Hyaluronan-modified core–shell liponanoparticles targeting CD44-positive retinal pigment epithelium cells via intravitreal injection

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Abstract

Retinal inflammation, a common process of posterior ocular diseases, may lead to severe vision loss or even blindness. Retinal pigment epithelium (RPE) cells are generally considered as the therapeutic target of inflammation pathogenesis. However, the lack of retina-specific distribution for general intravitreous drug delivery systems makes the anti-inflammation treatment inefficient. In the present study, a hyaluronan (HA)-modified core–shell liponanoparticles (HA-LCS-NPs) was designed to improve the treatment efficiency by increasing RPE-targeted distribution. Our in vitro RPE cell uptake study showed that a higher HA grafting density (5.8%) and a higher molecular weight (200–400 kDa) modification of HA improved the intracellular uptake of HA-LCS-NPs. In addition, in vivo distribution evaluation in experimental autoimmune uveitis (EAU) rats revealed that HA-LCS-NPs could specifically target RPE cells through the interaction between the CD44 receptor and the HA ligand, while chitosan nanoparticles (CS-NPs) were limited to the vitreous cavity and the core–shell liponanoparticles (LCS-NPs) only reached the inner layers of the retina. At 7 d post-injection, approximately 75% of the fluorescence of HA-LCS-NPs still remained in the RPE/choroid. In conclusion, HA-LCS-NPs might present a promising intraocular drug delivery system to achieve RPE-targeted distribution and prolonged intraocular residence.

1. Introduction

Inflammation plays an important role in the occurrence and development of many vision-threatening ocular diseases, such as age-related macular degeneration (AMD), diabetic retinopathy (DR), and posterior uveitis [1–3]. Retinal pigment epithelium (RPE) cells are key targets of inflammation pathogenesis. CD44, a member of the adhesion receptor family, is constitutively expressed on leukocytes and parenchymal cells including endothelial, epithelial, and smooth muscle cells. Crane et al. [4] has shown that CD44 expression on RPE cells is greatly enhanced in the in vivo eye, suggesting that CD44-positive RPE cells could be considered as an anti-inflammatory intervention target in inflammatory diseases.

Traditional topical formulations are unable to deliver therapeutic drugs to the posterior segment, while systemic administration of corticoids and/or immunosuppressive agents is frequently complicated by systemic side effects [5,6]. Thus, intravitreous injection is now a clinically available method to treat intraocular disease. However, retinal drug delivery is a challenging area of research because physiological barriers, such as the vitreous and the inner and external limiting membrane, limit the delivery of ocular drugs [7]. As a result, an ideal drug delivery system for posterior ocular disease treatment is needed, and it should possess such properties as (1) specific distribution to the inflamed posterior ocular tissues; (2) prolonged residence at the targeted site; and (3) biocompatibility and sustained drug release.

Chitosan nanoparticles (CS-NPs), with no toxicity and good biodegradability, can control drug release and be utilized as carriers for a variety of therapeutic agents such as genes, peptides, and small molecule chemical drugs [8]. However, due to the electrostatic interaction between the anionic vitreous gel and cationic nanoparticles, CS-NPs cannot effectively transport therapeutic agents to the retina across the vitreous barrier [9,10]. Hyaluronan (hyaluronic acid, HA), a high molecular weight linear glycosaminoglycan composed of repeating disaccharide units of β-1,4-deoxyglucuronic acid-β-1,3-N-acetyl-d-glucosamine, is an important
component of the extracellular matrix, which specifically binds to several plasma membrane receptors, including CD44 [11]. Interactions between HA and CD44 could endow active targeting of nanocarriers to the retina [12].

Thus, HA-modified core–shell liponanoparticles (HA-LCS-NPs) were designed and fabricated. With the enveloping of the lipid bilayer, the positive surface charges of CS-NPs would be shielded, ensuring free diffusion of NPs in the vitreous gel. Moreover, with subsequent modification of the HA ligand, HA-LCS-NPs would exhibit RPE-targeted distribution. To the best of our knowledge, the application of HA-modified core–shell nanoparticles to achieve RPE-targeted intraocular distribution has never been reported.

