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The movement of self-assembled amphiphilic polymeric nanoparticles in the vitreous and retina after intravitreal injection

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ABSTRACT

The purpose of this study is to determine the correlation between the distribution of nanoparticles in the vitreous and retina and their surface properties after intravitreal injection. For this purpose, we synthesized seven kinds of nanoparticles through self-assembly of amphiphilic polymer conjugates in aqueous condition. They showed similar size but different surface properties. They were labeled with fluorescent dyes for efficient tracking. After intravitreal injection of these nanoparticles into a rodent eye, their time-dependent distribution in the vitreous and retina was determined in stacking tissue images by confocal microscopy. The results demonstrated that the surface property of nanoparticles is a key factor in determining their distribution in the vitreous and retina after intravitreal injection. In addition, immunohistochemistry and TEM images of retina tissues suggested the important mechanism related with Müllller cells for intravitreally administered nanoparticles to overcome the physical barrier of inner limiting membrane and to penetrate into the deeper retinal structures. Therefore, we expect that this study can provide valuable information for biomedical researchers to develop optimized nanoparticles as drug or gene carriers for retinal and optic nerve disorders such as glaucoma, age-related macular degeneration, and diabetic retinopathy.

1. Introduction

In spite of state-of-the-art ocular cares accomplished by recent advances in biomedical technology, many peoples still suffer from their vision loss due to various ocular diseases, including glaucoma, age-related macular degeneration, and diabetic retinopathy. Most vision-threatening eye conditions are related to the retina. Many kinds of ocular drug delivery methods to the retina such as topical administration, systemic administration, trans-scleral delivery and intravitreal injection have been researched for several decades. However, in clinic, only the intravitreal injection is currently most favorable to deliver therapeutic agent to the retina as well as reducing the systemic side-effects because of the intrinsic limitations of the other methods such as limited uptake or ocular penetration [1,2]. Intravitreal injection of anti-vascular endothelial growth factor (anti-VEGF), such as ranibizumab, is currently the first line treatment in exudative age-related macular degeneration (AMD) [3] and efficacious in many ocular diseases such as diabetic retinopathy, retinal vein occlusion, and choroidal vascularization by other causes like high myopia [4].

Despite the proven efficacy, the most important issues unresolved regarding intravitreal drug delivery are (1) the intraocular retention time and (2) the effective drug delivery into the target cells. Current intravitreal injections of anti-VEGF agents required monthly dosing are vulnerable to ocular and systemic complications. Longer half-life of a drug formulation such as particulate forms could enable less frequent intravitreal injections, lower cost and less injection-related complications such as sight-threatening endophthalmitis and retinal detachment [5,6]. As the half-life of intravitreal drugs is correlated to the size, nanoparticles that are larger than the size of original drugs can lead to longer intraocular retention. Second, as the amount of solution that can be injected intravitreally is limited (less than 0.1 mL), effective delivery of materials into target cells is essential to achieve adequate

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therapeutic concentrations in the target site like sub-retinal space in cases of AMD or retinitis pigmentosa. Various nano-sized drug delivery systems fabricated with different materials have been studied extensively to overcome the limitation of the intravitreal administration [7–9]. However, the distribution of intravitreally administered various nano-sized drug delivery systems depending on different surface properties is not yet well understood. Without understanding key factors influencing the movement of nanoparticles in the vitreous and retina, it is difficult to develop an optimized drug delivery system to target a specific vitreous and retina tissue and treat different retinal disease by controlling constituents and surface properties of nanoparticles.

