In vitro Gene Delivery to HepG2 Cells with a Novel Galactosylated Polyornithine

Heebeom Koo, Geun-woo Jin, and Jong-Sang Park*

School of Chemistry & Molecular Engineering, Seoul National University, Seoul 151-747, Korea
**E-mail: pfjspark@plaza.snu.ac.kr*
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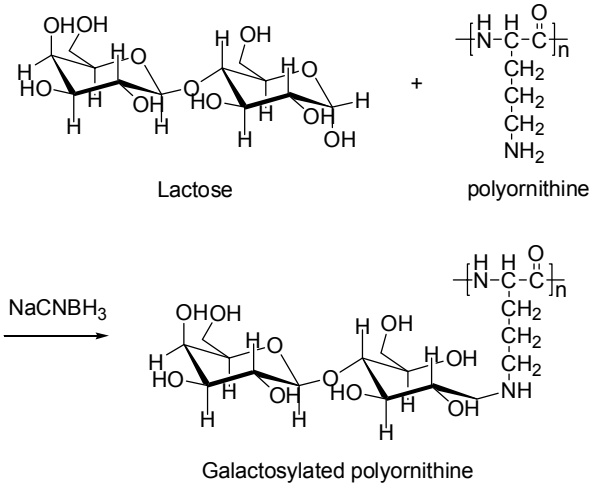
Various non-viral gene delivery carriers including cationic lipids,[1] polypeptides,[2] and polymers[3] have been synthesized as alternatives to viral vectors, because they have many advantages such as low immune response and easy large-scale production. Among these non-viral gene carriers, using natural or artificial polypeptides such as polylysine (PLL),[4] polyarginine,[5] polyhistidine[6] and polyornithine (PO)[7] is considered as promising approach. Polylysine is one of the first gene transfer polymers to be used, but polyornithine is more effective in the gene transfer into mammalian cells *in vitro*.[8]

To enhance the transfection efficiency of these carriers to a specific cell, a targeting moiety against a specific receptor in cell membrane has been bound to various gene carriers to deliver plasmid DNA by a receptor-mediated endocytosis. For instance, galactose have been modified to PLL,[9] chitosan,[10] and PEI[11] for the selective delivery of plasmid DNA to hepatocytes having specific galactose-binding receptors in their cell membrane, asialoglycoprotein receptor. From this point of view, we reported how to improve the transfection efficiency of polyornithine to HepG2 cells by galactosylation in this paper.



Scheme 1. Synthetic scheme of galactosylated polyornithine.

The galactose modification of primary amines in polyornithine is performed by reductive amination with lactose according to the literature (Scheme 1).[12] The ratio between polyornithine and lactose determines the degree of galactosylation and sodium cyanoborohydride was used as the complex metal hydride in this reaction. The reaction was confirmed by ^{1}H-NMR (supporting information) and multi angle laser light scattering (MALLS) (Table 1). The increased molecular weight means successive modification of the polymers with lactose. The degree of modification was calculated from the amount of molecular weight increase in MALLS data considering the molecular weight of lactose and ornithine unit. The molecular weights of polymers are between 10 kDa and 25 kDa, the range of the molecular weights suitable for gene delivery.

Because the electrostatic polyplex formation between cationic polymers and anionic DNA is necessary for transfection, the polyplex formation ability of polyornithine and galactosylated polyornithines were compared each other using agarose gel electrophoresis assay (Figure 1).[13] The numbers in the figure represent the weight ratios between cationic polymer/plasmid DNA. DNA migration disappeared when unmodified polyornithine was mixed at weight ratios of 2 and above. This disappearance means that plasmid DNA completely forms polyplex with polymer. In case of galactosylated polyornithine, the mobility of DNA with polymer was retarded at weight ratio around 3 or 4, and it shows the complex formation abilities of galactosylated polyornithines are slightly weaker than that of initial unmodified polyornithine. It is due to the binding of lactose to primary amine groups of polyornithine and resulting decrease of amine density.

The transfection on the human hepatoblastoma HepG2 cell line was performed with the polymer-DNA complex (Figure 2).[14] The transfection increase by galactosylation is remarkable in HepG2 cell line and further modification increased trans-

Table 1. The molecular weight measurement of polyornithine and galactosylated polyornithines.

	M_n	M_w	PD (M_w/M_n)	Modification (%)
Polyornithine	14420	15740	1.092	
Glactosylated polyornithine 1	16530	19220	1.163	14%
Glactosylated polyornithine 2	20050	22140	1.104	26%

Modification (%) means the percentage of modified amine/total amine.

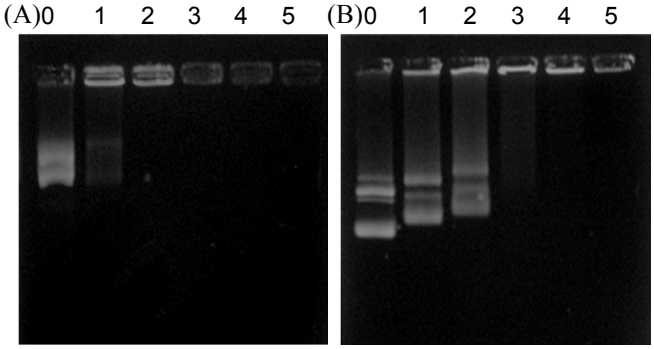


Figure 1. Agarose gel retardation assay of plasmid DNA and unmodified polyornithine (A) and galactosylated polyornithine (B). The number represents weight ratios (polymer/DNA).