In this study, LCS-NPs were prepared by hydrating a dry lipid film with a CS-NPs solution. HA-LCS-NPs were obtained by covalent linkage of the positive glucuronic acid moiety of the targeting ligand HA to the primary amine of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) in the preformed liposome shell. The CD44-targeting ability of HA-LCS-NPs was evaluated in vitro using cultured ARPE-19 cells. Finally, vitreous and retinal distributions of the nanosystems were investigated in vivo in both normal rats and experimental autoimmune uveitis (EAU) rats.

2. Materials and methods

2.1. Materials

Chitosan (Mn 2 × 10^5; degree of deacetylation, approximately 90%) was obtained from Golden-Shell Biochemical Co. Ltd. (Zhejiang, China). Sodium hyaluronate (10–100 kDa and 200–400 kDa) and egg phospholipid (PC-98T, PC-98H, PC-98K, PC-98T, PC-98R) were kindly provided by Q.P. Co. (Tokyo, Japan). 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) was obtained from Nippon Fine Chemical Co. Ltd. (Tokyo, Japan). Tripolyphosphate sodium (TPP), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHSS), and hexadecyltrimethyl ammonium bromide (CTAB) were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). All other chemicals were of analytical grade.

2.1.1. Cell culture

Human retinal pigment epithelial cells (ARPE-19, ATCC C2502) were grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and F12 nutrient mixture (Gibco, Invitrogen Ltd.) supplemented with 10% fetal bovine serum (FBS), 10 IU/ml penicillin, and 10 μg/ml streptomycin (Invitrogen Ltd.). The cell incubator was kept at 37 °C in a humidified atmosphere containing 5% CO₂.

2.2. Preparation and characterization of HA-LCS-NPs

CS-NPs were prepared using the ionotropic gelation method, as first developed by Calvo [13]. Briefly, chitosan was dissolved (0.15% w/v) in 1% acetic acid, and TPP aqueous solution (0.1% w/v in distilled water) was added dropwise to the chitosan solution at 1000 rpm (IKA RCT basic, IKA, China) at room temperature. Spontaneously formed NPs were then concentrated by centrifugation (Tomos 2-16A, Tomos, Cambridge, UK) at 15,000g for 30 min. After discarding the supernatants, the CS-NPs were resuspended in distilled water for further study.

LCS-NPs were prepared by hydrating a dried lipid film with the CS-NPs solution according to the procedure developed by Grenha et al. [14]. Briefly, PC-SBT: cholesterol/DOPE at a mass ratio of 15:4:1 was dissolved in 15 ml of chloroform, and the mixture was evaporated to dryness under reduced pressure using a rotary evaporator (IKA RV 10 digital, IKA, China), providing a homogeneous thin lipid film. The resulting thin film was then hydrated with 20 ml of the CS-NPs suspension (lipids:CS-NPs = 6:1, w/w) at ambient temperature for 1 h to obtain large multilamellar vesicles. Finally, LCS-NPs were obtained by extrusion (Emulsi Flex-C5, Avestin Inc., Canada) through polycarbonate membranes (0.2-μm pore diameter; Whatman Inc., UK) several times until the desired vesicle size of approximately 200 nm was obtained.

HA-LCS-NPs were prepared using a modified method reported by Yerushalmi et al. [15]. In brief, samples of HA with different molecular weights were hydrated in water (pH 4.0) overnight to allow swelling and complete solubilization. After the addition of the coupling agents EDC and NHSS to activate HA, the pH of the solution was back-titrated to 4.0 using 1 M HCl. The solution was then incubated for an additional 2 h at 37 °C before being transferred to an equal volume of LCS-NPs (pH 8.6). The mixture was stirred for 12 h at 37 °C. The resulting HA-LCS-NPs were separated from free HA and excess reagents by centrifugation (150,000 g, 4 °C, and 30 min) and washed three times with distilled water.

