates the cornea to reach intraocular tissues [13].

The cornea is a transparent membrane located in the central part of the ocular surface and is mainly composed of three layers: epithelium, stroma, and endothelium (schematic images of an eye and a cornea are shown in Supplementary Information, Fig. S1) [14]. A corneal epithelium is a stratified cell membrane and its apical tight junctions between surface epithelial cells are considered to be the most prominent barrier for corneal absorption. A corneal stroma is a hydrated fibrous tissue with dispersed cells and it has a hydrophilic environment limiting the penetration of highly lipophilic compounds. A corneal endothelium is a monolayer of cells with large intercellular junctions, which presents a leaky lipophilic barrier. Due to the dual nature of the cornea, with a lipophilic epithelium and a hydrophilic stroma, the epithelium appears to be rate limiting to the movement of hydrophilic compounds, whereas for lipophilic compounds, the stroma is rate limiting [15]. Thus, an epithelium and a stroma, which present different physicochemical properties, act as a strong

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barrier to the entry of drugs into the ocular globe via the cornea [12,16–18].

At present, using the hydrolysis reaction that converts a drug polarity from lipophilic/hydrophobic to hydrophilic is a widely employed strategy to enhance drug penetration through the cornea, and such drugs are referred to as prodrugs [2]. Prodrugs are drugs with attached functionalities to obtain favorable structural natures and they regenerate their active parent form by enzymatic or chemical reactions [12].

One of the attractive approaches for increasing ocular drug penetration is using nanoparticles. Even though the lipophilic/hydrophobic drug molecule itself is not soluble in water, it can be regarded as showing water solubility by means of forming nanoparticles in aqueous medium with stable water dispersion [4,19]. Furthermore, an advantage of a nanoparticle formulation is the expected increase in ocular drug penetration based on a small particle size. For example, increased ocular drug penetration is expected by means of decreasing the particle size of a steroid, which has less water solubility, resulting in micron-sized sedimentation in currently used drug formulations [20,21]. Thus, in an effort to improve the quality of aqueous dispersion of lipophilic/hydrophobic drugs, there has been increasing interest in developing smaller sized particles.

Nanocarrier based approaches, which are useful for increasing the water solubility of lipophilic and hydrophobic drugs by containing these drugs within capsule carriers, are being widely investigated at present [13,19,22–24]. These approaches also allow for site specific targeting in drug delivery. However, nanocarriers, such as the nanosphere, liposome, and micelle, may lead to cytotoxicity and show insufficient drug levels or the uncontrolled release of drugs [25–29]. Furthermore, nanocarriers are not always ideal for increasing the ocular penetration of drugs [30]. Recently, the use of nanocarrier-free organic nanocrystals for photodynamic cancer therapy and bioimaging was reported, where stable water dispersion and high nanocrystal tissue uptake was achieved [31,32]. The nanocrystals were prepared by the reprecipitation method [31,32].

This article demonstrates a method for enhancing eye drop ocular penetration using nanoparticles of hydrolyzable dye. The nanoparticles were prepared by the reprecipitation method [31–33]. The two beneficial aspects of the nanoparticles are small size, which is expected to support stable water dispersion and high ocular penetration, and dye polarity transformation from lipophilic to hydrophilic in *in vivo* hydrolysis reactions, which may overcome the corneal barrier consisting of a lipophilic epithelium and hydrophilic stroma, which is similar to a prodrug approach. Thus, the nanoparticles are presented in this study as a candidate for the production of superior eye drops with stable water dispersion, nanocarrier-free status, and deeper ocular penetration.

