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All-*trans*-retinoic acid (ATRA)-grafted polymeric gene carriers for nuclear translocation and cell growth control

Kyong Mi Park [a,1], Han Chang Kang [b,1], Jung Kyo Cho [a], Ik-Joo Chung [c], Sang-Hee Cho [c], You Han Bae [b], Kun Na [a,*]

[a] Department of Biotechnology, The Catholic University of Korea, 43-1 Yeokgok 2-dong, Wonmi-gu, Bucheon-si, Gyeonggi-do 420-743, Republic of Korea
[b] Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, 421 Wakara Way, Suite 318, Salt Lake City, UT 84108, United States
[c] Department of Hematology/Oncology, Chonnam National University Medical School and Chonnam National University Hwasun Hospital, Republic of Korea

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ABSTRACT

Polyethyleneimine (PEI)-g-All-trans-retinoic acid (ATRA) (designated as PRA) was synthesized as a gene carrier. ATRA at its low concentration is known to be linked to nuclear translocation and cell cycle control (either proliferation or growth arrest) depending on its binding protein in cells. The cytotoxicity of PRA conjugates was lower than that of PEI and was gradually reduced as increasing ATRA graft ratios. The resulting nanosized and positively charged PRA/pDNA complexes showed lower transfection efficiency than the PEI/pDNA complexes (N/P = 10) against NIH3T3 which is less sensitive to ATRA in cell growth and more sensitive HeLa cells. However, when a mixed gene complex of PEI and PRA was applied in an effort to reduce the ATRA contents, their NIH3T3 transfection evidenced effective nuclear translocation and induced 2- to 4-fold better transfection efficiency as compared with the PEI/pDNA complexes. When the PEI/pDNA complexes were utilized to transfect HeLa cells, free ATRA treatment reduced their cellular uptake and transfection efficiency. These findings show that the NIH3T3 cells against ATRA-mediated growth arrest would not damage the PRA-mediated transfection enhancement resulting from the facilitated nuclear translocation of polyplexes or pDNA. The more ATRA-sensitivity in growth arrest of HeLa cells would reduce the transfection efficiency of ATRA-incorporated polyplexes. The transfection capability of gene by newly synthesized PRA conjugates to cells is differentiated by their ATRA-sensitivity to nuclear translocation and cell growth control.

1. Introduction

Along with safety concerns regarding viral vectors such as immunogenicity, tumorigenicity, and mutagenesis [1,2], increasing interest in safer gene carriers such as polymeric gene carriers has prompted investigations of their chemical modifications for receptor-mediated cellular internalization, endosomal disruption, and facilitated nuclear import [3]. As asialoorosomucoid-grafted polylysines were initially reported in the targeting of asialoglycoprotein receptors on the plasma membranes of hepatocellular carcinoma in 1987 [4], a variety of cellular receptor–ligand (*e.g.*, transferrin receptor–transferrin [5], folate receptor–folate [6], and sulfonylurea receptor–sulfonylurea [7]) or antigen–antibody interactions have been investigated extensively. These specific interactions improve the transfection efficiency of delivered transgenes at the cells of interest due both to cell targetability and facilitated cellular internalization. After the polyplexes are internalized, the endolysosomal escape of polyplexes or transgenes is crucial for the prevention of transgene degradation from lysosomal enzymes and can be conducted by polymeric materials evidencing proton buffering or pH-modulated conformational changes (*e.g.*, polyethyleneimines (PEIs) [8], polyhistidines [9], poly(2-alkylacrylic acid) [10], and oligomeric sulfonamides [11]). In addition, for the facilitated nuclear import of polyplexes or transgenes released into the cytoplasm, nuclear localization signals (NLSs) have been assessed, because NLS can dilate nuclear pores and transport pharmaceutical cargos via the classical nuclear import pathway. However, NLS-linked gene carriers or transgenes have yielded controversial results in terms of transfection efficiency [12–17]. A few studies have noted dexamethasone (Dex)-conjugated transgenes or polymeric carriers for nuclear import of plasmid DNA (pDNA) and their use evidenced noticeable enhancements of transfection [18–21]. These effects could be mediated by the

* Corresponding author. Tel.: +82 2 2164 4832; fax: +82 2 2164 4865.
E-mail address: kna6997@catholic.ac.kr (K. Na).
[1] These authors contributed equally to this work.

