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A novel gene therapy vector based on hyaluronic acid and solid lipid nanoparticles for ocular diseases

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ABSTRACT

The introduction of therapeutic genes in target tissues is considered as a novel tool for the treatment of several diseases. We have developed nanoparticles consisting on SLNs, protamine (P) and hyaluronic acid (HA) as carrier for gene therapy. Stable complexes positively charged and with a particle size ranging from 240 nm to 340 nm were obtained. Transfection studies in ARPE-19 and HEK-293 cells showed the versatility of vectors to efficiently transfect cells with different division rate, widening the potential applications of SLN-based vectors. In ARPE 19 cells, the incorporation of P and HA induced almost a 7-fold increase in the transfection capacity of SLNs. The CD44 inhibition studies suggested the participation of this receptor in the internalization of the vectors in this cell line. The intracellular disposition of DNA showed that the HA is able to modulate the high degree of condensation of DNA due to the protamine inside the cells; an important fact, if the vector is uptaken via non-degradative endocytosis. Besides, the therapeutic plasmid which encodes the protein retinoschisin was employed achieving a positive transfection in ARPE-19 cells, showing a promising application of this new non-viral system for the treatment of X-linked juvenile retinoschisis by gene therapy.

1. Introduction

The introduction of therapeutic genes in the target tissues is considered as a promising alternative to conventional drug products for the treatment of several chronic diseases. However, despite the recognized value of DNA-based therapeutics, important problems, such as poor cellular uptake, a rapid in vivo degradation, limited transport to the target, and low effective delivery of the genetic material to the cell nucleus, need to be solved before this therapeutic strategy becomes viable (de la Fuente et al., 2008a). Therefore, a key challenge to realizing the broad potential of DNA-based therapeutics is the need for safety and effective delivery methods. A broad diversity of materials is under exploration to address the challenges of delivery, including, viral vectors, inorganic particles, polymeric-, cationic lipid-, and peptide-based vectors (Gascón et al., 2013). Despite the delivery successes known by some of these carriers, advances are necessary to allow the fullest application of DNA in the clinic. In this regard, non-viral delivery systems possess features that result advantageous for their use in gene therapy: safety, low-cost production, high-reproducibility and no limit size of DNA to transport (del Pozo-Rodríguez et al., 2011).

Solid lipid nanoparticles (SLNs), are a promising non-viral vector for gene therapy due to their capacity to condense and protect the genetic material, besides they show efficacy in the cell internalization, and once inside, release the genetic material (Gascón et al., 2012a, 2012b), as well as the ability of SLNs for transfection *in vitro* in several cell lines, and *in vivo* after intravenous and ocular administration as it has been previously demonstrated (del Pozo-Rodríguez et al., 2010; Delgado et al., 2012a,2012b). In order to improve the capacity of SLNs to transfect, several ligands have been incorporated on the nanoparticle surface; for instance, cell penetration peptides (del Pozo-Rodríguez et al., 2009), protamine (Delgado et al., 2011), dextran (Delgado et al., 2012b) or oligochitosans (Delgado et al., 2013), among others.

Hyaluronic acid (HA), a high molecular linear glycosaminoglycan composed of repeated disaccharide units of β-1,4-D-glucuronic acid-β-1,3-N-acetyl-D-glucosamine, is an attractive polymer in the field of pharmaceutical technology due to its biocompatibility, biodegradability and mucoadhesive character (Aragona, 2004). HA

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presents several advantages from a biological point of view, since it promotes the adhesion and proliferation in mammalian cells, and it is involved in biological processes like cell signalling (de la Fuente et al., 2008b). Furthermore, as it is a hydrophilic polymer, it could prevent opsonin adsorption by steric repulsion allowing mononuclear phagocyte system uptake to be reduced (Wojcicki et al., 2012). Another property of HA is the well-known capacity to interact with the CD44 receptor, which is expressed in different tissues, including human cornea and conjunctive, and participates in a wide variety of cellular functions, like the receptor-mediated internalization between others. Therefore, the interaction of HA with CD44 and other HA-specific receptors facilitates cell internalization of different systems (Ruponen et al., 2001); moreover, its capacity to favour the nuclear entry is well known, and the role as a transcription activator has also been suggested (de la Fuente et al., 2008b). All these properties make HA a very useful compound for transfection approaches (Yamada et al., 2013).

With this premise in mind, we developed nanoparticles consisting on SLNs, protamine and HA as a carrier for gene therapy. We prepared different complex compositions, with regard to the molecular weight of HA and HA–protamine–SLN mass ratios, and studied transfection efficiency, cell uptake and intracellular trafficking pathways in two cell models: ARPE-19 and HEK-293. Finally, it was also studied the capacity of the vectors to transfect ARPE-19 cells with the therapeutic plasmid that encodes the protein retinoschisin, which is responsible of a retinal degenerative disorder, the X-linked juvenile retinoschisis.

2. Material and methods

2.1. Preparation of the hyaluronic acid–protamine–DNA–SLN vectors (HA–P–DNA–SLN)

The SLNs were produced by a previously described solvent emulsification-evaporation technique (del Pozo-Rodríguez et al., 2007). The solid matrix of the particles was composed by the lipid Precirol® ATO 5 which was kindly gifted by Gateffossé. The cationic lipid N-(1-(2,3-Dioleoyloxy) propyl)-N,N,N trimethyl ammonium methyl sulfate (DOTAP), purchased from Avanti Polar Lipids, was used to form the external phase of the nanoparticles, and the surfactant Tween 80 (Panreac) was selected to obtain the initial emulsion. To prepare the SLNs the lipid Precirol® ATO 5 was dissolved in the organic solvent dichloromethane (Panreac) (5%, w/v) and emulsified by sonication in the aqueous phase containing DOTAP (0.4%, w/v) and Tween 80 (0.1%, w/v). Dichloromethane was then evaporated by magnetic stirring followed by vacuum conditions, so that the inner lipid Precirol® ATO 5 precipitated and nanoparticles were obtained.