The amount of free HA in the supernatants was quantified using the hexadecyltrimethyl ammonium bromide (CTAB) turbidimetric method [16]. Briefly, 50 μl of HA standard solutions (0.2–2 mg/ml) or supernatants were added to a 96-well plate and the samples were incubated with 50 μl of 0.2 M sodium acetate buffer for 10 min at 37 °C. Then, 100 μl of 10 mM CTAB solution was added to the mixture, and the absorbance of the precipitated complex was read within 10 min against the blank at 570 nm using a microplate reader (BioTek synergy 4 Instruments, Inc., Winooski, VT, USA). The amount of conjugated HA was calculated by subtracting the

![Fig. 1. Particle sizes and zeta potentials of different NPs (n = 5, mean ± SD).](image-url)
The particle size and the zeta potential of the nanosystems were determined using a dynamic light scattering (DLS) analyzer (Zetasizer Nano-ZS, Malvern Instruments, UK) at 25 °C. Each measurement was made in triplicate. For transmission electron microscopy (TEM) observation, the sample was stained with an aqueous solution of phosphotungstic acid (1%, w/v) for approximately 2 min [14]. A drop of sample was then dipped on the carbon-coated copper grid, and the excess solution was drawn off using filter paper. The grid was allowed to air-dry thoroughly, and the sample was imaged with a TEM (CM-200, Philip, Netherlands) at 160 kV.

2.3. Cell viability assay

The cytotoxicity of various NPs on ARPE-19 cells was evaluated using the XTT method. ARPE-19 cells (8 × 10⁴ cells/ml) were cultured in a 96-well plate and incubated for 24 h at 37 °C. The cells were treated with various preparations of a series of concentrations for 4 h at 37 °C. After aspirating the treatment media, 100 µl of growth medium and 25 µl of XTT solution were added to the cells. The negative control was treated with medium alone without NPs. Plates were read in a microplate reader (SpectraMax M2e, Molecular Devices, USA). The isothiocyanate group of red fluorescence RITC can react with the primary amino group of chitosan [17], so we labeled CS-NPs with RITC in the cellular uptake study. RITC was first dissolved in methanol (1 mg/ml) and mixed with chitosan in 0.1 M HAc (acetic acid, chitosan:RITC ratio = 25:1, w/v). After reacting for 3 h in the dark at room temperature, RITC–CS was precipitated by adjusting the pH to 8–9 with 12 M NaOH. Unconjugated RITC was removed by repeated centrifugation and washing until no fluorescence (λex = 550 nm, λem = 580 nm) could be detected in the supernatant by a microplate reader (SpectraMax M2e, Molecular Devices, USA). RITC–CS was then redissolved in 1% HAc. We used the above-mentioned method to prepare RITC-labeled CS-NPs, LCS-NPs, and HA-LCS-NPs.

Cellular uptakes of the RITC-labeled NPs were observed by confocal laser scanning microscopy (CLSM, FV1000, Olympus, Japan). ARPE-19 cells were seeded in 96-well plates and incubated for 24 h. The cells were treated with various NPs for 4 h at 37 °C, washed three times with ice-cold PBS, and then harvested for the analysis by flow cytometry (FACSCount, BD Bioscience, USA). Cells incubated with medium alone without any NPs served as a negative control. To identify the nuclei using CLSM. We then used the above-mentioned method to prepare RITC-labeled CS-NPs, LCS-NPs, and HA-LCS-NPs.

2.4. Cellular uptake study

The expression level of CD44 on the retina of EAU rats was evaluated using immunohistochemistry. At 12 h post-injection, one rat from the HA-LCS-NPs3 group was killed and 10 µl of CD44 antibody (1:100 dilution; sc-53069, Santa Cruz Bioscience, USA) was injected into the vitreous cavity of rat eyes using a 30-gauge needle. The eyes were enucleated at 12 h or 7 d post-injection. The eyes were fixed with 4% paraformaldehyde. Sections were embedded in paraffin and stained with hematoxylin and eosin. The severity of ocular inflammation was graded according to the grading system reported previously by Caspi [18].

2.5. Intravascular behavior of intravitreally administered NPs

2.5.1. Animals

Six to eight-week-old Lewis rats (weighing approximately 200 g) were provided by the Animal Experimental Center of Shanghai Institute of Materia Medica. The animals had free access to rat chow and water. All of the animal experiments were carried out according to the Institutional Animal Care and Use Committee (IACUC) guidelines of Shanghai Institute of Materia Medica.

