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Evaluation of cystamine-modified hyaluronic acid/chitosan polyplex as retinal gene vector

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ABSTRACT

Purpose: A successful gene therapy approach can prevent or treat congenital and acquired diseases. However, there is still no ideal non-viral vector for gene delivery in a safe and timely manner. In this report the anionic polymer hyaluronic acid (HA) was investigated as a potential vector for gene therapy. Due to its intrinsic characteristics it constitutes an excellent candidate to deliver therapeutic genes, pending the modification of its surface charge.
Methods: To modify its charge, HA was modified with cystamine. Several formulations were prepared using modified HA combined with sodium sulfate, sodium triphosphate, K-carrageenan and chitosan. Vectors were characterized with respect to size, charge, DNA load and its protection, and effect on cell viability. The better performing formulations were further evaluated *in vitro* for their transfection efficiency in HEK293T and ARPE-19 cells.
Results: Cell viability assays showed low cytotoxicity for both polymers. Gene transfer efficiency depended on cell line and formulation, but no increased transfection efficiency was observed with the modified polymer.
Conclusions: HA has great potential as a gene therapy vector, but further optimization, including incorporation of a higher percentage of positive groups in HA, is needed before its use as a gene delivery vector.

1. Introduction

Gene therapy is an area that has been growing rapidly and revolutionizing the world of research [1]. An essential requirement for the success of this technique is an efficient method for gene transfer, since DNA molecules cannot enter cells efficiently because of their large size, hydrophilic nature and susceptibility to degradation mediated by nucleases [2,3]. Considering the great diversity of diseases targeted by gene therapy, it is implausible that a single gene delivery system (vector) is suitable for all applications. However, the main requirements are common to all systems: vectors should transfer the genetic material to the tissue of interest and induce the proper level of therapeutic gene expression with no side effects [4].

Currently, vectors used in gene therapy can be divided into two main categories: viral and non-viral. Recombinant viruses such as retrovirus, lentivirus, adenovirus, adeno-associated viruses, and herpes viruses have been widely used for genetic material transfer [2]. Viruses have as main advantage their high transfection efficiency, however, viral vectors entail many intrinsic problems such as difficulties in production, limitations concerning repeated administrations that can lead to acute inflammatory responses, immune responses of the host to the virus and induction of mutagenesis by some viruses that integrate into the genome of host cells [2]. On the other hand, non-viral vectors have a higher safety profile due to their low toxicity and low immunogenicity. Additionally, non-viral vectors have the ability to carry larger genes and present lower production costs. Despite this appealing feature from the safety standpoint, non-viral vectors have little clinical importance due to low transfer and expression of transgene [2]. However, due to problems in clinical trials using viral vectors in the recent past, the interest in non-viral technologies has been renewed, particularly in the release properties of the non-viral vectors that resemble traditional drugs [4].

Non-viral vectors include synthetic or naturally occurring chemical compounds, such as lipids and cationic polymers. These can form complexes with the negatively charged DNA through electrostatic interactions that allow the therapeutic transgene across the cell membrane *via* endocytosis [2,3,5]. The complexes protect DNA from nuclease-mediated degradation and facilitate cell entry as well as gene transferring into the nucleus [2].

Abbreviations: CS, chitosan; DNA, deoxyribonucleic acid; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HA, hyaluronic acid; $HASSNH_2$, hyaluronic acid-*co*-*N*-cystaminyl-hyaluronamide; HASH, hyaluronic acid-*co*-*N*-cysteaminyl-hyaluronamide; MW, molecular weight; RPE, retinal-pigmented epithelia.

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Polymers have chemical flexibility, which enables the design and construction of polymers with multiple functionality, and thus more efficient in gene delivery, while maintaining the characteristics of biocompatibility, easy production and stable formulation [4]. One of the key points for the construction of more efficient vectors relates to various biological barriers of cells that must be overcome to achieve higher transfection rate. These barriers include the attachment of the polymer to the cell surface, then entering the cell through the cell membrane, displacement throughout the cytoplasm, escape endosomal degradation and ending with the passage of the nuclear envelope and nuclear entry [3]. In order to overcome different barriers, functional groups can be introduced into polymers, such as ligands to enhance cell entry *via* receptor-mediated endocytosis, membrane peptides to allow endosomal release and nuclear localization signals to increase nuclear entry of the transgene [3].

In this paper, hyaluronic acid (HA) was evaluated as a possible retinal gene therapy vector. HA is a biocompatible, non-toxic, non-immunogenic, non-inflammatory anionic biopolymer that has been widely used in various biomedical applications [6]. Multiple studies on the biological function of HA have revealed that there is a strong relationship between the presence of HA and the migration and proliferation of cells as well as an involvement in wound healing, cell motility, angiogenesis, and extracellular matrix formation. Another important feature of HA for its use as a vector of therapeutic genes is the ability to interact with various cell receptors [6]. The negatively charged carboxyl group of HA is responsible for the interaction with membrane receptors allowing the connection with HA [7].

