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Specific uptake of folate-decorated triamcinolone-encapsulating nanoparticles by retinal pigment epithelium cells enhances and prolongs antiangiogenic activity

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ABSTRACT

We are proposing folate-decorated polymeric nanoparticles as carriers of poorly soluble drug molecules for intracellular and prolonged delivery to retinal pigment epithelium (RPE) cells. RPE is a monolayer of epithelial cells that forms the outer blood-retinal barrier in the posterior segment of the eye, and is also implicated in the pathology of, such as neovascularization in age-related macular degeneration (AMD). In this study, folate-functionalized poly(ethylene glycol)-b-polycaprolactone (folate-PEG-b-PCL) were synthesized for assembling into nanoparticles of ~130 nm. These nanoparticles were internalized into ARPE-19 (human RPE cell line) via receptor-mediated endocytosis, and the cellular uptake was significantly higher than particles without folate modification. Triamcinolone acetonide (TA) was efficiently encapsulated (>97%) into the folate-decorated nanoparticles and was slowly released over a period of 4 weeks at pH 5.5 and 8 weeks at pH 7.4. The enhanced uptake and controlled release resulted in prolonged anti-angiogenic gene expression of RPE cells. In cell culture, the down-regulation of vascular endothelial growth factor (VEGF) and up-regulation of pigment epithelium derived factor (PEDF) lasted for at least 3 weeks. Unlike benzyl alcohol, the surfactant found in commercial formulation, folate-modified nanoparticles were non-toxic. Furthermore, TA became less cytotoxic by being encapsulated in the nanoparticles. Our findings suggest that folate-PEG–PCL nanoparticles are promising drug carriers for RPE targeting.

1. Introduction

Retinal pigment epithelium (RPE) is a target site for drug delivery to the posterior segment of the eye. The dysfunction of RPE plays an important role in the pathological development of ocular diseases, including retinitis pigmentosa (RP) and age-related macular degeneration (AMD). In AMD, abnormal RPE cells trigger choroidal neovascularization (CNV) in the subretinal macular region by the secretion of vascular endothelial growth factor (VEGF) [1,2]. Anatomically, RPE forms the outer blood-retinal barrier to tightly regulate the transport between neuro-retina and choroidal blood vessels. Thus, when nano-drug carriers are injected into the vitreous, RPE represents an important gate keeper before they are cleared dynamically into the blood circulation. On the other hand, folic acid (FA) is actively transported by RPE cells via folate-receptors, which are only present in this cell layer in retina [3]. Based on this knowledge about RPE, we formulate here nanoparticles to take advantage of folate-receptors as specific portals to deliver therapeutics to RPE cells. Although polymeric nanoparticles delivering drugs and genes to RPE have been reported before [4,5]; to our best knowledge, there has not been any investigation using folic acid as the targeting moiety against RPE cells.

We have chosen triamcinolone acetonide (TA), a lipophilic corticosteroid, as the model drug in our study. Intravitreal injection of TA is a common treatment for inflammation in the back of the eye. In recent years, this has also been used to treat wet AMD and proven successful in stabilizing the development of choroidal neovascularization [6]. TA suppressed VEGF and upregulate PEDF expression in RPE cells. [7,8]. It has been well known that VEGF promotes ocular angiogenesis [9,10] while PEDF is a potent inhibitor of angiogenesis [11–13]. There are problems, however, associated with the intravitreal injection of TA. First, TA has short half-life in vitreous [14]. Frequent and repeated injections are needed especially for chronic conditions; and such procedures may lead to serious complications such as cataract, glaucoma and retinal detachment [15]. Second, because of the poor solubility of TA, benzyl alcohol (BA) is added in the commercial formulation of TA suspension. There are concerns about cytotoxicity caused by the crystalline form of aggregated TA, as well as the surfactant itself [16]. Both problems can be addressed by encapsulating TA in nanoparticles made of slowly degradable biocompatible materials.

In this work, folate-modified polyethylene glycol-b-polycaprolactone (folate-PEG-b-PCL) is used as the building block of nanoparticles. The amphiphilic diblock copolymers self-assemble into micelles with the hydrophilic PEG as the corona and PCL as the core [17]. Driven by

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hydrophobic interaction, TA molecules are sequestered in the PCL-rich core. Both PEG and PCL are well known for their biocompatibility [18,19]. And since PCL undergoes slow degradation by hydrolysis [20], TA encapsulated is expected to be slowly released into the external medium in its soluble form. Our design incorporates folate acid on the nanoparticles surface, in order to enhance uptake and retention in RPE cells. We examined cellular uptake of these nanoparticles using fluorescence microscopy. The therapeutic effect of TA-loaded particles was investigated by analyzing antiangiogenesis-related gene expression of cultured RPE cells [7]. The cytotoxicity of the new nanoparticles was measured and compared with benzyl alcohol and TA suspension.

2. Materials and methods

2.1. Materials

Allyl alcohol, ethylene oxide, ε-Caprolactone, ethylenediamine, 3-mercaptopropionic acid, folic acid, N,N-diisoprorylethylamine (DIPEA), diphenylmethyl potassium (DPMK), N,N,N′,N′-Tetramethyl-O-(7-azabenzotriazol-1-yl) uronium hexafluorophosphate (HATU), triamcinolone acetonide (TA) and Nile red (NR) were obtained from Sigma-Aldrich, MO, USA. Dichloromethane (DCM), dimethyl sulfoxide (DMSO), benzyl alcohol, diethyl ether, tetrahydrofuran (THF) and N, N-dimethylformamide (DMF) were HPLC grade, obtained from Sigma-Aldrich, MO, USA and were used without further purification.