2.5.2. Induction and scoring of EAU

Custom peptide R14 (IRBP 1169–1191, PTARSVGAAADGSSWEGVGVVPDV) was purchased from GL Biochem (Shanghai, China). Twenty-five Lewis rats were immunized subcutaneously into both hind legs with a total volume of 200 µl of emulsion containing 50 µg of peptide R14 in complete Freund’s adjuvant, fortified with mycobacterium tuberculosis strain H37RA (Biolead, Beijing, China) to a final concentration of 2.0 mg/ml. Animals were examined daily after R14 immunization to assess the onset of inflammation using a slit-lamp biomicroscope. For histological analysis, one rat was killed on day 17 after immunization. The eyes were removed and fixed in 4% paraformaldehyde. Sections were embedded in paraffin and stained with hematoxylin and eosin. The severity of ocular inflammation was graded according to the grading system reported previously by Caspi [18].

2.5.3. Imaging of intravascular distribution of NPs using CLSM

Both normal and EAU rats were randomly divided into three groups (n = 6 per group). The rats were anesthetized with 0.4 ml (1.5% w/v) of sodium pentobarbital by intraperitoneal injection. Pupils were dilated with a topical application of 5% tropicamide, and one drop of 1% tetracaine was used for local anesthesia. Ten microliters of RITC-labeled CS-NPs, LCS-NPs, or HA-LCS-NPs3 was injected into the vitreous cavity of rat eyes using a 30-gauge needle. The rats were euthanized and the eyes were enucleated at 12 h or 7 d post-injection. The eyes were fixed in 4% paraformaldehyde. Sections were cut using a cryostat (Leica CM 1950) and then fixed with 4% paraformaldehyde. The sectioned eyes were stained with DAPI to identify the nuclei using CLSM. The coated eyes were enucleated at 12 h or 7 d post-injection. The eyes were embedded and frozen in optimal cutting temperature compound (OCT, Sakura Finetechanical Co., Ltd.). Frozen eye sections (20 µm) were cut using a cryostat (Leica CM 1950) and then fixed with 4% paraformaldehyde. The sectioned eyes were stained with DAPI to identify the nuclei using CLSM. The coated eyes were enucleated at 12 h or 7 d post-injection. The eyes were embedded and frozen in optimal cutting temperature compound (OCT, Sakura Finetechanical Co., Ltd.). Frozen eye sections (20 µm) were cut using a cryostat (Leica CM 1950) and then fixed with 4% paraformaldehyde. The sectioned eyes were stained with DAPI to identify the nuclei using CLSM. The coated eyes were enucleated at 12 h or 7 d post-injection. The eyes were embedded and frozen in optimal cutting temperature compound (OCT, Sakura Finetechanical Co., Ltd.). Frozen eye sections (20 µm) were cut using a cryostat (Leica CM 1950) and then fixed with 4% paraformaldehyde. The sectioned eyes were stained with DAPI to identify the nuclei using CLSM.
with goat anti-mouse IgG-FITC (1:100 dilution; Santa Cruz Biotechnology, Inc., USA) for 30 min at room temperature. They were then counterstained with DAPI and observed using CLSM.

2.5.5. Fluorescence intensity measurement

After intravitreal injection with various NPs, two rats from each group were executed at the designated time points. The cornea, vitreous, retina, and RPE/choroid of the excised eye were dissected and homogenized on an ice bath with 600 μl of PBS (containing 2% Triton X-100). The obtained supernatants after centrifugation at 10,000 rpm (Tomos 2-16A, Tomos, China) for 20 min were analyzed by spectrofluorometry at excitation and emission wavelengths of 550 and 580 nm, respectively. The fluorescence intensities of different tissues were expressed as the fluorescence intensity per mg of tissues. Each assay was performed in triplicate.

3. Results

3.1. Characterization of NPs

3.1.1. Particle size, zeta potential, and TEM

As shown in Fig. 1, the mean particle size of LCS-NPs was about 190 nm, which was similar to that of naked CS-NPs. After modification with the high molecular weight polymer HA, the particle size of HA-LCS-NPs increased to about 320 nm. The zeta potential of naked CS-NPs was approximately +36 mV. After build-up of the lipid shell on the CS-NPs core, the zeta potential shifted from positive values (+36 mV) to negative values (−10 mV), which indicated that either all or a part of the CS-NPs was coated by a lipid bilayer [19]. With the surface modification by HA, the zeta potential of HA-LCS-NPs decreased greatly to approximately −25 mV.