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nuclear translocation of Dex–glucocorticoid receptor (GR) complexes, in addition to the Dex-induced dilation of nuclear pores to 60–140 nm in diameter [22,23].

Like the nuclear translocation of Dex-bound GRs, this study notes that all-*trans*-retinoic acid (ATRA) binds to specific cytosolic proteins (*e.g.*, cellular retinoic acid binding protein II (CRABP-II) and fatty acid binding protein 5 (FABP5)), after which the formed ligand–protein complexes translocate into the nucleus [24,25]. The nuclear translocation characteristic of ATRA-bound complexes could improve the bioavailability of pDNA in the nucleus when ATRA-incorporated polycations carry pDNA. In addition, ATRA inhibited or activated cell growth depending on its dose and exposure time, as well as cell type [26–30]. Schug et al. very recently revealed a possible reason for this—notably, that the balance between CRABP-II and FABP5 modulates the ATRA-activated cellular proliferation or apoptosis (*i.e.*, cell growth control) [24]. Thus, this study newly synthesizes ATRA-conjugated PEI and attempts to determine whether the polymer improves the nuclear translocation of delivered pDNA. Additionally, we attempted to understand how ATRA-induced changes in cell growth affect transfection efficiency.

2. Materials and methods

2.1. Materials

Branched PEI (MW: 25 kDa), ATRA, thiazolyl blue tetrazolium bromide (MTT), 1,3-dicyclohexylcarbodiimide (DCC), and *N*-hydroxysuccinimide (HoSu) were purchased from the Sigma Chemical Company (St. Louis, MO). YOYO-1 was bought from Invitrogen, Inc (Carlsbad, CA). Plasmid pGL3 encoding for the firefly luciferase gene (pDNA) was isolated and purified using QIAfilter Midi Kits (Qiagen, Hilden, Germany). The luciferase assay kit was purchased from Promega (Madison, WI).

2.2. Synthesis and characterization of PEI-g-ATRA (PRA) conjugates

The PRA conjugates were synthesized by a conventional condensation reaction between the primary amines of PEI and a carboxyl acid of ATRA, as shown in Fig. 1. In brief, PEI (0.04 mmol) and a mixture of ATRA (*e.g.*, 0.17 mmol), DCC (1.2× ATRA in moles), and HoSu (1.2× ATRA in moles) were dissolved separately in dimethyl sulfoxide (DMSO) (20 mL) and the solutions were stirred thoroughly for 3 h prior to the condensation reaction. Two reactant solutions were mixed and stirred at room temperature. After 24 h, the reaction solution was filtered to remove insoluble by-products (*e.g.*, dicyclohexylurea) and the filtrate was dialyzed using a dialysis membrane (MWCO: 3500 Da) against deionized water for 2 d. The final products (PRA conjugates) were lyophilized. PRA synthesis was verified by ^{1}H NMR and IR spectra. The ATRA contents in the PRA conjugates were measured by UV–vis spectroscopy.

2.3. Cytotoxicity of PRA conjugates

PRA-mediated cytotoxicity was evaluated via MTT-based assays and compared with PEI as a control polymer. Mouse embryonic fibroblast NIH3T3 cells were seeded in 96-well plates at a density of 3.0×10^4 cells/well (0.1 mL of culture medium) and were cultured for 24 h in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics under humidified air containing 5% CO_2 at 37 °C. After adding PRA conjugates, the cells were incubated for an additional 24 h. Then, MTT solution (20 μL; 0.5 mg/mL) was added to the cells. After 4 h incubation, MTT-containing medium was removed and the formazan crystals produced by living cells were dissolved by DMSO (150 μL). The absorbance of the formazan crystals was measured at 595 nm. Cell viability (%) was calculated via the following equation:

$$\text{Cell Viability } (\%) = \frac{\text{Abs}_{\text{Sample}} - \text{Abs}_{\text{DMSO}}}{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{DMSO}}} \times 100$$