Despite its advantageous features, HA exists in the form of an aqueous gel, has a short lifetime and quickly degrades after administration. In order to increase the lifetime of HA for long-term clinical applications, several strategies have been developed, particularly modifications of the polymer at the level of carboxyl and hydroxyl groups. These new polymers, although they have different physico-chemical properties compared to unmodified HA, they maintain essential biological properties that allow their use in non-viral therapies [6]. Among possible chemical modifications to perform, the more relevant for gene therapy is the inclusion of amine groups [6]. A higher amount of amine groups may lead to greater DNA loads and increase the efficiency of transfection and transgene expression. In this study, HA was modified with cystamine through a coupling reaction with the carboxyl groups of HA. This modification with cystamine not only adds amine groups to the polymer but it also contains a disulfide bond that can be cleaved in the presence of intercellular glutathione, thereby promoting a more rapid release of genetic material [8,9].

In this work we compare the transfection efficiency between modified and unmodified HA using two types of cells: human embryonic kidney (HEK 293T) cells and retinal pigment epithelium cells (ARPE-19). The former are a commonly used cell line in transfection studies, while the latter are a model of retinal pigmented epithelial (RPE) cells, our target in the retina, due to their important role in the support of the retinal homeostasis and involvement in several retinal diseases [8].

2. Materials and methods

2.1. Materials

Chitosan (MW of 80 kDa) with a degree of de-acetylation of 83% was purchased from Polysciences, Inc., USA. Hyaluronic acid, with MWs of 132 and 214 was purchased from Lifecore Biomedical. All other reagents were analytical grade and used without further purification.

2.2. Plasmid constructs and cell lines

A plasmid expressing enhanced green fluorescent protein driven by the cytomegalovirus promoter (kindly provided by Jean Bennett, University of Pennsylvania, USA) was amplified in Top 10 bacteria and purified using a Plasmid Maxi kit (Qiagen, California, USA) following manufacturer guidelines. Plasmid DNA (DNA) was dissolved in TE buffer, and the concentration was evaluated using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA) at 260 nm.

Two cell lines were used for transfection and cytotoxicity evaluation: HEK 293T cells (kindly provided by Guilherme Ferreira, University of Algarve, Portugal) and a human retinal pigment epithelial cell line (ARPE-19, kindly provided by Francisco Ambrósio, University of Coimbra). All cell culture reagents were purchased from Sigma-Aldrich® (St. Louis, MO/USA).

2.3. Methods

2.3.1. Modification of hyaluronic acid with cystamine ($HASSNH_2$)

The modification reaction was performed in an adaptation of what was previously described elsewhere and depicted in Fig. 1 [10–12]. Briefly, 500 mg of hyaluronic acid was dissolved in 100 ml of distilled H_2O, a one and a half molar excess (relative to the carboxylic acid groups in HA) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC, 0.378 g, 1.974 mmol), and three molar excess of cystamine (0.889 g, 3.947 mmol) were added to the solution. The mixture was stirred at room temperature for 72 h. The reaction mixture was dialyzed against 4 g/l NaCl (MW cut off 2000) for 6 h and then against distilled H_2O for 24 h. The final product – $HASSNH_2$ – was lyophilized for 3 days and stored at room temperature until use.

2.3.2. Determination of the thiolation extent

In order to determine the extent of the modification, the thiol groups were quantified by the Ellman's test. Firstly, 50 mg of $HASSNH_2$ was dissolved in distilled H_2O and treated with 30 molar excess of dithiothreitol (DTT, 0.418 g, 2.712 mmol) in order to expose the thiol groups. The mixture was stirred under N_2 atmosphere for 24 h, at room temperature. The reaction mixture was dialyzed against 4 g/l NaCl (MW cut off 2000) for 6 h and then against distilled H_2O for 24 h. The final product HASH was lyophilized for 2 days and stored at room temperature until use. Secondly, for the Ellman's test, 3.8 mg of HASH was dissolved in 1 ml of 0.1 M Tris buffer, pH 8.0. For each measurement, a tube containing 100 μl of Ellman's reagent (10 mM of 5,5′-dithio-bis-(2-nitrobenzoic acid) in 0.1 M Tris buffer), 1800 μl of 0.1 M Tris buffer and 100 μl of sample was prepared, incubated at room temperature for 15 min and then the absorbance at 412 nm was measured [13].

2.3.3. Potentiometric titration of $HASSNH_2$

In order to characterize the protonation behavior of the modified polymer, a titration was performed [14]. Polymer solutions, HA and $HASSNH_2$, were prepared with a concentration of total titratable carboxyl group concentration of 10 mM in a volume of 10 ml. The initial pH was adjusted to 2–3 by adding 2.00 M HCl prior to the dropwise addition of the titrant 0.08 M NaOH to the polymer solutions under constant stirring. Potentiometric measurements were made using a pH measurement electrode (Orion 9157BN) connected to an Orion pH meter (Thermo Orion 4 Star pH-ISE Benchtop; ThermoFisher Scientific (Waltham, MA USA).

2.3.4. Polyplex preparation

Polyplexes were prepared as previously described by our lab [8,15]. Vector formulations were prepared at various ratios by adding anionic solutions to the $HASSNH_2$ solution, as indicated in Diagram 1. Briefly, polymer solutions (1 mg/ml in MilliQ H_2O, pH 5.5) and sodium sulfate solutions were separately heated to 55 °C. Equal volumes of both solutions were quickly mixed together, vortexed for 30 s, placed on ice and stored at 4 °C. Alternatively, HA and other anionic species (TPP and κ-carrageenan) were tested by combining them to the sodium sulfate solutions. Different weight ratios were tested keeping constant the $HASSNH_2$ amount (250 μg) and varying the HA amount. In order to use