Biomaterials 32 (2011) 9483–9493

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Nanoparticle-mediated gene transfer specific to retinal pigment epithelial cells

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ARTICLE INFO

Article history:
Received 20 June 2011
Accepted 19 August 2011
Available online 1 September 2011

Keywords:
Gene therapy
Retinal pigment epithelium
DNA nanoparticle
Subretinal injection
Polyethylene glycol (PEG)

ABSTRACT

Previously, we demonstrated that CK30PEG10k-compacted DNA nanoparticles (NPs) efficiently target photoreceptor cells and improve visual function in a retinitis pigmentosa model. Here, we test the ability of these NPs in driving transgene expression in the retinal pigment epithelium (RPE), using an RPE-specific reporter vector (VMD2-eGFP). NPs, uncompacted plasmid, or saline were subretinally delivered to adult BALB/c mice. NP-based expression was specific to RPE cells and caused no deleterious effects on retinal structure and function. eGFP expression levels in NP-injected eyes peaked at post-injection day 2 (PI-2), stabilized at levels ~3-fold higher than in naked DNA-injected eyes, and remained elevated at the latest time-point examined (PI-30). Unlike naked DNA, which only transfected cells at the site of injection, NPs were able to transfect cells throughout the RPE. Subretinal injections of rhodamine labeled NPs and naked DNA showed comparable initial uptake into RPE cells. However, at PI-7 and -30 days significantly more fluorescence was detected inside the RPE of NP-injected eyes compared to naked DNA, suggesting NPs are stable inside the cell which could possibly lead to higher and sustained expression. Overall, our results demonstrate that NPs can efficiently deliver genes to the RPE and hold great potential for the treatment of RPE-associated diseases.

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1. Introduction

Historically, non-viral gene therapy approaches have been limited by transient transgene expression, low transfection efficiency, limited uptake into some cells and low transgene expression levels [1–3]. However, recent advancements in vector engineering and delivery technologies have overcome these limitations, making non-viral gene therapy a promising approach for the treatment of human and animal diseases. Nanoparticles (NPs) containing a single molecule of plasmid DNA compacted with 30-mers of polylysine conjugated to 10 kDa polyethylene glycol (CK30PEG10k) is an example of a novel non-viral delivery approach [4]. This technology has been used to efficaciously deliver genes to cells of the lung, brain and retina [4–12] and yielded positive results in a phase I clinical trial for cystic fibrosis [13]. Our proof-of-principle experiments have demonstrated that DNA delivered via NPs can mediate phenotypic improvement in the structure and function of photoreceptors in the *retinal degeneration slow* mouse model of retinitis pigmentosa [5,6]. In addition, others working with similarly compacted NPs have shown therapeutic benefits when delivered to a rat model of Parkinson's disease [12].

Many blinding inherited ocular diseases target the retinal pigment epithelium (RPE), a monolayer of cuboidal epithelial cells lining the back of the retina which functions to nourish photoreceptor cells, facilitate attachment of the retina, and metabolize retinoids [14]. Mutations in RPE-specific genes involved in the visual cycle and retinoid metabolism have been reported to cause several visual defects in patients [14]. For example, mutations in RPE65, bestrophin, lecithin retinol acyltransferase (LRAT), and retinol dehydrogenase (Rdh) genes have been shown to result in multiple ocular diseases including retinitis pigmentosa, Leber's Congenital Amaurosis (LCA), and Best's disease [15–18]. Viral vectors have shown promise in multiple RPE-associated LCA clinical trials (NCT00481546, NCT00516477, NCT00643747, www.clinicaltrials.gov) using recombinant adeno-associated viral (rAAV) vectors [19–23]. In spite of the striking positive results currently obtained with AAV-based vectors in the RPE, expanding the available repertoire of effective gene delivery agents is critical. Several of the disease-associated RPE-specific genes are too large to be packaged in AAV [24,25], a limitation not present in CK30PEG10k NPs which can effectively deliver plasmids up to at least 20 kb [26]. Although, AAV vectors are advantageous with great deal of tissue tropism, preexisting immunity due to prior exposure to wild-type (WT) AAV could limit therapeutic benefits [27,28]. Furthermore, neutralizing antibodies to the capsid was detected in the serum of

