Effect of hyaluronic acid-binding to lipoplexes on intravitreal drug delivery for retinal gene therapy

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ABSTRACT

Intravitreal administration of nanomedicines could be valuable for retinal gene therapy, if their mobility in the vitreous and therapeutic efficacy in the target cells can be guaranteed. Hyaluronic acid (HA) as an electrostatic coating of polymeric gene nanomedicines has proven to be beneficial on both accounts. While electrostatic coating provides an easy way of coating cationic nanoparticles, the stability of electrostatic complexes in vivo is uncertain. In this study, therefore, we compare electrostatic with covalent coating of gene nanocarriers with HA for retinal gene therapy via intravitreal administration. Specifically, DOTAP:DOPE/plasmid DNA lipoplexes coated with HA are evaluated in terms of intravitreal mobility using a previously optimized ex vivo model. We find that both electrostatic and covalent HA coating considerably improve the mobility of the lipoplexes in the vitreous humor of excised bovine eyes. In addition we evaluate in vitro uptake and transfection efficiency in ARPE-19 cells. Contrary to PEGylated lipoplexes it is found that HA coated lipoplexes are efficiently internalized into ARPE-19 cells. Covalent HA-coated lipoplexes had an 8-fold increase of transgene expression compared to the uncoated lipoplexes. We conclude that covalent HA-coating of gene nanomedicines is a promising approach for retinal gene therapy by intravitreal administration.

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1. Introduction

A wide variety of retinal disorders, often leading to blindness or severely affecting vision, are potential therapeutic targets for retinal gene therapy (Trapani et al., 2014). While most clinical successes have been achieved by subretinal injection of viral vectors, this procedure is very invasive and requires the expertise of vitreoretinal surgeons, limiting its application on a large scale. Due to the local retinal detachment induced during the injection, photoreceptor cell death can occur, resulting in a loss of visual function (Zulliger et al., 2015). In addition, even though viral vectors reach high transfection efficiencies in vivo, gene expression is usually limited to the immediate surroundings of the injection site (Igarashi et al., 2013). Furthermore, viral vectors are expensive to produce, and are associated with potential immunogenic

reactions and neurotropic dissemination (Kumar-Singh, 2008; Provost et al., 2005). In light of this, intravitreal injection of non-viral vectors could be a more suitable alternative for the delivery of therapeutic nucleic acids (NAs) to the retina.(Adijanto and Naash, 2015) Intravitreal injection of therapeutics is nowadays being performed on a daily basis in the clinic such as for the treatment of wet AMD with anti-VEGF medication like Lucentis®. In contrast to subretinal injection, it can be performed by trained personnel with barely any post-injection complications (Englander et al., 2013). Even though intravitreal injection has been associated with increased ocular pressure (IOP) and a small risk of endophthalmitis, these risks can be easily managed and cannot be compared to the risks of other intraocular administration routes. Furthermore, non-viral vectors offer several advantages over viral vectors, being (i) cheaper to produce on a large scale, (ii) less immunogenic and (iii) higher cargo capacity (Issa and MacLaren, 2012). Especially the latter is important for gene therapy as some hereditary disorders require delivery of a therapeutic gene larger than the cargo capacity of AAV vectors (e.g. ABCA4 for Stargardt syndrome). Typically, non-viral vectors lack the transfection efficiency of their viral

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counterparts, nor can they bring about stable transfection. Nonetheless, efficient delivery routes could aid in increasing the transfection efficiency of non-viral vectors at the target site.

There is an immense variety of non-viral vectors, differing in composition and surface features. They can be subdivided in two major classes, being polymeric and lipid nanocarriers (Koirala et al., 2013). Both types of nanocarriers usually have a positive charge, which allows them to spontaneously complex with anionic NAs. This results in the formation of spherical particles with sizes ranging around 100 nm to 500 nm with versatile surface characteristics depending on the functionalization of the non-viral vector used (Remaut et al., 2007). We, and others, have previously shown that intravitreally injected nanoparticles can be hampered en route to the retina by the vitreous humor itself. Especially cationic charges and hydrophobicity were shown to be detrimental for intravitreal mobility (Kim et al., 2009; Martens et al., 2013; Peeters et al., 2005; Pitkänen et al., 2003; Xu et al., 2013). We demonstrated that this impaired mobility can be alleviated by surface decoration with polyethylene glycol (PEGylation), though it is also known to be detrimental for cellular interactions (Mishra et al., 2004; Sanders et al., 2007). With the aim to combine optimal vitreal mobility with efficient retinal cell uptake, we have previously proposed to use hyaluronic acid (HA) as an alternative coating strategy for PEG (Martens et al., 2015).

HA is a glycosaminoglycan ubiquitously found in mammals and is a major macromolecular component of the vitreous humor. In recent years, its use in drug delivery has surged due to its biocompatible and non-immunogenic nature, combined with its inherent anionic and viscoelastic properties (Raemdonck et al., 2013). HA molecules have several sites appropriate for chemical modification (*e.g.* hydroxyl, carboxyl, *N*-acetyl) which adds to its attractiveness for use in drug delivery. Since HA is a ligand for various cell receptors, most notably CD44, HA-conjugation is abundantly investigated for drug targeting to CD44-over-expressing (tumor) tissues (Arpicco et al., 2013). Also in the field of ocular drug delivery, HA is gaining attention as a drug delivery additive (Apaolaza et al., 2016, 2014; Gan et al., 2013; Koo et al., 2012; Martens et al., 2015). Indeed, we have previously shown that an electrostatic coating of HA was able to increase intravitreal mobility of cationic polymeric gene complexes while maintaining cellular uptake and transfection efficiency (Martens et al., 2015). Also in other recent reports electrostatic HA-coating has been found to improve *in vitro* transfection efficiency of gene polyplexes in various retinal cell types (Apaolaza et al., 2014; Ruiz De Garibay et al., 2015). However, electrostatic coating of HA may be unstable in contact with extracellular matrices or tissues. It is therefore of interest to evaluate covalent HA coating as a more stable alternative to the electrostatic coating for retinal gene therapy *via* intravitreal administration.