The morphological characteristics of different NPs were directly visualized by TEM (CM-200, Philip, Netherlands). CS-NPs appeared as dispersed black spots (Fig. 2A). HA-LCS-NPs demonstrated a spherical shape, exhibiting a black spot surrounded by a gray rim, which corresponded to the lipid membrane modified with HA molecules (Fig. 2B). The TEM images confirmed the expected core–shell structure of HA-LCS-NPs.

Fig. 3. Viability of ARPE-19 cells after incubation with various NPs (n = 5, mean ± SD). *: statistical difference versus the CS-NP group (P < 0.05); **: statistically significant difference versus the CS-NP group (P < 0.01).

Fig. 4. Effect of HA grafting density on cellular uptake of HA-LCS-NPs. (A) Confocal images of ARPE-19 cells incubated with various NPs. (B) The flow cytometry histogram displays the relative fluorescence intensity of control ARPE-19 cells (filled curve) or cells treated with CS-NPs (blue curve), LCS-NPs (red curve), HA-LCS-NPs1 (green curve) and HA-LCS-NPs2 (orange curve). (C) Quantitative analysis of the cellular uptake. mean ± SD, n = 3. *: statistical difference (P < 0.05); **: statistically significant difference (P < 0.01). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
3.1.2. HA grafting density evaluation

To investigate the effect of the HA molecular weight and grafting density on cellular internalization and molecular targeting, three kinds of HA-LCS-NPs were prepared in this study. CTAB is a cationic surfactant that readily precipitates acidic polysaccharides to form a complex in solution with low ionic concentration [20]. The precipitated complex had a strong light absorption at 570 nm, which correlated to the HA concentration in solution. When the initial amount of HA (10–100 kDa) added to the LCS-NP suspension increased from 2 to 4 mg, the amount of HA conjugated to the LCS-NPs increased proportionally from approximately 2.3%–5.8% (Table 1).

3.1.3. Cytotoxicity of NPs

The ARPE-19 cell viability was measured after incubation with various formulations for 4 h. At lower concentrations, no significant cytotoxicity was observed for any of the NPs ($P > 0.05$). For CS-NPs, the cell viability decreased gradually with the elevation of NPs concentration, which dropped to approximately 70% when the concentrations increased to 5.0 mg/ml. LCS-NPs or HA-LCS-NPs exhibited better biocompatibility. Especially for HA-LCS-NPs, no significant cytotoxicity could be found even at the highest concentration (Fig. 3).

3.2. CD44-targeted cellular uptake studies

The cellular uptakes of HA-LCS-NPs1 and HA-LCS-NPs2 were studied to identify the relationship between HA grafting density and targeting activity of HA-LCS-NPs. As shown in the confocal images, more intracellular red fluorescence was found in the HA-LCS-NPs1 and HA-LCS-NPs2 groups, and HA-LCS-NPs2 showed significantly greater red fluorescence associated with RPE cells (Fig. 4A). Quantitative analysis by flow cytometry showed that cellular uptakes of HA-LCS-NPs1 and HA-LCS-NPs2 were significantly greater than that of LCS-NPs. Moreover, the cellular uptake of HA-LCS-NPs2 was approximately 60% greater than that of HA-LCS-NPs1 ($P < 0.01$), as shown in Fig. 4B and C. These findings suggested that LCS-NPs grafted with greater HA density can improve the cellular uptake.

To study the effect of the HA molecular weight on the targeted delivery efficacy, 10–100 kDa and 200–400 kDa HA molecules were used to modify the LCS-NPs. The same concentration was used when HA-LCS-NPs1 and HA-LCS-NPs3 were added to ARPE-19 cells for cellular internalization studies. The confocal images showed that cells treated with HA-LCS-NPs3 exhibited more red fluorescence than the other groups (Fig. 5A). Quantitative flow cytometric analysis showed that the cellular uptakes of HA-LCS-NPs1 and HA-LCS-NPs3 were significantly greater than that of LCS-NPs (Fig. 5C). With the higher molecular weight HA modification, HA-LCS-NPs3 exhibited approximately 40% greater cellular uptake than that of HA-LCS-NPs1 ($P < 0.01$).