To prepare the HA–P–DNA–SLN vectors, firstly a solution of protamine (P) was mixed with an aqueous solution of pCMS–EGFP plasmid (0.2 μg/μl), which encodes the enhanced green fluorescent protein (EGFP), at a fixed ratio of 2:1 (w:w) during 5 min. Then, an aqueous solution of HA was added to form the HA–P–DNA complex at the desired ratios. After 15 min, the HA–P–DNA complex was mixed with a suspension of SLNs during 20 min at room temperature, and electrostatic interactions between the HAPDNA complexes and SLNs led to the formation of HA–P–DNA–SLN vectors. All the vectors were brought up to a final volume of 300 μl with HBS buffer. HA of three different molecular weights purchased from Sigma–Aldrich were used for the preparation of the vectors: 150 kDa (HA_{150}), 500 kDa (HA_{500}), and 1630 kDa (HA_{1630}). The pCMS–EGFP plasmid was purchased from BD Biosciences Clontech (Palo Alto, California, USA) and amplified by Dro Biosystems S.L. (San Sebastian, Spain).

As control, a DNA–SLN vector was prepared by mixing 50 μl of an aqueous solution of pCMS–EGFP plasmid (0.2 μg/μl) together with 150 μl of the SLNs suspension (1 μg/μl). The incubation time for the electrostatic binding was 20 min at room temperature. In all cases, the SLN to DNA ratio, expressed as the ratio of DOTAP to DNA (w:w), was fixed at 5:1.

Finally, vectors bearing the pCEP4-RS1 plasmid were prepared as explained above. This plasmid encodes retinoschisin protein, and was obtained from the Institute of Human Genetics of the University of Regensburg (Germany). The pCEP4-RS1 plasmid was formulated in HA–P–DNA–SLN vectors prepared with HA_{150} and HA_{500} at different w:w ratios.

2.2. Studies with the pCMS–EGFP plasmid

2.2.1. Size and Zeta potential measurements

Sizes of SLNs, DNA–SLN and HA–P–DNA–SLN vectors were determined by photon correlation spectroscopy (PCS). Zeta potentials were measured by Laser Doppler Velocimetry (LDV). Both measurements were performed on a Zetasizer Nano series-Nano ZS (Malvern Instruments, Worcestershire, UK). All samples were diluted in Milli-Q™ water.

2.2.2. Electrophoresis on agarose gel

Binding efficiency of DNA by HA–P–DNA–SLN complex at different HA ratios, protection from DNAse I (Sigma–Aldrich) digestion and DNA plasmid release from the vectors were performed using a 0.7% agarose gel (Sigma) containing Gel Red™ (Biotium) for visualization during 30 min at 120 V. The bands were observed with an Uvitec Uvidoc D-55-LCD-20M Auto transilluminator.

Complexes were diluted in Milli-Q™ water up to a final concentration of 0.03 μg DNA/μL. A concentration of 1 U DNase I/2.5 μg DNA was added to DNA–SLN and HA–P–DNA–SLN vectors, and the mixtures were then incubated at 37 °C for 30 min; afterwards, 4% SDS solution was added to the samples to a final concentration of 1% to release the DNA from the SLNs. Finally, the integrity of the DNA in each sample was compared to a control of untreated DNA.

2.2.3. Preparation of cell cultures

In vitro assays were performed with human retinal pigment epithelial (ARPE-19) cells and human embrionic kidney (HEK-293) cells, both obtained from the American Type Culture Collection (ATCC).

ARPE-19 cells and HEK-93 cells were cultured in Dulbecco's Modified Eagle's Medium–Han's Nutrient Mixture F-12 (1:1) medium (D-MEM/F-12, Gibco) and in Eagle's Minimal Essential with Earle's BSS and 2 mM L-glutamine (EMEM, LGC Promochem), respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% Normocin™ antibiotic solution (Invivogen). Cells were incubated at 37 °C in 5% CO_2 atmosphere and subcultured every 2–3 days using trypsin–EDTA (Lonza).

ARPE-19 cells were seeded on 12 well plates (3×10^4 cells/well) and HEK-293 cells were seeded on 24 well plates (1.5×10^5 cells/well). Before the treatment with vectors both cell lines were allowed to adhere overnight. Vectors were diluted in HEPES Buffer Solution (HBS) and added to the cell cultures. In all cases, an amount of vectors equivalent to 2.5 μg of DNA was added to each well. Cells were incubated in presence of the vectors for 4 h at 37 °C, after which the medium containing the complexes was refreshed with 1 mL of complete medium. Transfection efficacy was quantified at 24 h, 48 h, 72 h, 96 h and one week.

2.2.4. Transfection evaluation by flow cytometry and fluorimetric assay

At the established times, the cells were washed once with 300 μL of Phosphate Buffered Saline (PBS), and then detached with 300 μL of 0.05% trypsin–EDTA. The cells were then centrifuged at