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A novel penetratin-modified complex for noninvasive intraocular delivery of antisense oligonucleotides

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ABSTRACT

Inhibition of gene expression by nucleic acids is a promising strategy in the treatment of ocular diseases. However, intraocular delivery of nucleic acids to the posterior ocular tissues remains a great challenge due to the presence of various biological barriers. To circumvent this problem, we established a novel penetratin (P) modified poly(amidoamine) dendrimer (D)/hyaluronic acid (H) complex to deliver antisense oligonucleotides (ASOs, O). Complexes (D/O, HD/O and PHD/O) were easily prepared and modification layers (hyaluronic acid and penetratin) were respectively absorbed on the surface via electrostatic interaction. Complexes with different outer layers were characterized as spherical particles with reversed charges. *In vitro* cellular uptake of ASOs in PHD/O complex was significantly increased than those in other formulations. *In vivo* studies were carried out after topical instillation of the complexes in the conjunctival sac of mice. Compared with D/O and HD/O, PHD/O exhibited much more distribution in the posterior segment of the eyes and prolonged retention time of ASOs in retina for more than 8 h. Taken together, these results indicated that PHD/O complex possessed substantially improved ocular permeability and distribution in the posterior ocular tissues. This work provided a promising noninvasive intraocular delivery strategy for nucleic acids via topical administration.

1. Introduction

Gene therapy is a treatment strategy aiming at delivering nucleic acid drugs into cells and achieving medical purpose by regulating the expression of target proteins and genes (Flotte, 2007). With the well-defined anatomy as well as characteristics such as immune-privilege, the eye is an ideal organ for gene therapy (Chaum and Hatton, 2002). To date, inhibition of gene expression by nucleic acids has become a promising strategy in the treatment of numerous ocular diseases (Garoon and Stout, 2016). Ophthalmic application of nucleic acids such as antisense oligonucleotides (ASOs), aptamers or siRNAs, has gained tremendous interests in recent decades (Solinis et al., 2015; Campbell et al., 2016; Takanashi et al., 2015). Among the promising strategies of ocular gene therapy, antisense oligonucleotides have won a foothold for their easy synthesis and direct effect to down-regulate expression of target genes. Antisense oligonucleotides are developed to bind with specific messenger RNA (mRNA), thus undesirable protein synthesis will be shut down as a consequence (Stein and Cheng, 1993; Hagigit et al., 2012).

However, for most ocular diseases, the target sites of nucleic acids are in the posterior segment of the eye (Diebold et al., 2007). Ocular gene delivery to the posterior segment of the eye remains a great challenge due to the presence of various biological barriers (Tian et al., 2012). Specifically, cornea, conjunctiva and tear film, act as biological barriers to prevent topically instilled formulations from penetrating to the posterior segment of the eye (Vandenberghe and Auricchio, 2012). Therefore, most nucleic acids are generally administered via intravitreal injections, which leads to poor patient compliance (Fagan and Al-Qureshi, 2013). Moreover, repeated injections might cause further complications, such as cataracts, vitreous hemorrhages and retinal detachment (Jager et al., 2004). Thus, there is an urgent need to develop noninvasive ocular delivery systems for nucleic acids to overcome these barriers and to reach posterior segment of the eye.

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Guttae ophthalmicae (eye drops) are the most popular dosage forms of ophthalmic medicine. Nevertheless, eye drops administrated via topical route are inefficient for delivery of ASOs due to their hydrophilicity, high molecular weight and the presence of abundant negative charges (Berdugo et al., 2003). Cationic carriers are often utilized because of their protective effects on encapsulated nucleic acids while enabling cellular uptake. Several gene delivery systems have been developed for ocular delivery of ASOs but mainly focused on diseases locating in anterior segment of the eye (Ko et al., 2011; Ma et al., 2014; de la Fuente et al., 2010). To date, ocular gene therapy via topical administration to the posterior segment of eye is still restricted owing to the lack of efficient delivery systems (Solinis et al., 2015).

Polyamidoamine dendrimer (PAMAM) was first used as transfection agents in 1996, condensing ASOs for *in vitro* gene-silencing investigation (Bielinska et al., 1996). Since then, PAMAM dendrimers have been extensively used for their ability to completely condense ASOs into complexes (PAMAM/ASO) with positively charged groups under physiological condition. Positive charges of the complexes may facilitate attachment of the PAMAM/ASO complex to the cell surface and subsequent endocytosis (Kang et al., 2005). However, when applied for topical ophthalmic researches, PAMAM/ASO complexes are inefficient in permeation across the cornea or sclera after topical administration (Souza et al., 2014). To further improve permeability of the PAMAM/ASO complexes, complementary modification is indispensable. Moreover, most studies involving topical application of ASO complexes remain on the level of *in vitro* experiments and are lack of *in vivo* validation (Marano et al., 2004, 2005).