To further verify if the uptake of HA-LCS-NPs is specific to CD44 receptors, competitive binding experiments were performed by pretreating ARPE-19 cells with a saturable amount of free HA (200–400 kDa) before incubation. Referring to that reported by Qhattal et al., the 200–400 KDa HA was chosen to for the pretreatment

Fig. 5. Effect of HA molecular weight on cellular uptake. (A) Confocal images of ARPE-19 cells incubated with various NPs. (B) The flow cytometry histogram displays the relative fluorescence intensity of control ARPE-19 cells (filled curve) or cells treated with CS-NPs (blue curve), LCS-NPs (red curve), HA-LCS-NPs1 (green curve) and HA-LCS-NPs3 (orange curve). (C) Quantitative analysis of the cellular uptake. mean ± SD, n = 3. *, statistical difference ($P < 0.05$); **: statistically significant difference ($P < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
[20], as the chain of 200–400 KDa HA was long enough to bind CD44 thoroughly. As shown in Fig. 6, ligand pretreatment did not change the cellular uptakes of CS-NPs and LCS-NPs, while the cellular uptake of HA-LCS-NPs was significantly reduced. These results indicated that the free ligand competed with HA-LCS-NPs for receptor binding sites (Fig. 6A, B). Thus, HA cell surface receptors, mainly CD44, mediated the interaction.

3.3. Histopathology

EAU, an animal model sharing many essential features and mechanisms with posterior uveitis, is often used to establish the parameters of diagnostic evaluations and therapies for ocular inflammation. The normal rat retina was orderly composed of several layers (Fig. 7A). As shown in Fig. 7B, after induction with the IRBP peptide, typical EAU histopathological changes, such as leukocyte infiltration and destruction of the photoreceptor and the outer nuclear layer, were observed (uveitis score of 2).

3.4. RPE-targeted distribution of HA-LCS-NPs in vivo

Fig. 8 illustrates the posterior distribution of CS-NPs, LCS-NPs, and HA-LCS-NPs3 at different post-injection times in normal and EAU eyes. At 12 h post-injection, most CS-NPs were filtered and could not penetrate the vitreous in normal rat eyes. Moreover, the intensity of red fluorescence signals in the vitreous 7 d post-injection was very weak, indicating the fast elimination of intra-vitreous CS-NPs. LCS-NPs and HA-LCS-NPs3 passed through the

Fig. 6. Effect of HA blocking on cellular uptake of CS-NPs, LCS-NPs, and HA-LCS-NPs. (A) Confocal images display cellular association and uptake of each formulation before and after HA pretreatment. Images are at 60× magnification. (B) Quantitative analysis of cellular association and uptake of each NP before and after HA pretreatment. The bars represent mean ± SD of each treatment, n = 3. **: statistically significant difference versus the CS-NP group (P < 0.01).
vitreous barrier and reached the inner limiting membrane with parts of NPs getting to the ganglion cell layer of the retina.

A similar distribution of CS-NPs was found in the EAU eyes. Most of the LCS-NPs reached the inner layers of the retina, and some even got to the outer nuclear layers in the EAU eyes. Obviously, red fluorescence of HA-LCS-NPs3 was found in the RPE layer in the inflamed eyes, which was not observed in the LCS-NP group.

The immunohistochemistry results showed that CD44 was highly upregulated on RPE cells in EAU eyes. CD44 was positively stained both on infiltrating leukocytes and RPE cells. Many HA-LCS-NPs were found to be colocalized with CD44-positive RPE cells. In the amplified images, HA-LCS-NPs were found in the cytoplasm of RPE cells (Fig. 9).

