Journal of Controlled Release 192 (2014) 40–46

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Efficient *in vitro* gene delivery by hybrid biopolymer/virus nanobiovectors

Rahul Keswani [a], Kai Su [b,c], Daniel W. Pack [a,b,c,*]

[a] Department of Chemical and Biomolecular Engineering, University of Illinois, Urbana, IL 61801, USA
[b] Department of Chemical and Materials, University of Kentucky, Lexington, KY 40506-0046, USA
[c] Department of Pharmaceutical Sciences, University of Kentucky, Lexington, KY 40536-0596, USA

ARTICLE INFO

Article history:
Received 16 April 2013
Accepted 30 June 2014
Available online 8 July 2014

Keywords:
Chitosan
Gene therapy
Gene delivery
Retrovirus
Hybrid vectors

ABSTRACT

Recombinant retroviruses provide highly efficient gene delivery and the potential for sustained gene expression, but suffer from significant disadvantages including low titer, expensive production, poor stability and limited flexibility for modification of tropism. In contrast, polymer-based vectors are more robust and allow cell- and tissue-specific deliveries via conjugation of ligands, but are comparatively inefficient. The design of hybrid gene delivery agents comprising both virally derived and synthetic materials (nanobiovectors) represents a promising approach to development of safe and efficient gene therapy vectors. Non-infectious murine leukemia virus-like particles (M-VLPs) were electrostatically complexed with chitosan (χ) to replace the function of the viral envelope protein. At optimal fabrication conditions and compositions, ranging from 6 to 9 μg chitosan/10^9 M-VLPs at 10×10^9 M-VLPs/ml to 40 μg chitosan/10^9 M-VLPs at 2.5×10^9 M-VLPs/ml, χ/M-VLPs were ~300–350 nm in diameter and exhibited efficient transfection similar to amphotropic MLV vectors. In addition, these nanobiovectors were non-cytotoxic and provided sustained transgene expression for at least three weeks *in vitro*. This combination of biocompatible synthetic agents with inactive viral particles to form a highly efficient hybrid vector is a significant extension in the development of novel gene delivery platforms.

1. Introduction

Human gene therapy relies upon the introduction of exogenous genetic material to diseased cells or tissue for the replacement of damaged and mutated genes, introduction of new genes, or supplementation of deficient gene expression [1]. Thus, gene therapy has the potential to treat inherited diseases including cystic fibrosis [2,3], muscular dystrophy [3–5] and severe combined immunodeficiency (SCID) [3], as well as acquired diseases such as cancers [6], cardiovascular diseases and viral infections [7,8]. This technology has yet to deliver on its promise, however, due in large part to the lack of safe and efficient means of delivering therapeutic genes [9].

Delivery of nucleic acids to mammalian cells can be achieved via recombinant viruses or synthetic materials including cationic lipids and polymers [10]. Recombinant retroviruses, in particular, are common vectors in the laboratory and in clinical trials, and are capable of integrating their genetic cargo with the target cell genome with remarkable efficiency. However, the lack of cell-specific targeting, immunogenicity, risk of insertional mutagenesis followed by oncogene activation, as well as poor stability and expensive and low-titer production of retroviruses have been critical limitations [11]. In order to move retroviral vectors toward clinical application, safety has been enhanced by introduction of features such as chromatin insulators, conditional and cell type-specific gene expression, targeted transduction, and site-specific integration [12].

In the last two decades, numerous synthetic materials have been developed as alternatives for delivering genetic material. Two prominent classes of these synthetic materials are polycations, such as poly-L-lysine (PLL) [13], polyethylenimine (PEI) [14] and polyamidoamine (PAMAM) [15], and various cationic lipids [16]. These materials electrostatically bind and condense nucleic acids forming nanometer-scale particles [10,17]. Such synthetic vectors typically offer safety, flexibility, functionality for attachment of cell-specific targeting ligands, and more facile manufacturing, but are comparatively inefficient and typically provide short duration of gene expression [18].

