# Dual modal gene expression and silencing using viral/nonviral chimeric nanoparticles for synergistic gene therapy

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## ABSTRACT

Combining the advantages of virus (e.g., high transduction) with that of nonviral materials (e.g., low immunogenicity) is considered to be a logical approach to develop ideal gene delivery vector. In this context, adenoassociated virus (AAV) was shelled with an aciddegradable polyketal (PK) layer to produce viral/nonviral nanoparticles (ChNPs). chimeric ChNPs showed significantly enhanced transduction of human lymphoma B cells, compared with unmodified free AAVs even at low virus titer, while simultaneously silencing a target gene. As hypothesized, the gene delivery efficiency of the ChNPs was not affected by AAV-neutralizing antibodies. Confocal microscopy studies revealed facilitated nuclear translocation of the AAV core and efficient release of siRNA from the PK shell into the cytoplasm. Furthermore, ChNPs with pro-apoptotic AAV (Bim) core and prosurvival siRNA (MCL1) shell induced synergistic apoptosis in leukemia cells.

*Keywords*: adeno-associated virus, gene transfer, simultaneous expression and silencing, stimuli-responsive delivery, viral/nonviral chimeric nanoparticle

# **1 INTRODUCTION**

Development of efficient, safe, and versatile gene delivery carriers is a pivotal technological challenge in gene delivery. Viral vectors' superior gene delivery efficiency has been undermined by their major limitations such as high immunogenicity [1]. In addition, it is a daunting task to engineer viral vectors to carry multiple kinds of therapeutic and diagnostic molecules due to their limited flexibility for modifications. In contrast, nonviral (synthetic) vectors offer a high level of flexibility for structural and functional modifications but with relatively low gene delivery efficiency, particularly in vivo. Many attempts to improve the limitations of viral vectors by incorporating synthetic materials (e.g., polymers and peptides) have often led to significantly compromised gene delivery efficiency or minimally reduced/newly developed immunogenicity [2,3].

In this study, viral/nonviral chimeric nanoparticle (ChNP) was prepared by shielding an adeno-associated virus (AAV) with an acid-degradable polyketal (PK) shell. Incorporation of siRNA into shell allows co-delivery of viral DNA and siRNA for simultaneous expression and silencing. Co-delivery of AAV and siRNA was evidenced using reporter genes (GFP and fire-fly luciferase) and synergistic gene therapy was demonstrated using therapeutic genes (Bim and MCL1). ChNPs reported in this study can further be decorated with targeting moieties to achieve targeted and enhanced gene therapy.

# 2 RESULTS AND DISCUSSION

# 2.1 Design and Synthesis of ChNPs

ChNP was prepared using photopolymerization of ketal monomer. The PK shell was designed to 1) protect the AAV core from immune recognition, 2) assist the intracellular trafficking of the AAV (i.e., rapid endosomal release into the cytoplasm), and 3) encapsulate siRNA for simultaneous gene expression and silencing for synergistic gene therapy. In addition, the PK shell is a convenient platform for anchoring targeting and imaging moieties.



Figure 1: Core-shell structure of ChNPs and release of AAV core upon acid-hydrolysis.



Figure 2. DCBCL-Val/Luc cells incubated with free AAVs and ChNPs. (A) Simultaneous GFP transduction (bars) and Luc silencing (dots). (B) Relative viability of the cells after incubation with free AAVs and ChNPs. (C) GFP transduction by free AAVs and ChNPs in the presence of anti-AAV antibodies.

AAV core held advantages over other recombinant viruses owing to its small size (~ 20 nm), allowing high siRNA loading while maintaining overall sizes smaller than 200 nm for efficient utilization of EPR effect. AAV is also known for its low risk of mutagenesis and lysines on the surface of AAV capsids facilitate easy conjugation of initiators. Use of photo-initiator eosin-5-isothiocyanate allowed surface-initiated polymerization by visible light eliminating the need to use the more damaging UV light. DLS measurements determined ChNPs to be 165 nm in size which was in agreement with the size observed by TEM (Fig. 1).

Incubation of ChNPs at an endosomal pH of 5.0 indicated the presence of a core-shell structure. Release of AAV was observed in TEM. Complete AAV isolation (white arrows) as well as partial hydrolysis (yellow arrows) was observed. Further incubation of hydrolyzed ChNPs in AAV lysis buffer released viral ssDNA (data not shown). This stimuli-responsive release of siRNA and viral ssDNA support the hypothesis that siRNA is released into the cytoplasm upon endosomal escape and the released AAV core translocates to nucleus where capsid disassembly occurs and viral ssDNA is released.