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0142-9612/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.biomaterials.2011.08.062

animals subretinally treated with AAV vectors [29]. In one study, some humoral response was also observed upon re-administration in a partner eye when injected intra-vitreally [30]. Importantly, NPs exhibit a positive safety profile. NPs carrying a variety of different vectors did not elicit an inflammatory response *in vivo* when delivered to the lungs of mice [31], the nares of human cystic fibrosis patients [13], the brains of rats [9], or the subretinal space of mice [5,32]. CK30PEG10k NPs are non-immunogenic even after multiple subretinal injections (Z. Han, A. Koirala et. al., manuscript submitted). In this study, we evaluate the ability of CK30PEG10k NPs to efficiently transfect and drive gene expression in adult murine RPE cells *in vivo* as a potential therapeutic strategy for RPE-associated diseases using an eGFP reporter construct driven by a 600 bp fragment of the RPE-specific human vitelliform macular dystrophy 2 (VMD2) promoter previously shown to transduce mice RPE cells [33].

2. Materials and methods

2.1. Plasmid construction and nanoparticle formulation

pEGFP-1 plasmid (GenBank accession #U55761) was purchased from Invitrogen (Carlsbad, CA). The 600 bp human VMD2 promoter was PCR amplified from human DNA samples and cloned into the EcoRI and BamHI multiple cloning sites upstream of the eGFP sequence (Fig. 1a). pEGFP-1 encodes a red shifted variant of wild-type GFP whose excitation maximum is 488 nm and emission maximum is 505 nm. Prior to NP formulation, plasmid DNA was processed for endotoxin removal by Aldevron (Fargo, ND) at <5 EU/mg DNA. Compaction of this vector into CK30PEG10k NPs was performed in the presence of acetate counterions and were processed with tangential flow filtration to remove excess polycations, solvent exchanged to normal saline, and concentrated to ~4.3 mg/ml DNA as described previously [10,26]. DNA NPs were then characterized with a panel of quality control tests [4] and had an endotoxin level of <5 EU/mg DNA. The minor diameter of the rod-like NPs was determined by transmission electron microscopy to be 8–11 nm (Fig. 1b). Electron microscopy was performed using carbon-coated grids and counterstained with uranyl acetate, as previously described [8].

Rhodamine labeling of endotoxin purified VMD2-eGFP plasmid was performed using *Label* IT® TM-Rhodamine labeling kit (Mirus Bio LLC, Madison, WI) with slight changes from the manufacturer's protocol. The number of Rhodamine molecules bound to DNA was quantified using the formula- base: dye = $(A_{base} * \varepsilon_{dye})/(A_{dye} * \varepsilon_{base})$ and was determined to be 1 per 20 bp DNA (ε = Extinction coefficient, A = absorbance). Contribution of the dye to the absorbance was corrected by using the formula- $A_{base} = A_{260}-(A_{dye} * C.F._{260})$. $C.F._{260} = 0.27$ and $\lambda_{max} = 576$ nm for TM-Rhodamine as suggested by the manufacturer's protocol. TM-Rhodamine labeled DNA was compacted into rod-like CK30PEG10k NPs in the presence of acetate at a concentration of 3.6 mg/ml as described above.

2.2. Animals

One-month-old BALB/c female mice (25–30 g) were purchased from Harlan Laboratories (Indianapolis, IN). Before use, mice were maintained in the breeding colony for at least one week under cyclic light (12L:12D) conditions; cage illumination was ~7 foot-candles during the light cycle. All experiments and animal maintenance were approved by the local Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center and were performed under the guidelines of the Association of Research in Vision and Ophthalmology (Rockville, MD).

2.3. Subretinal injections

Trans-corneal or trans-scleral (only for fundus imaging) subretinal injections were performed as previously described [34]. Briefly, Balb/c mice were anesthetized by intramuscular injection of ketamine (80 mg/kg) and xylazine (14 mg/kg) in saline. Eyes were dilated with 1% Cyclogyl® (Alcon Laboratories, Ft. Worth, TX), animals were placed on a 38 °C regulated heating pad under a surgical microscope (Carl Zeiss Surgical, NY) and an insulin syringe with a beveled 26 G needle was used to puncture a hole in the cornea. Next, a 33 G blunt-end needle attached to a 10 μl Nanofil® syringe controlled by a UMP3 pump controller (World Precision Instruments, Sarasota, FL) was positioned toward the superior nasal portion of the retina at which point 1 μl of either NPs, uncompacted naked DNA (both at 4.3 mg/ml), or saline alone (vehicle) was injected into the subretinal space. The needle was retracted 10–15 s after injection when a bleb of retinal detachment was visible. Following complete removal of the injection needle, the eye was carefully observed for any indication of post-surgical complications such as iris and subretinal bleeding, pronounced retinal detachment or damage, or excessive vitreous loss. Careful evaluation of the severity of these acute post-surgical complications and subsequent long-term complications including eye infection, loss of visual function and atrophy were used to determine if animals would be excluded from the study. In the absence of any severe complications the procedure was deemed successful and the animal remained in the study. All injections were performed by a single individual. The success rate for injections was ~ 80%. Lastly, Gonak™ hypromellose (2.5%; Akorn Inc., Decatur, IL) was applied to the corneal surface and the animal returned to the regulated heating pad until fully recovered.