Developments in nanobiotechnology provide the means to generate hybrid vectors, comprising viral and synthetic components, for efficient and safe gene delivery. For example, murine leukemia virus-like particles (M-VLPs) are similar in composition, size and morphology to murine leukemia virus (MLV) but lack the envelope protein (Env), which is an important cause of many of the limitations of retroviral vectors including their instability, which leads to relatively low titers and difficult

* Corresponding author at: Department of Chemical and Materials Engineering and Department of Pharmaceutical Sciences, University of Kentucky, 467 Biological Pharmaceutical Building, 789 South Limestone, Lexington, KY 40536-0596, USA. Tel.: +1 859 218 0907; fax: +1 859 257 7585.
E-mail address: dan.pack@uky.edu (D.W. Pack).

http://dx.doi.org/10.1016/j.jconrel.2014.06.060
0168-3659/

purification, and difficulty in cell-specific targeting [19–21]. Env is responsible for binding receptors on target cells and initiating infection, making M-VLP non-infectious [22]. Gene delivery is possible, however, if a synthetic agent associated with M-VLP provides entry of the viral particle into the cell and release of the viral cargo into the cytosol. Once the M-VLPs are internalized, native viral mechanisms lead to transport of viral genes to the cell nucleus and highly efficient transgene expression.

We have reported transfection employing nanobiovectors comprising M-VLPs with the aide of cationic polymers [21,23] and liposomes [24]. Here we have expanded upon the polymer parameter space using a more biocompatible polymer, chitosan (χ), to form nanobiovectors with improved physicochemical properties and reduced cytotoxicity. This paper demonstrates assembly of the chitosan/virus-like particle (χ/M-VLP) nanobiovectors, highly efficient gene delivery and sustained transgene expression in a human cell line *in vitro*.

2. Materials and methods

2.1. Cell lines

Human embryonic kidney cells, HEK293, were purchased from the American Type Culture Collection. The MLV producer cell line, GP293Luc, expressing the MLV viral *gag-pro-pol* genes and a viral packaging sequence encoding neomycin resistance and luciferase reporter genes was purchased from Clontech. Both cell lines were grown in DMEM supplemented with 10% FBS (Gemini Bio-Products) and cultured at 37 °C in 5% CO_2. Dulbecco's Modified Eagle's Medium (DMEM) and phosphate-buffered saline (PBS) were produced by the Cell Culture Media Facility, School of Chemical Sciences, University of Illinois.

2.2. M-VLP production and quantification

M-VLPs were produced in GP293Luc cells seeded at 2×10^6 cells in a 10 cm dish. The cells were cultured for four days before the M-VLP containing supernatant was collected and filtered through a 0.45 μm surfactant-free cellulose acetate syringe filter. M-VLP supernatant was either used immediately or stored at 4 °C for short-term storage (<1 month) or −80 °C for long term storage.

The concentration of M-VLPs in the supernatant was measured using quantitative reverse-transcriptase PCR [23]. RNA standards were obtained from the Clontech q-PCR Retroviral Quantification Kit and stored at −80 °C before further use. Viral RNA was extracted using the QIAGEN Viral RNA Extraction kit and stored in a 60 μl eluate at −80 °C before further use. Standards and viral RNA samples were prepared for reverse transcription using Taqman reverse transcription reagents (Applied Biosystems, Carlsbad, CA). Twenty microliter samples were mixed in 200 μl PCR tubes with 250 nM sequence-specific primers. Thermal cycling was carried out on a Peltier Thermocycler (PTC-100, MJ Research).

Real-time PCR of the cDNA standards and samples was carried out in triplicate in 10 μl/well samples on a 384-well plate in a Taqman 7900 Real-Time PCR Machine (Applied Biosystems) and analyzed using SDS software (Applied Biosystems). The final reaction mixture ratio of the components was 5:1:1:3 (2X SYBRGreen real-time PCR reagent:forward primer:reverse primer:cDNA volume). The final concentration of the sample RNA was calculated using the calibration curve obtained via the cDNA standards. Each viral particle has two RNA copies, which enabled us to calculate the total number of M-VLPs in a given volume of supernatant. Two RNA extracts were collected for each M-VLP sample and quantified using three dilutions of each cDNA sample.