As a model compound for demonstrating this concept, a hydrolyzable lipophilic dye, fluorescein diacetate (FDA), was selected. FDA is a non-fluorescent, non-polar fatty acid ester that is membrane permeable and can enter the cell [34]. FDA is hydrolyzed by esterases to produce hydrophilic fluorescein, a fluorescent compound that tends to accumulate intracellularly [34]. We selected FDA for this study because FDA is a lipophilic compound and is thus nearly water insoluble [35,36]. Also, FDA is suitable for fabricating nanoparticles based on the precipitation procedure in water by means of the reprecipitation method. Furthermore, FDA has been widely used for biological applications and the fluorescein it produces is currently used for ocular diagnostic treatments. Another advantage is that the resulting fluorescein dye can be used as a useful fluorescent tracer to determine the depth of ocular penetration. The final factor in the selection of FDA was the fact that FDA eye drops, including their fluorescein derivatives, can be administered to rats without producing abnormal behaviors.

We investigated whether FDA nanoparticle eye drops can penetrate through the corneal epithelium, which has tight junctions that usually act as a strong barrier to keep foreign substances from entering the ocular globe. We also determined whether they can overcome the physicochemical property barrier consisting of a lipophilic epithelium and a hydrophilic stroma. Nanoparticle eye drops were administrated to rats for qualitative observation by fluorescence microscopy and confocal laser fluorescence microscopy as well, as quantitative analysis. The nanoparticles and micron-sized FDA particles were compared with investigate the effect of particle size. Comparison was also made between FDA and its fluorescein derivatives to assess the effect of drug polarity.

2. Material and methods

2.1. Materials

FDA, fluorescein, sodium fluorescein (the chemical structures of these substances are shown in Fig. S2, Supplementary Information), polyvinylpyrrolidone (PVP, K30), acetone (>99.5%), dimethyl sulfoxide (DMSO: >99.0%, non fluorescent solvent), sodium chloride (NaCl) and sodium hydroxide (NaOH) were obtained from Wako Pure Chemical Industry (Japan), and used without further purification. Seamless cellulose tubing with a pore size of 50 Å was purchased from Viskase Corporation (USA). Syringe filters with a pore size of 0.22 μm were obtained from Millipore Corporation (USA).

2.2. Animals

Specific-pathogen free male Sprague–Dawley rats, aged 4 to 5 weeks, were obtained from Japan SLC Inc (Japan). All animals were managed in accordance with the rules of the Animal Experiment Committee in Tohoku University.

2.3. Preparation of eye drops

FDA nanoparticle eye drops were prepared using the reprecipitation method [31–33]. The reprecipitation method, which is a solvent displacement method, provides a very simple and versatile way to prepare organic nanoparticle dispersions. The method involves the rapid mixing of a small amount of concentrated stock solution of the target compound dissolved in a good solvent with excess of a poor solvent. In the reprecipitation method, nanoparticles can be successfully prepared without using carriers that are usually necessary for nanoparticle formation. Typically, 200 μl of FDA acetone solution (25 mg/ml) was rapidly injected into a magnetically stirred (1400 rpm) PVP (2%, w/w) containing water solution (10 ml) using a microsyringe at room temperature. Then, water soluble PVP, which is commonly used for current ophthalmic drug formulation as a stabilizer, acted as a stabilizer surrounding the nanoparticles to increase the water stability of the aqueous dispersion of the nanoparticles. The resulting dispersion of nanoparticles in water was dialyzed for 6 h to remove acetone using seamless cellulose tubing. The FDA nanoparticle water dispersion was then filtered by the syringe filter with a pore size of 0.22 μm. The filtered FDA nanoparticle water dispersion was mixed with sterile NaCl water solution (9%, w/w) in a volume ratio of 9:1. Finally, the FDA nanoparticle water dispersion containing NaCl solution (0.9%, w/w) was prepared as eye drops.

FDA microparticle eye drops were prepared as follows. First, FDA powder (25 mg) was dissolved in acetone (1 ml). The solution was evaporated for recrystallization. The recrystallized powders were milled in a mortar. The FDA powder (5 mg) was then mixed with 2% (w/w) PVP water solution (10 ml). Finally, the FDA powder solution was mixed by vortex-induced vibration for 1 min. This was followed by 1 min of sonication to achieve a uniform dispersion of the particles. Only part of the resulting stable dispersion without precipitated aggregates was collected. This FDA microparticle water dispersion was mixed with sterile NaCl water solution (9%, w/w) in a volume