ARPE-19 cells were a gift from Dr. Amy Lo's Group, Eye Institute, The University of Hong Kong. Cell culture medium DMEM-F12 (1:1), fetal bovine serum (FBS), L-Glutamine and penicillin/streptomycin were purchased from Gibco (Invitrogen, Gaihersburg, MD, USA). Endocytosis inhibitor methyl-β-cyclodextrin was purchased from Sigma, MO, USA. Laminin 24-well Multiwell Plates was purchased from BD Biosciences, NJ, USA.

2.2. Synthesis of block copolymers

Allyl-(polyethylene glycol)-OH and methoxy-(polyethylene glycol)-OH were prepared according to reported methods [21]. With allyl-PEG–OH or mPEG–OH as the initiator, allyl-PEG-b-PCL and mPEG-b-PCL were prepared respectively following procedures adapted from Meier [22].

Allyl-PEG-b-PCL-OH was converted to COOH-PEG-b-PCL by the radical addition reaction using 3-mercaptopropionic acid, as reported by Li et al. [21].

COOH-PEG-b-PCL was converted to NH_2-PEG-b-PCL by conjugating with ethylenediamine.

NH_2-PEG-b-PCL was converted to folate-PEG-b-PCL by conjugating folic acid to the amino terminus following a protocol by Park et al. with modification [23]. A summary on the synthesis scheme of FA-PEG-b-PCL were stated below (Fig. 1).

2.2.1. Characterization of polymers

^{1}H NMR spectra were obtained on a DMX 500 MHz spectrometer with $CDCl_3$ as the solvent. Gel permeation chromatography (GPC) was performed on a Waters HPLC system (Waters, Milford, USA) with a G1310A pump and a G1362A refractive index detector, using tetrahydrofuran (THF) as eluent with an elution rate of 1.0 mL/min. One Styragel columns (HR 3 THF Waters, Milford, USA) were calibrated by polystyrene standards (Polymer Source).

2.3. Preparation of PEG-b-PCL nanoparticles

PEG-b-PCL nanoparticles were prepared by a nano-precipitation [24]. Briefly, 5 mg PEG-b-PCL block copolymers and triamcinolone acetonide (10:1 wt/wt) were dissolved in 1 ml of dimethylformamide (DMF). The organic solution was added dropwise into 10 ml of distilled water under stirring at room temperature. The solution was dialyzed overnight against double deionized water using regenerated cellulose membrane with molecular weight cutoff of 10 kDa (Spectrum Laboratories, Houston, USA) to remove DMF and free TA. Nile Red (NR)-loaded nanoparticles were prepared by replacing the drug with the dye in the aforementioned procedure. Solutions of TA-loaded or NR-encapsulated nanoparticles were then characterized and utilized in experiments immediately. The size of NPs was controlled by varying the concentration of PEG-b-PCL copolymers dissolved in DMF. Higher concentration was correlated with larger particle size.

2.4. Characterizations of nanoparticles

2.4.1. Size and zeta potential analysis of the nanoparticles

Mean diameter, size distribution and zeta potential were measured by Dynamic Light Scattering (DLS) with a Brookhaven BI-9000AT instrument (Brookhaven Instruments Corporation, NY, USA).

2.4.2. Drug loading content and encapsulation efficiency

Drug-loaded nanoparticles were first dissolved in dimethyl sulfoxide to release all the drug content. The concentration of TA was determined from UV absorbance at 269.5 nm by correlation with a standard TA solutions [25] (Ultrospec 4300 pro UV/Visible Spectrophotometer, GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) Standard TA solution is prepared by dissolving 1 mg TA in 1 ml DMSO solution. The stock solution was serially diluted to 1:10 to obtain 0.1 mg/ml 10 μg/ml, 1 μg/ml and 100 ng/ml TA solution. The following equations were applied to calculate the drug loading content and encapsulation efficiency.

2.5. Measurement of drug release profile from nanoparticles

To measure drug released from nanoparticles, 0.5 mg/ml TA-loaded nanoparticles in 0.1 M phosphate buffered saline (PBS) were placed into a pre-swelled dialysis bag (Spectra/Por Biotech Regenerated Cellulose Dialysis Membranes of molecular weight cutoff at 10 kDa, Spectrum Laboratories, Houston, USA.) and immersed in 25 ml of 0.1 M PBS (pH 7.4) or 0.1 M sodium acetate/acetic acid buffer (pH 5.5) at 37 °C with gentle agitation. 2 ml of samples was withdrawn from the incubation medium and measured for TA concentration by UV spectroscopy. After sampling, old incubation medium was replaced by fresh buffer. The concentration of TA released from the nanoparticles was expressed as a percentage of the total TA in the nanoparticles and plotted as a function of time.

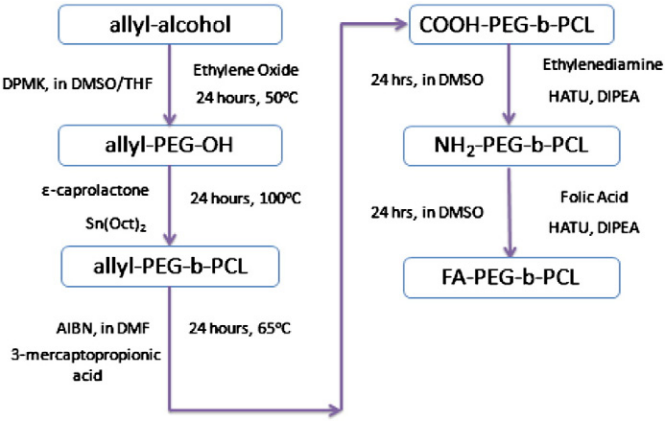

Fig. 1. Synthesis scheme of Folate-PEG-b-PCL copolymer.