2.3. Assembly of polymer/M-VLP hybrid vectors

PEI (750 kDa, Sigma–Aldrich, 1 mg/ml in ultrapure water), PLL (150–300 kDa, Sigma–Aldrich, 1 mg/ml in ultrapure water) or chitosan (190–310 kDa, Sigma–Aldrich, 1 mg/ml dissolved overnight at 55 °C in 0.6% acetic acid and filtered through a 0.22 μm surfactant-free cellulose acetate syringe filter) was added drop-wise to the required volume of M-VLP supernatant while vortexing to achieve the desired polymer: M-VLP ratio. The hybrid vectors were then incubated at 4 °C for 4 h.

2.4. Transfections

HEK293 cells were seeded 18–24 h prior to transfection at 4×10^5 cells/well in 12-well plates. Growth media containing serum was replaced with serum-free DMEM prior to drop-wise addition of vectors and replaced again with normal growth media 4 h post-transfection. For serum studies, the transfection media contained 0–50% fetal bovine serum. For uptake inhibition studies, growth media was replaced with serum-free DMEM along with predetermined concentrations of drugs 1 h prior to addition of the hybrid vectors.

2.5. Luciferase expression assay

Luciferase expression was quantified 48 h post-transfection using the Promega luciferase assay system following the manufacturer's protocol. Luciferase activity was measured in relative light units (RLU) using a Lumat LB 9507 luminometer (Berthold, GmbH, Germany). Lysate protein concentration was then determined by BCA assay (Thermo Scientific) to standardize expression values.

2.6. Cellular uptake

Twenty milliliters of M-VLP supernatant was centrifuged at 60,000 *g* for 2 h at 4 °C and the pellet was resuspended in 50 mM HEPES with 145 mM NaCl to obtain a M-VLP density of 10^{10} M-VLPs/ml. 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD) (Invitrogen, 1 mM in DMSO, Ex = 644 nm, Em = 665 nm) was added to the resuspended M-VLP to give a final concentration of 2 μM DiD. The mixture was incubated for 1 h at room temperature. Labeled M-VLPs were separated from the free dye through a PD-10 gel filtration column (GE Healthcare). HEK293 cells were seeded in 12-well plates at 4×10^5 cells/well 18–24 h prior to transfection. Hybrid vectors composed of polymer and DiD-labeled M-VLP were assembled as described above and used immediately for transfection. The target cells were washed with PBS containing 0.001% SDS 2 h post-transfection to remove surface-bound, uninternalized vectors followed by a PBS wash. Cells were then trypsinized followed by neutralization with 50 μl of FBS, collected, and analyzed by flow cytometry using a Becton Dickinson LSR II Flow Cytometer with a 633 nm laser.

2.7. Negative stain transmission electron microscopy

M-VLP and χ/M-VLP vectors were fixed by adding 1 ml of Karnovsky's fixative to 3 ml of sample. The sample was then centrifuged at 60,000 *g* for 2 h at 4 °C. The pellet was resuspended in a small volume of 5% glucose and diluted with the fixative. A 40 μl drop of the sample was placed on a parafilm sheet, and a copper grid was placed face down on the sample drop and incubated for 30 min. Excess sample was wicked off the grid using filter paper. The grid was then placed grid-face down on a drop of 7% uranyl acetate stain for 1 min. Excess stain was again wicked off using filter paper and the grid was dried for 15 min before viewing in a Hitachi H600 Electron Microscope.

2.8. Size and zeta potential measurements

Vectors were prepared as described above and diluted 10-fold in 5% glucose. The sizes of the resulting complexes and the constituents were measured using dynamic light scattering with a Brookhaven 90Plus Particle Size Analyzer (Brookhaven Instruments). Light scattering was measured 10 times at 10-second intervals for each sample.