In the present study, we prepared electrostatic and covalent HA-coated lipid gene nanomedicines and compared their performance in terms of vitreal mobility and capacity to transfect retinal cells *in vitro*. Lipid gene nanomedicines containing plasmid DNA (pDNA) were composed of the cationic lipid 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) and the fusogenic lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). Intravitreal mobility of both HA-coated lipoplexes was evaluated using our previously published *ex vivo* eye model, using cadaveric bovine eyes and single particle tracking microscopy(Martens et al., 2013). Cellular uptake and transfection was evaluated in an *in vitro* ARPE-19 cell line, representative for the retinal pigment epithelium (RPE) cell layer (Strauss, 2005).

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium supplemented with nutrient mixture F12 (DMEM:F12 (1:1), OptiMEM™, Trypan Blue, L-glutamine, fetal bovine serum (FBS), penicillin-streptomycin solution

(5000 IU/mL penicillin and 5000 μg/mL streptomycin) (P/S), and Dulbecco's phosphate-buffered saline (DPBS 1×, with or without Ca^{2+}/Mg^{2+}) were supplied by GibcoBRL (Merelbeke, Belgium). 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) chloride salt was obtained from Avanti Polar Lipids (Alabaster, AL, USA), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) from Lipoid GmbH (Ludwigshafen, Germany), 1-ethyl-3-[3-dimethyl)aminopropyl] carbodiimide hydrochloride (EDC) from Sigma-Aldrich (Saint-Quentin Fallavier, France) and high-molecular-weight HA (1,600,000 Da) from Fluka (Sigma-Aldrich Chemie, Buchs, Switzerland). All other reagents were purchased from Sigma-Aldrich (Bornem, Belgium), unless otherwise stated.

2.2. Plasmids

The plasmid constructs pGL4.13 (4641 bp) and gwiz-GFP (5757 bp) (Promega, Leiden, The Netherlands) were amplified in transformed *E. coli* bacteria and isolated from a bacteria suspension with a Purelink™ HiPure Plasmid DNA Gigaprep kit K2100 (Invitrogen, Merelbeke, Belgium). Concentration and purity were determined by UV absorption at 260 nm and 280 nm on a NanoDrop 2000c (Thermo Fisher Scientific, Rockford, IL, USA). Finally, the plasmids were suspended at a concentration of 1 μg/μL and stored in 25 mM HEPES, pH 7.2, at −20 °C. For fluorescent labeling of pGL4.13 plasmids with YOYO-1™ (λ_{ex} = 491 nm, λ_{em} = 509 nm, Molecular Probes, Merelbeke, Belgium), YOYO-1 iodide (1 mM in DMSO) was added to the plasmid at a mixing ratio of 0.15:1 (v:w), resulting in a theoretical labeling density of 1 YOYO-dye molecule per 10 base pairs. The mixture was incubated at room temperature for 4 h in the dark. To remove the DMSO and free YOYO-1, the labeled plasmid was purified with ethanol precipitation and the fluorescently labeled plasmid was finally resuspended in 25 mM HEPES, pH 7.2. The concentration of the plasmid was again determined by UV absorption at 260 nm, and adjusted to 1 μg/μL.

2.3. Conjugation of DOPE to hyaluronic acid

The HA-DOPE conjugate was synthesized as reported by Surace et al. (Surace et al., 2009) based on a modified reaction described by Yerushalmi and Margalit (Yerushalmi and Margalit, 1998). In brief, HA was dissolved in water overnight and preactivated for 2 h at 37 °C by incubation with EDC at pH 4, which was adjusted by titration with 0.1 N HCl. Afterwards, DOPE suspension was added to the HA solution and pH was adjusted to 8.6 with 0.1 M borate buffer. The reaction was allowed to proceed for 24 h at 37 °C. The conjugate was purified by ultrafiltration using a membrane with a molecular weight cut-off of 100,000 Da (Amicon Ultrafiltration, Millipore, Billerica, MA). Purity of the conjugate was proven by thin layer chromatography. The successful conjugation was shown by ^{1}H NMR. The conjugate was lyophilized and stored at −25 °C until further use. The coupling degree was determined to be 1.081% w/w (weight DOPE/weight conjugate).

2.4. Liposomes and lipoplexes

To prepare uncoated liposomes, a thin lipid film was obtained by evaporation under vacuum of a chloroformic solution of an equimolar mixture of DOTAP and DOPE using a rotary vacuum evaporator. This lipid film was rehydrated with 1 mL pure ethanol, for a final molar concentration of 15 mM, and liposomes were further prepared *via* the ethanol injection method published by Nascimento et al. (Nascimento et al., 2015). For liposome preparation, 400 μL of ethanolic lipid solution was rapidly injected into 2.6 mL MilliQ water under stirring with a magnetic bar to obtain a final lipid concentration of 2 mM. HA-modified liposomes were prepared by diluting an aqueous stock solution of the HA-DOPE conjugate (1 mg/mL) to different concentrations in MilliQ water before injection of the ethanol-lipid mixture. The content of HA-DOPE conjugate is expressed in percentage molar ratio HA-DOPE/