Acta Biomaterialia 17 (2015) 89–97

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Enhancement of chitosan-mediated gene delivery through combination with phiC31 integrase

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ARTICLE INFO

Article history:
Received 1 September 2014
Received in revised form 9 January 2015
Accepted 11 January 2015
Available online 17 January 2015

Keywords:
Polymers
Chitosan
Gene therapy
Gene delivery
Integrase

ABSTRACT

Gene transfer efficiency and expression stability are key factors to a successful gene therapy approach. In the present work we have developed a combined system for gene transfer that integrates well established non-viral polymeric vectors based on chitosan particles with the properties of phiC31-integrase that promotes a relatively non-immunogenic, site-specific integration, with sustained gene expression. Simultaneously, to overcome one of the major limitations in adeno-associated virus mediated gene transfer – the delivery of large genes – we have tested the capacity of our non-viral vectors to incorporate a large (8 Kb) transgene. Polyplexes were extensively characterized for their size, surface charge, morphology, pDNA complexation, transfection efficiency and transgene expression *in vitro* using HEK293 cells. Co-transfection with integrase was done by complexation in a single polyplex preparation or the use of two separate polyplex preparations. Transgene expression, GFP and CEP290 (1 Kb and 8 Kb, respectively), was evaluated by fluorescence microscopy, flow cytometry and Western blot analysis. DNA complexation efficiency, particle size and morphology were consistent with gene delivery for all formulations. In contrast, transfection efficiency and transgene expression varied with polymer and polyplex size. Following delivery by chitosan polyplexes, high levels of GFP expression were still visible 16 weeks post-transfection and over-expression of the large transgene was detected at least 6 weeks post-transfection. Polyplexes incorporating phiC1 integrase demonstrate prolonged gene expression of both small (GFP, 1 Kb) and large genes (CEP290, 8 Kb). This approach, using a combined strategy of polymers and integrase may overcome the size limitation found in commonly used adeno-associated virus mediated gene transfer techniques, while maintaining a high safety profile and prolonged, sustained gene expression, thus constituting an alternative for gene delivery.

1. Introduction

Gene therapy shows great promise regarding treatment of several inherited diseases and, in recent years, gene therapy has been featured in numerous clinical trials with encouraging outcomes [1–4]. Currently, there are two general methodologies used to deliver transgenes: a viral mediated and a non-viral mediated approach. The first has shown great success through the use of viruses such as adeno-associated viruses while the latter uses, for example, cationic polymers and lipids to deliver genes with so far modest results.

Despite the success of viral vectors, the use of viruses still raises several issues. Retroviral vectors can randomly integrate into the host genome and promote long-term gene expression but are unable to transduce non-dividing cells and can cause insertional mutagenesis [5]. Alternatively, lentivirus can be used to transduce non-dividing cells; however insertional mutagenesis is also a concern [5]. Adenoviruses and adeno-associated viruses (AAV) have also been evaluated for gene delivery but have limited use due to cytotoxicity and limited transgene size packing, respectively [5]. The great advances in ocular gene therapy in the past decade can be attributed to the use of AAV vectors. As such AAV vectors have been prominently used in the most recent gene therapy clinical trials, especially in retinal disease, that have shown overwhelming safety and in some trials, efficacy [3,4,6]. The main limitation of this virus considering ocular applications is its limited packaging capacity (less than

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http://dx.doi.org/10.1016/j.actbio.2015.01.013
1742-7061/

5 kb), although some clinical trials have used lentivirus to overcome the limited packaging issue of AAV, safety and efficacy concerns continue. Also, AAV transduction of terminally differentiated post-mitotic cells, like retinal cells, is limited by insufficient dsDNA production. Despite the fact that these dsDNA genomes can integrate into the host cell, integration occurs at low frequency and randomly and so transgene expression tends to decline with time due to silencing or degradation of the episomal agent [5].

In contrast, non-viral vectors are biocompatible, with low or no immunogenicity, have less handling safety requirements [7] and have greater potential to deliver large genes. However, their lower efficiency and short-term transgene expression compared to viruses have limited their use. This is the major rationale behind our study, which aims to demonstrate that the aforementioned shortcomings can be improved on.

Any successful gene therapy strategy requires long-term sustained transgene expression. Recently, several strategies have been evaluated in order to promote safe integration and long-term expression. One of the most promising exploits a site-specific recombinase, the phage phiC31 integrase. PhiC31 integrase is a member of the serine site-specific recombinase family that catalyzes recombination between attachment sites on phage and bacterial genomes, attP and attB, respectively [8,9]. Recombination at these sites results in the formation of hybrid sites, known as attL and attR that prevent further interaction with the integrase recombinase. In the absence of an excisionase protein, the reaction results in unidirectional integration [9]. PhiC31 integrase has also been shown to promote a safer, site-specific integration in mammalian cells using pseudo attP sites endogenous to the mammalian genome [8,10]. Recent studies have shown that the phiC31 integrase can be successfully included in gene therapy strategies promoting integration and prolonged expression *in vivo* in mouse lungs [11], mouse liver [12], rat retina [13] and human skin [14,15].