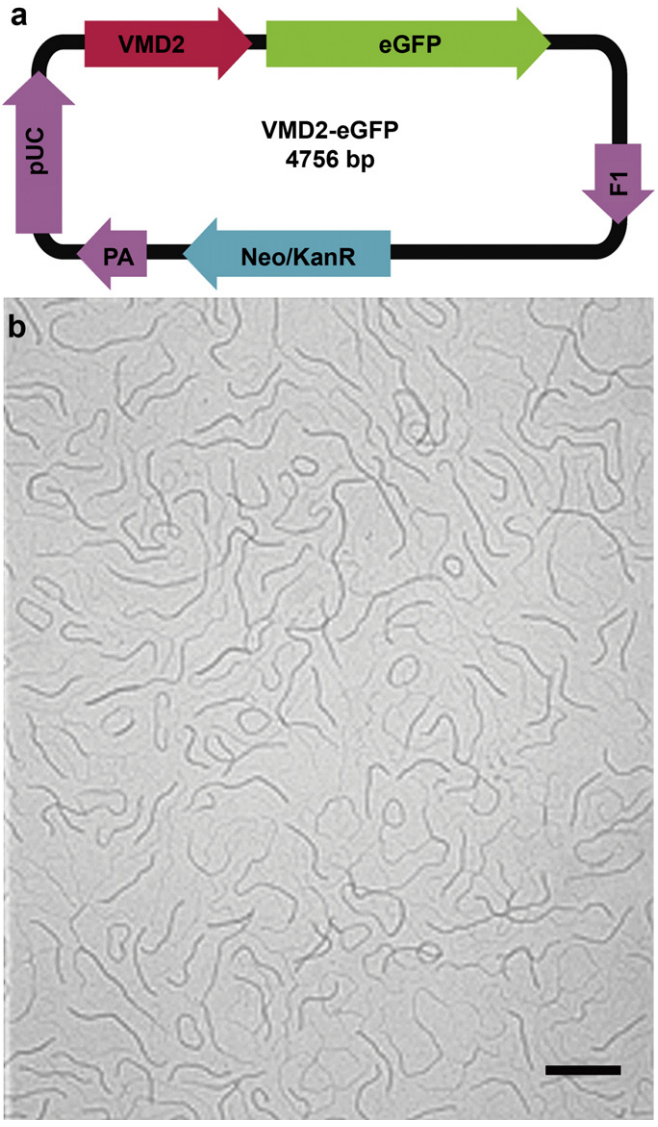

Fig. 1. Vector construction and Nanoparticle EM. (a) Vector map depicting the organization of the expression cassette. VMD2 promoter was cloned into EcoRI and BamHI sites in the pEGFP-1 vector purchased from Invitrogen. (b) EM image of NP formulation at 40,000×. The rod shaped particles were determined to have diameter of ~8–11 nm. Scale bar = 200 nm.

2.4. RNA isolation and quantitative real-time PCR

Total RNA was extracted from the enucleated whole eye using Trizol® reagent (Invitrogen, Carlsbad, CA) as described by the manufacturer. Total RNA (3 μg) was digested by RNase free DNase-I (Invitrogen, Carlsbad, CA) and purified using a phenol chloroform extraction procedure. Total purified RNA (1 μg) was used for cDNA preparation using Superscript® III reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA was further treated with RNase H (Invitrogen, Carlsbad, CA) to ensure degradation of any residual RNA. qRT-PCR using SYBR green was performed on a Bio-Rad C1000 Thermal Cycler (Hercules, CA). Relative expression was calculated using hypoxanthine-guanine phosphoribosyltransferase (HPRT) as an internal control according to the following formula: relative expression = $2^{-\Delta Ct}$, where ΔCt = (*Gene* Ct− HPRT Ct). None of the samples were HPRT negative. A total of 4–7 independent