# 2.2 Simultaneous Gene Expression and Silencing

Free AAVs and ChNPs were incubated with DLBCL-Val/Luc cells (Fig. 2). Free AAVs transduced cells in concentration-dependent manner. ChNPs showed either comparable or even higher transduction (~ 3-fold) efficiency especially in lower virus titer. This finding supports the hypothesis that PK layer mediates intracellular trafficking. ChNPs also showed simultaneous gene silencing in a concentration-dependent manner up to 50 % Luc silencing. Loading more siRNA in the shell is anticipated to increase the gene silencing efficiency. ChNPs with non-degradable cationic shell (ND-ChNPs) were also prepared as a control. ND-ChNPs showed minimal GFP transduction and Luc silencing suggesting the importance of acid-degradable layer (data not shown). ChNPs showed comparable or slightly reduced cell viability with overall cell viability higher than 70 %.

As hypothesized, the transduction efficiency of ChNPs was not compromised by neutralizing antibodies. While free AAV transduction was greatly reduced by increasing the concentration of anti-AAV antibodies, transduction efficiency of ChNPs was retained even in very high antibody concentrations (10  $\mu$ g/mL). This finding



Figure 3. Intracellular release and distribution of (A) free AAVs and (B) ChNPs incubated with DLBCL-Val/Luc cells. AAVs were labeled with Alexa Fluor 488 (green dots) and siRNA was labeled with Dye547 (red dots). Yellow dots indicate co-localization of AAV and siRNA in unhydrolyzed ChNPs.

furthersuggests complete encapsulation and protection of core AAVs by PK shells.

## 2.3 Efficient Intracellular Trafficking

AAVs labeled with Alexa Fluor 488 (green) and Dye547-siRNA (red) were used to prepare ChNPs for intracellular trafficking study (Fig. 3). Free AAVs and ChNPs were incubated with DLBCL-Val/Luc cells for 10 h followed by wash and a subsequent 14 h incubation. Free AAVs distribution inside the cell was not altered significantly from 10 h incubation to 24 h. Green dots were mainly found in the cytoplasm. Majority of ChNPs' AAVs and siRNA were found co-localized in unhydrolyzed ChNPs represented by yellow dots at 10 h. At 24 h, complete release of AAVs and siRNA was observed as indicated by separate green and red dots. More interestingly, while siRNA was found exclusively in the cytoplasm, significant portion of AAVs were found in the nucleus. This result, in combination with the in vitro studies in the previous section, strongly suggest that PK shell facilitates efficient endosomal escape leading to increased nuclear localization for enhanced transduction. Intracellular trafficking of ND-ChNPs further demonstrated the importance of acid-degradable PK layer as AAVs and siRNA were not released even after 24 h (data not shown).

#### 2.4 Synergistic Gene Therapy

Cancer cells often over-express a pro-survival gene while suppressing pro-apoptotic signals. Therefore, simultaneously promoting pro-apoptotic gene expression and silencing the expression of the pro-survival gene is a promising approach to synergistic cancer gene therapy. ChNPs with the pro-apoptotic Bim-encoding AAV core and the pro-survival MCL1 siRNA-encapsulating PK shell (ChNP\_Bim/MCL1, red line) was incubated with K562 cells to induce efficient apoptosis in leukemia cells (Fig. 4). Control ChNPs with Bim AAV and scrambled siRNA (ChNP\_Bim/scRNA, blue line), Null AAV and MCL1 siRNA (ChNP\_Null/MCL1, green line), and Null AAV and scrambled siRNA (ChNP\_Null/scRNA, purple line) were also prepared. Free Bim AAV (yellow line) and free MCL1 siRNA (orange line) were incubated independently as positive controls. Until day 5 of incubation, all conditions showed similar cell growth profile except for untreated samples (PBS, black line) and free Bim AAVs which showed higher cell growth. At day 7, total viable cell number of ChNP\_Bim/MCL1 was around 50 % of untreated cells suggesting efficient apoptosis induced synergistically by simultaneously expressing Bim and silencing MCL1.