3.5. Longer residence of HA-LCS-NPs in vivo

To identify the amount of NPs that were distributed in the anterior and posterior segments of the EAU eyes, the cornea, vitreous, retina, and RPE/chorioid of the excised eye were dissected and homogenized at different post-injection times. The fluorescence intensity of different tissues was measured and is shown in Fig. 10. At 12 h, the vitreous of the CS-NPs group exhibited the greatest fluorescence intensity, which indicated that most of the CS-NPs were slowed down in the vitreous. Almost no fluorescence of CS-NPs could be detected in the posterior segment. As for the LCS-NPs group, the retina showed the greatest fluorescence intensity (approximately 500 fluorescence intensity units/mg). Only in the HA-LCS-NPs group did RPE/chorioid exhibit approximately 250 fluorescence intensity units/mg. At 7 d post-injection, almost 80% of the CS-NPs were cleared from the vitreous, with only 250 fluorescence intensity units/mg left. For LCS-NPs, approximately one-third of the total fluorescence intensity was cleared, with most of the fluorescence left in the retina. While for HA-LCS-NPs, approximately 75% of the fluorescence still remained in the RPE/choroid.

4. Discussion

RPE cells, serving as the outer blood-retinal barrier, are involved in various inflammatory diseases [21–24]. It has been shown that CD44 expression on RPE cells is greatly enhanced in inflamed human eyes [4] and that high expression of CD44 receptors is present on ARPE-19 cells, a widely used RPE cell line [25]. Therefore, in the present study, we designed a intraocular drug delivery carrier, HA-LCS-NPs, to target CD44-positive RPE cells. We first investigated the influence of HA modification density and molecular weight on ARPE-19 cells. As reported by Qhattal [20], a critical density of the ligand is required on the surface of the lipid bilayer to bind to the CD44 receptor effectively. In our study, a grafting density of approximately 5.8% (w/w) HA, with a relatively low molecular weight (10–100 kDa), enhanced ARPE-19 cell internalization of HA-LCS-NPs. Further addition of HA did not dramatically improve the surface coverage of HA but increased the viscosity of the particles. Fig. 5 shows that the amount of HA-LCS-NPs3 (modified with 200–400 kDa HA) internalized by ARPE-19 cells was 40% greater than that of HA-LCS-NPs1 (modified with 10–100 kDa HA). An upward trend of internalization was obtained when the molecular weight of HA was increased. Similar results have been reported by other researchers [26,27]. As the number of binding sites is proportional to the chain length of HA [28], internalization was mainly caused by the increased chances of HA-LCS-NPs to contact with CD44 when a longer polymeric chain was bound to the surface of the LCS-NPs. However, intracellular uptake of NPs was reduced when the molecular weight was greater than 1000 kDa. This phenomenon might be due to the fact that the high system viscosity restricted the mobility of NPs to the cellular surface.

In vitro cytotoxicity assays showed that after exposing ARPE-19 cells to HA-LCS-NPs for 4 h, these cells exhibited relatively high cell viability. Moreover, no changes of retinal morphology and ocular appearance were found after the intravitreal injection of HA-LCS-NPs in normal rats’ eyes during the intracocular distribution experiment. We speculated that HA-LCS-NPs did not cause toxicity when the intravitreal injection of HA-LCS-NPs was performed.

Intravitreal delivery of nanoparticles into the retina is promising for the treatment of many severe retinal diseases. However, the lack of specific distribution for ocular posterior segment tissues and quick clearance of nanoparticles has made the efficiency of these kinds of nanocarriers unsatisfactory in previous studies [29,30].

The surface charge plays a major role in the mobility of nanoparticles in the vitreous. The vitreous is a gel-like material that is made of an ordered three-dimensional network of collagen fibrils bridged by proteoglycan filaments (which contain negatively charged glycosaminoglycans, GAGs) [7,31]. It can serve as a barrier of negatively charged GAGs, thereby decreasing their zeta potential and inducing aggregation; and (c) destabilizing the nanocarriers...
through GAG interactions that can impede cellular uptake and/or intracellular trafficking [7]. Therefore, only particles bearing negative or neutral charges could diffuse freely in the vitreous after intravitreous injection. In this work, the ionotropic gelation method allowed the preparation of positively charged CS-NPs. After coating with the lipid bilayer, the NPs shifted to a negative charge, indicating that there was a lipid bilayer coating around the positively charged CS-NPs core. After covalent conjugation of HA, the zeta potential of the HA-LCS-NPs became much lower, indicating successful binding of the negatively charged polysaccharide.