Hyaluronic acid (HA) exists naturally in ocular tissues with high biocompatibility as well as mucoadhesive and biodegradable properties (Zeng et al., 2016). As a result, HA is a common modification component in ophthalmic preparations. Solid lipid nanoparticles were prepared with protamine and HA modification, trying to deliver nucleic acids to treat ocular diseases (Apaolaza et al., 2014). And complexes constructed with PAMAM and HA via electrostatic interaction (HA/PAMAM) was developed to enhance the intraocular delivery of ASOs (Apaolaza et al., 2014). While the above studies were both conducted only in cellular experiments. *In vitro* results suggested HA modification could be a promising strategy for gene delivery. However, *in vivo* application of HA-modified complexes for ASO delivery to the posterior segment of eye may face biological obstacles. Addition of negatively charged HA to the cationic complex could neutralize the positive surface charges and impair tissue penetrating ability of the complex.

Aiming at improving permeability of PAMAM/ASO while taking advantage of ocular biocompatibility of HA, we developed a novel PAMAM/ASO complex double modified with HA and penetratin. Penetratin is a cell-penetrating peptide derived from a non-viral protein. In our previous work, penetratin showed high ocular permeability and wide distribution in the posterior segment of the eye with low toxicity to ocular tissues via eye drops (Liu et al., 2014). Therefore, we modified the surface of HA/PAMAM complex non-covalently with penetratin for intraocular delivery of ASOs. Three complexes, PAMAM/ASO (D/O), HA/PAMAM/ASO (HD/O) and Penetratin/HA/PAMAM/ASO (PHD/O), were prepared and their physical properties were characterized. *In vitro* cellular uptake and *in vivo* ocular distribution of these complexes were evaluated after topical administration. We expect the facile complexes could deliver nucleic acid drugs from the surface to the posterior segment of the eye via topical instillation, and hence improve patient compliance as well as avoid the potential adverse effects of intraocular injection in further application.

2. Materials and methods

2.1. Materials

PAMAM G5-NH_2 dendrimer (M.W. ~28.82 kDa) was obtained from Sigma-Aldrich. Hyaluronic acid sodium salt (HA, 6 kD) was purchased from Freda Biochem (Shandong, China). Antisense oligonucleotides targeting TGF-β2 gene with sequence of 5′-CCGTGACCAGATGCAGGAT-3′ was labeled with 6-carboxyfluorescein (FAM-ASO) by Sangon Biotech (Shanghai, China). Penetratin (RQIKIWFQNRRMKWKKK) was synthesized by ChinaPeptides Co., Ltd. (Shanghai, China).

2.2. Cell lines and animals

Human conjunctival epithelial cells (NHC) and murine fibroblast cell line (L929 cells) were cultured at 37 °C in a 5% CO_2 humidified atmosphere and a DMEM medium supplemented with 10% fetal calf serum (FCS, Gibco), 4 mmol/L glutamine, 100 μg/mL streptomycin, and 100 units/mL penicillin. Spontaneously derived human corneal epithelial cells (SDHCEC) donated by Zhongshan Ophthalmic Center, Sun Yat-sen University (Guangzhou, China) were cultured in the same medium but with the addition of 5 mg/mL insulin, 10 ng/mL human epidermal growth factor, and 1% (w/v) hydrocortisone.

ICR mice of about 5 weeks age were purchased from the Experimental Animal Center, Fudan University. The mice were maintained in laboratory conditions (22 ± 2 °C and a 12 h light/dark cycle) for 1 week prior to experiments. All animal experiments were performed in accordance with guidelines evaluated and approved by the ethics committee of Fudan University.

All data are presented as means of triplicate determinations from representative experiments. Data analysis was performed using GraphPad Prism Software, and $p < 0.05$ (*) was considered statistically significant.

2.3. Synthesis of fluorescein-labeled PAMAM

PAMAM G5-NH_2 dendrimer was conjugated with fluorescein isothiocyanate (FITC) to obtain fluorescein-labeled PAMAM (FITC-PAMAM) for confocal imaging. In brief, PAMAM dissolved in methanol was dried under nitrogen on a water bath maintained at 40 °C and redissolved in phosphate buffered saline (PBS, pH 7.4) to a desired concentration. 1 mL FITC solution (containing FITC 2 mmol/L) in ethanol was added dropwise into 1 mL PAMAM solution (1 mmol/L, molar ratio of PAMAM:FITC was 1:2). The mixture was stirred overnight at room temperature in the dark to obtain FITC-PAMAM, followed by dialysis against deionized water to remove the unconjugated by-products. The purified product was then lyophilized for later use. Conjugation of rhodamin B isothiocyanate (RB) to PAMAM G5-NH_2 was carried out to obtain RB-PAMAM using the same preparation method of FITC-PAMAM as described above for fluorescence microscopic imaging.

2.4. Preparation of complexes

PAMAM/ASO complex (D/O) was prepared by adding 1 mL ASO solution (30 μg/mL) to 1 mL PAMAM solution (240 μg/mL, weight ratio of PAMAM to ASO was 8:1), followed by vigorous vortexing for 30s. Hyaluronic acid coated complex (HD/O complex) was obtained by adding 1 mL sodium hyaluronate solution (60 μg/mL) to the D/O complex mixture upon vigorous vortexing for 30 s at room temperature. Penetratin coated complex (PHD/O complex) was prepared by adding 1 mL penetratin solution (1.5 mg/mL) to the HD/O complex mixture upon vigorous vortexing for 30 s at room temperature. Three complexes, D/O, HD/O and PHD/O were