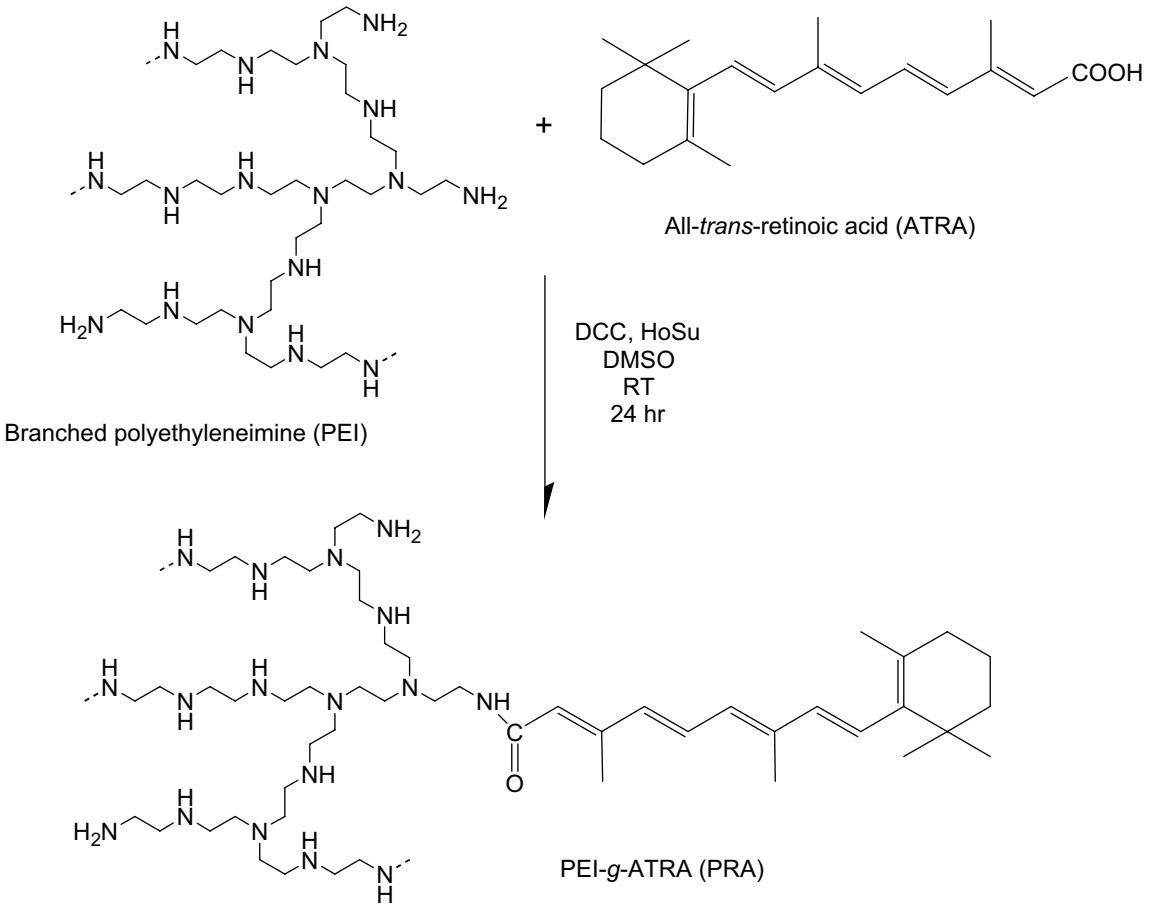

Fig. 1. Synthetic scheme of PEI-g-ATRA (PRA).