In this study, we sought to understand the movement of intravitreally administered nanoparticles in the vitreous and retina depending on their surface properties (Scheme 1A) [10]. Four different polymers such as polyethyleneimine (PEI), glycol chitosan (GC), hyaluronic acid (HA), and human serum albumin (HSA) were used as hydrophilic shell polymers of nanoparticles. Using these materials, we developed seven kinds of nanoparticles, PEI nanoparticle, GC nanoparticle, HA nanoparticle, HSA nanoparticle, PEI/GC heterogeneous nanoparticle, HSA/GC heterogeneous nanoparticle, and HSA/HA heterogeneous nanoparticle. All nanoparticles were fabricated using self-assembly method of amphiphilic polymer conjugates in aqueous condition with common hydrophobic 5β-cholanic acid groups to control other factors except the surface properties [11]. Then, we present the movement and distribution of different nanoparticles, with respect to both surface charge and components after intravitreal injection. Additionally, we demonstrated a possible mechanism for trans-retinal penetration of intravitreally administrated nanoparticles into the sub-retinal space based on tissue immunohistochemistry and transmission electron microscopy (TEM) images.

2. Materials and methods

2.1. Materials

Glycol chitosan (MW = 2.5×10^5 kDa, degree of deacetylation = 82.7%), human serum albumin, polyethyleneimine (MW = 25 kDa), 5β-cholanic acid, ethylenediamine, 1-hydroxybenzotriazole (HOBT), N-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were obtained from Sigma and used without further purification. Hyaluronic acid (Sodium hyaluronate, MW = 2.344×10^3 kDa) were purchased from Lifecore biomedical (Chaska, MN, USA), and was used after dialysis against distilled water and lyophilization. Anhydrous methanol and dimethyl sulfoxide (DMSO) were obtained from Merck (Darmstadt, Germany). Fluorescence dye, FPR-552 was purchased from Bioacts (Incheon, South Korea). All other chemicals were of analytical grade and used without further purification.

2.2. Synthesis of nanoparticles

All nanoparticles were fabricated by self-assembly of amphiphilic polymer conjugates in aqueous condition. To synthesize amphiphilic conjugate, 5.0 mg of 5β-cholanic acid was incubated with 4.0 mg of EDC and 2.4 mg of NHS (1.5 M equivalent of 5β-cholanic acid) in 5 ml of methanol for 1 h for activation. Then, 50 mg of each polymer except hyaluronic acid (HA) was dissolved in distilled water (50 ml), and added to the solution. After further stirring for 24 h, it was purified by dialysis against water and methanol, and lyophilized. In case of HA nanoparticle, 5β-cholanic acid was aminated by ethylenediamine with EDC and HOBT by same method in our previous paper [12]. Then, this aminated 5β-cholanic acid was conjugated to HA polymer in same condition with other nanoparticles. To make heterogeneous nanoparticles, two kinds of polymer conjugates (weight ratio = 1:1) was mixed in DMSO-water co-solvent (1:1), and sonicated for perfect dissociation of nanoparticles. Then, the resulting solution was dialyzed against distilled water for self-assembly again. For fluorescence labeling, 30 mg of nanoparticles were incubated with 0.5 mg of FPR-552 in DMSO-water co-solvent (1:1) for 2 h, and purified by dialysis against water and methanol. All nanoparticles were filtered through syringe filter membrane (cellulose acetate, Millipore) with pore sizes of 0.45 μm before characterization and intravitreal injection.

2.3. Characterization of nanoparticles

The size distribution and zeta potential of nanoparticles were measured using a Malvern Zetasizer Nano ZS 3000HAs (Malvern Instrument Ltd., Worcestershire, U.K.). Each nanoparticle was dissolved in PBS (pH 7.4) at 1.0 mg/ml, and measured five times at 37 °C. The morphologies of nanoparticles were observed using transmission electron microscopy (TEM, CM30, Philips, CA). Each nanoparticle was dissolved in distilled water, and loaded onto a 300-mesh copper grid coated with carbon. After 2 min, the copper grid was tapped with filter paper to remove distilled water and air-dried. Then, a droplet of 2% (w/v) uranyl acetate was added to each grid for negative staining, and TEM images were obtained at an acceleration voltage of 80 kV.