The use of non-viral gene delivery systems has gained importance over the years, especially with the use of natural cationic polymers, such as chitosan. Chitosan is a deacetylated derivative from chitin that effectively encapsulates and protects therapeutic nucleic acids [16], and has been used for gene delivery in prior *in vitro* [16–22] and *in vivo* [23–25] studies.

This study reports efficient gene transfer, by a novel combination of two well-reported methods, the non-viral phiC31-integrase mediated gene transfer with delivery to the cell using chitosan polyplexes. This method promotes stable transgene integration and sustained transgene expression. Additionally, we demonstrate the ability for stable and efficient gene transfer of large genes (>8 kb) that exceed the carrying capacity of commonly used adeno-associated viral vectors. This would enable gene therapy protocols for inherited degenerative diseases caused by mutations on large genes such as the blindness-leading Leber Congenital Amaurosis type 10 (LCA10) and Stargardt disease that affect the retina, among others.

2. Materials and methods

2.1. Materials

Chitosan CL 113 and 213 (C1 and C2, respectively), with a molecular weight (MW) of 80 and 260 kDa and deacetylation degree (DD) of 83% were purchased from Novamatrix (FMC BioPolymer AS, Norway). All other reagents were of analytical grade and used without further purification.

2.1.1. Plasmid DNA constructs

Three different plasmids (Fig. 1) were used (1) pCMVeGFP-attB, expressing the reporter gene green fluorescent protein (GFP); (2) pCMVCEP290attB, expressing the CEP290 gene (gene responsible for LCA 10), both driven by the cytomegalovirus (CMV) promoter and containing the attachment sites for integrase (attB); and (3) pCMVINT, an integrase expression plasmid (all kindly provided by Dr. Jean Bennett, University of Pennsylvania, USA) [8,26]. Plasmids were amplified in Top10 bacteria and purified using a Plasmid Maxi kit (Qiagen, USA) following manufacturer guidelines. Plasmid DNA (pDNA) was dissolved in TE buffer, and the concentration was determined by a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) at 260 nm.

2.2. Polyplex preparation

Chitosan polyplexes were prepared as described previously [22]. Briefly, a chitosan solution (1 mg/mL) in MilliQ water, pH 5.5) and a 25 mM of sodium sulfate solution were preheated separately to 55 °C. An equal volume of both solutions was quickly mixed together, spun for 30 s, placed on ice and then stored at 4 °C.

Polyplexes were prepared with chitosan (of 80 or 260 kDa) and combined with (1) plasmid coding for GFP (pCMVeGFP-attB) or (2) plasmid coding for CEP290 (pCMVCEP290attB). Each condition was also prepared in combination with the plasmid coding for integrase (pCMVINT). Another formulation was prepared with the plasmid coding for integrase (pCMVINT) (all formations described in Fig. 2). To prepare pDNA loaded polyplexes at a NH_3^+:PO_4^- ratio (N:P ratio) of 15:1, 250 μg of chitosan and 26.5 μg of pDNA were used. pDNA was mixed with the sodium sulfate solution and this solution mixed with the chitosan solution, as described above. When two plasmids were combined in the same polyplex the total pDNA amount was kept constant and a molecular ratio of 2:1 was used. Polyplexes were used without further purification.

2.3. Polyplex characterization

Dynamic light scattering (DLS) and non-invasive backscatter technology were used to determine the size of the polyplexes using a detection angle of 173°. Laser Doppler velocimetry and phase analysis light scattering technology were used to measure the zeta potential (Zetasizer Nano ZS, Malvern instruments, UK). The polydispersity index of polyplexes was obtained by DLS using the Zetasizer Nano Series software v 7.01 and all measurements were performed in ddH_2O at 25 °C. Morphological analysis was performed by transmission electron microscopy (TEM, JEOL JEM-1011 electron microscope, Japan). Samples were stained with a 2% (w/v) sodium phosphotungstate octadecahydrate solution and placed on copper grids with Formvar® films for observation.

2.4. Evaluation of pDNA-complexation

To evaluate the pDNA-complexation capacity by the polymer, pDNA retention in the polyplexes was assessed by a retardation assay. The retardation assay was performed using an agarose gel electrophoresis with 1% (W/V) agarose in TBE buffer with ethidium bromide. Gels were run at 80 mV for 1 h and further visualized under UV light.

2.5. Transfection studies

Human embryonic kidney 293 cells (HEK293 and HEK293T, kindly provided by Dr. Jean Bennett from University of Pennsylvania, USA) were used for the transfection studies. Cells were cultured at 37 °C, under a 5% CO_2 atmosphere, in Dulbecco's Modified Eagle's Medium (DMEM, Cellgro®, USA) supplemented with 10% of fetal bovine serum (FBS, Hyclone, USA) in standard cell culture conditions.