#### **3 EXPERIMENTAL**

#### **3.1** Synthesis and Characterization of ChNPs

Eosin-5-isothiocyanate was conjugated on the AAV capsids by mixing  $7 \times 10^{11}$  genome copies of AAVs in 2.5 mL of 10 mM sodium bicarbonate buffer with 3 mg of eosin-5-isothiocyanate in 30 µL of DMSO. Un-conjugated eosin was purified by PD 10 size-exclusion column. Polymerization was initiated by strong light upon adding 10 mg of ascorbic acid in 50 uL 10 mM HEPES buffer, premix of 10 mg amino ketal methacrylamide monomer [4] and 3 ug of siRNA in 100  $\mu$ L 10 mM HEPES buffer to 2  $\times$ 10<sup>11</sup> gc of eosin-conjugated AAVs in 1 mL 10 mM HEPES buffer. After 10 min of polymerization, pre-mix of 10 mg monomer and 4 mg crosslinker in 100 µL 10 mM HEPES buffer was added and further polymerized for 5 min. ChNPs were purified by centrifugal filtration (MWCO 10 kDa) at 2100 g at 4 °C for 30 min twice. Resulting ChNPs were resuspended in 1mL DI water, deposited on a carbon-coated



Figure 4. Total viable cell numbers counted following incubation of K562 cells with PBS (black), free AAV-Bim (yellow), free siRNA-MCL1 (orange), ChNP\_Bim/MCL1 (red), ChNP\_Bim/scRNA (blue), ChNP\_Null/MCL1 (green), and ChNP\_Null/scRNA (purple).

grid, stained with uranyl acetate and then examined using a Phillips CM20 transmission electron microscope.

Free AAVs and ChNPs were incubated with DLBCL-Val/Luc cells at various concentrations. Cells were plated at a density of  $2 \times 10^4$  cells/well in a 24-well plate 24 h prior to transduction, incubated with nanoparticles for 6 h followed by fresh media exchange. GFP expression by cells was measured using a Guava EasyCyte flow cytometer and luciferase expression was analyzed by Steady-Glo<sup>®</sup> luciferase assay normalized by total protein amount determined by BCA assay. Cell viability was measured using conventional MTT assay at 24 h of incubation.

DLBCL-Val/Luc cells were pre-incubated with anti-AAV antibodies at various antibody concentration 1 h prior to nanoparticle incubation to assess transduction inhibition by neutralizing antibodies. Free AAVs and ChNPs were incubated for 6 h in the presence of antibodies, washed with PBS twice and further incubated for 3 days followed by flow cytometry analysis.

## 3.3 Intracellular Trafficking of ChNPs

AAVs labeled with Alexa Fluor 488 and Dye547siRNA were used to prepare fluorescently labeled ChNPs for intracellular trafficking. ChNPs were prepared according to the procedure described in section 3.1. Free AAVs and ChNPs were incubated with DLBCL-Val/Luc cells in a Falcon 8-well culture slide for 10 h. Cells for 10 h condition were washed, fixed with 2 % formaldehyde in PBS, washed again, stained with 5  $\mu$ M DRAQ5 solution in PBS and washed. Fixed cells were suspended in 50 % (v/v) glycerol in PBS to hinder the movement of cells while imaging. 24 h condition cells were further incubated for 14 h after initial wash at 10 h and fixed and stained. Fluorescent cell images were observed using a Fluoview 1000 confocal scanning microscopy setup.

### 3.2 Gene Transduction and Silencing

### **3.4** Therapeutic Efficacy

Free AAV-Bim, free siRNA-MCL1, ChNPs and PBS were incubated with K562 cells plated at density of  $5 \times 10^4$  cells/well in a 12-well pate. Cell suspension was aliquoted everyday following incubation, mixed with equal volume of trypan blue and counted using a microscope.

#### 4 CONCLUSION

ChNPs comprised of AAV core and siRNAencapsulating, acid-degradable PK shell was prepared to co-deliver DNA and siRNA. By encapsulating two types of nucleic acids into one delivery vehicle, simultaneous expression and silencing was achieved resulting in synergistic gene therapy. PK shell not only protected AAVs and siRNA from neutralizing antibodies and enzymatic degradation, but also facilitated efficient intracellular trafficking resulting in higher gene transduction. ChNPs developed in this study can also be further decorated with targeting and imaging moieties using free surface amines for targeted, controlled and efficient gene delivery.

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