2.4. Intravitreal injection of nanoparticles

All experiments with live animals were performed in compliance with the relevant laws and institutional guidelines of Korea Institute of Science and Technology (KIST) and institutional committees have approved the experiments. Long Evans rats (6-week old) were purchased from Institute of Medical Science (Tokyo). Each nanoparticle suspended in 5 μl of PBS (0.5 mg/ml, pH 7.4) was administered into the vitreous of rat eyes by a 32 gauge needle (Hamilton, Reno, NV, USA). The eyes were enucleated and fixed immediately with 4% paraformaldehyde solution (PFA) 6, 24, or 72 h post-injection ($N = 3$ per each group).

2.5. Determination of the vitreal and retinal distribution of nanoparticle

The eye was opened by circumferential incisions at the surgical limbus after overnight fixation. After removal of the anterior segment and lens, the posterior segment of the eye was embedded in 7% agaros type XI gel (Sigma, St. Louis, MO, USA) and sectioned into 200 μm-thick slices with a vibrating blade microtome (Leica, Bannockbum, IL, USA). The eye sections were incubated overnight in DAPI solution (1:1000) diluted with ICC buffer and then washed three times with ICC buffer for 3 h. The eye sections were embedded in mounting medium (Vectashield, Vector Laboratories Inc., Burlingame, CA, USA) and sealed under microscope cover glass slides. Then, they were viewed on a Nikon laser scanning confocal microscope.

2.6. Immunohistochemistry

A rabbit anti-human von Willebrand factor antibody (DAKO, Carpinteria, CA, USA) and a mouse anti-glutamine synthetase (CHEMICON International Inc., Temcula, CA, USA) were utilized for immunohistochemistry staining of retinal blood vessels and Müller cells, respectively [8]. The 200 μm-thick eye sections were incubated in 5% goat serum overnight. Double staining was performed using rabbit anti-human von Willebrand factor antibody (dilution 1:150) and mouse anti-glutamine synthetase (diluation 1:150) with 2% goat serum ICC buffer as a primary antibody solution. The primary antibodies, anti-von Willebrand factor and anti-glutamine synthetase, were detected using a secondary antibody solution containing Alexa Fluor® 488 goat anti-rabbit IgG (dilution 1:150), Alexa Fluor® 633 goat anti-mouse IgG (dilution 1:150), and DAPI (dilution 1:1000) in ICC buffer.

2.7. Transmission electron microscopy (TEM) images of ocular tissues

2.0 ul of the HSA nanoparticle solution (0.5 mg/ml) were injected intravitreally to a rodent eye. 6 h post-injection, the both eye were enucleated and fixed in 2% glutaradehyde and 2% paraformaldehyde solution overnight. Then, the eyes were washed twice with 0.1% cacodelate buffer for 15 min. The anterior segment of the eye including the lens was removed and the retina was cut into four parts. The retinas were prepared as described previously [13]. The retinas were trimmed, post-fixed in 1% osmium in 0.1M sodium cacodylate buffer, 1% tannic acid (gallotannin, $C_{14}H_{10}O_9$), and 1% paraphenylenediamine (OTAP method) and embedded in epoxy resin (PolyBed 812; Polysciences, Warrington PA). One-μm-thick sections were cut with a Leica Ultramicrotome (Ultracut UCT, Leica Mikrosysteme AG, Vienna, Austria) and stained with 1% toluidine-O-blue. These sections were examined and photographed with a 40× planapochromat objective on an Eclipse 80i microscope (Nikon Instruments Inc., Melville NY).

3. Results and discussion

3.1. Development and characterization of nanoparticles

Herein, we synthesized four kinds of nanoparticles with different polymers and three more heterogeneous nanoparticles with two kinds of polymers. Among them, PEI and GC are cationic polymers; while HA and HSA are anionic ones. To make these polymers amphiphilic, hydrophobic 5β-cholanic acid groups were conjugated to these hydrophilic polymers by amide coupling reaction (Scheme 1B). As our previous papers, the resulting