RPE-targeted intraocular distribution of HA-LCS-NPs was investigated in both healthy Lewis rats and EAU rats. After intravitreous administration, CS-NPs were assembled and aggregated in anionic gels because the cationic surface property restricted their

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**Fig. 8.** Distribution of intravitreally administered CS-NPs, LCS-NPs, and HA-LCS-NPs at 12 h and 7 d in normal (A) and EAU (B) rats. (Red color = RITC-conjugated nanoparticles, blue color = DAPI-stained retinal cell nuclei). All images were captured at 20× magnification. GCL, INL, ONL, and RPE represent the ganglion cell layer, inner nuclear layer, outer nuclear layer, and retinal pigment epithelium, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
free distribution in the vitreous [32]. In normal eyes, both HA-LCS-NPs and LCS-NPs diffused from the vitreous body and accumulated along the inner limiting membrane. We found that the lipid bilayer-coated nanoparticles with or without HA modification could move freely in the vitreous gel, but they were intercepted by the inner limiting membrane due to their large particle size. Similar results have been reported by Iezzi et al. that the polyamidoamine nanodevice they explored had a pathology-dependent biodistribution when intravitreally administrated. The normal retina was hard to penetrate due to layers of barriers [33]. No vitreous opacity or inflammation response could be seen in normal rat eyes after intravitreal injection, which confirmed its excellent compatibility and biological safety.

In EAU eyes, both HA-LCS-NPs and LCS-NPs reached the outer layer of the retina, which might attribute to the disintegration of inner limiting membrane when EAU developed [34,35]. Zhang et al. also found that the fluorescent tacrolimus liposomes were detected along the inner limiting membrane in early stage and then reached the outer layers of retina with time during the treatment of EAU [36]. One interesting finding was that HA-LCS-NPs could be observed targeting RPE cells. With immunohistochemical staining of CD44, RITC-labeled HA-LCS-NPs were found to be colocalized with CD44-positive RPE cells, with some carriers getting into the cytoplasm of RPE cells. Thus, the RPE-targeting ability of HA-LCS-NPs might be associated with the ligand—receptor interaction between HA and CD44.

In normal rat eyes, the inner limiting membrane of the retina, the ganglion cell layer, the inner plexiform layer, and the interphotoreceptor matrix, confirmed by immunohistochemistry, is hyaluronan positive. Although both CD44 and HA were upregulated in the EAU eye, the colocalization of CD44 and HA was hardly observed [37]. It can be drawn that little free HA existed in CD44 positive RPE layer. Moreover, similar results has been got that the active targeting ability of HA-modified nanoparticles on CD44 positive cancer cells was not affected by the endogenous HA or even upregulated HA [38–40]. In this work, HA-modified core–shell liponanoparticles (HA-LCS-NPs) was designed for ocular posterior segment delivery. After intravitreally injected, HA-LCS-NPs showed enough mobility in the vitreous gel, which was contributed to its negative surface charge. HA-LCS-NPs diffused from the vitreous to...
RPE layer when EAU happened, where CD44 was upregulated and specifically binding with HA-LCS-NPs, which endowed the RPE targeting ability of HA-LCS-NPs.

The fluorescence intensity in various ocular tissues was evaluated to further study the temporal changes of distribution among the three groups. For CS-NPs, approximately 80% of the doses were cleared from the vitreous at 7 d post-injection, the fast elimination of which might be due to its agglomeration in the vitreous gel. The cationic CS-NPs are easily recognized and internalized by activated retinal Müller glial cells, ocular tissue resident macrophages, and rare infiltrating activated macrophages [41]. In our study, HA-LCS-NPs exhibited obviously slow elimination in the retina and RPE/choroid, which might contribute to the protective effect of both the liposome shell and the HA polymer [41].

5. Conclusions

In cases where a posterior segment disease occurs and intraocular injection is adopted, nanocarriers should be able to penetrate the vitreous barrier and accumulate on the neural retina. In this study, HA-modified core–shell nanoparticles could successfully reach the retina and target RPE cells after intravitreal injection. Moreover, an extended intracellular residence time was also achieved. In conclusion, this kind of HA-LCS-NPs is a prospective intraocular drug delivery system for posterior disease treatment.

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References