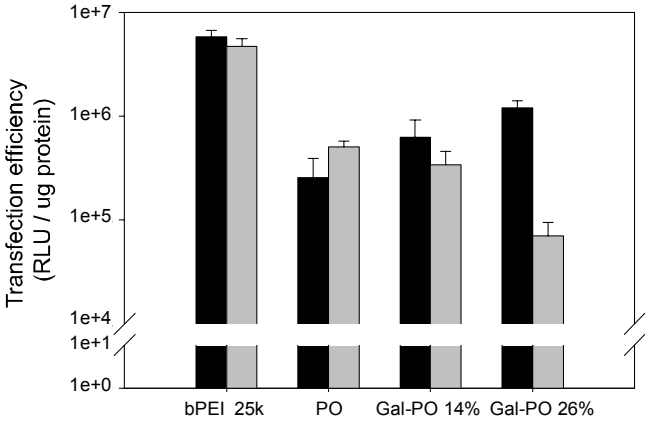


Figure 2. The comparison of the transfection efficiency on HepG2 (balck bars) and NIH3T3 (gray bars) cells between branched PEI 25k, unmodified polyornithine (PO), and galactosylated polyornithine (Gal-PO) at their optimum ratios. Branched PEI 25k was used as a positive control gene carrier. Data were expressed as mean standard (n = 3).

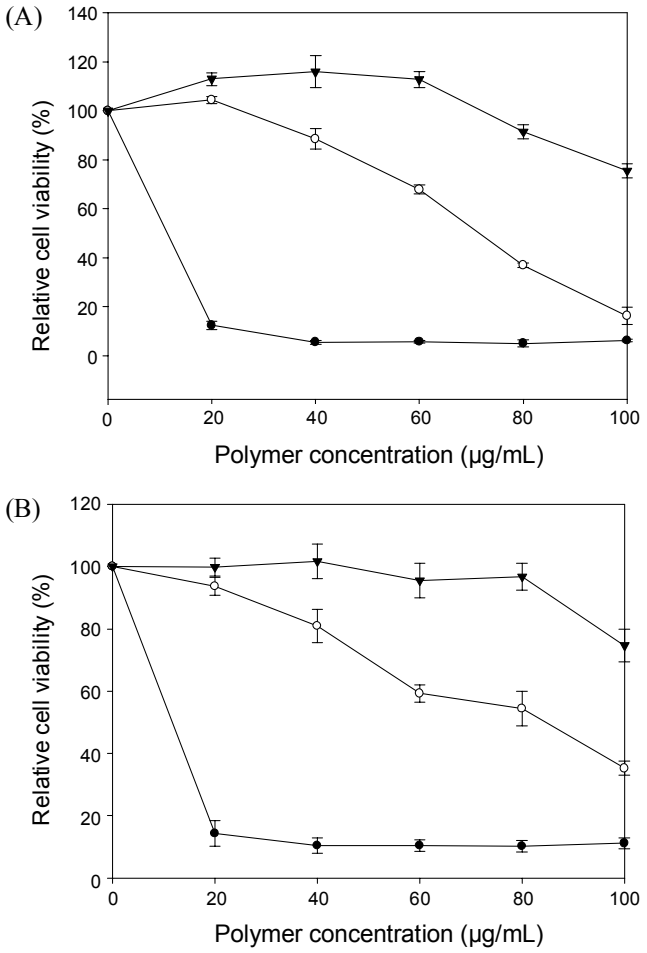


Figure 3. The cytotoxicity on HepG2 (A) and NIH3T3 (B) cells of branched PEI 25 kDa (●), polyornithine (○), and galactosylated polyornithine 26% (▼). Branched PEI 25k was used as a negative control. Data were expressed as mean standard (n = 5).

fection efficiency more (black bars). Galactosylated polyornithine 14% showed about three-fold higher transfection efficiency than unmodified polyornithine. Moreover, the transfection efficiency of galactosylated polyornithine 26% was about five times higher than that of unmodified polyornithine. It certainly showed that the more galactoses were modified, the higher transfection efficiency is achieved. Next, the change of transfection efficiency by galactosylation was also observed in mouse embryo fibroblast NIH3T3 cell line lacking asialoglycoproptein receptor (gray bars). The transfection efficiency of galactosylated polyornithine was similar with unmodified polyornithine (in case of 14%) and even decreased to about tenth (in case of 26%) in NIH3T3. This opposite results supported that the increased transfection efficiency of galactosylated polyornithine in HepG2 cells was because of receptor-mediated pathway. In case of non-hepatoma cell lines lacking asialoglycoproptein receptor, the large galactosyl coat would be obstacle in binding with plasmid DNA and transfection for loss of cationic charge density.

Finally, the cytotoxicities of polyornithine polymers were compared according to the galactosylation by MTT assay (Figure 3).[15] Branched PEI 25k was used as negative control. The polyornithine showed cytotoxicity at high concentration, but it is much lower than PEI 25k. The IC_{50} value of unmodified polyornithine is about 70 μg/mL in HepG2 (A) and 85 μg/mL in NIH3T3 (B). But galactosylated polyornithine showed very lower cytotoxicity than unmodified. Even at 100 μg/mL, more than 70% of cells were alive in both cell lines. This may be attributed to the masking effect of cationic charges in the polymer against cells by large galactose. Therefore, the introduction of galactose units binded to primary amines lowered the overall cytotoxicity of polyornithine used for gene carriers.

In summary, we synthesized galactosylated polyornithines with various degree of modification for gene delivery systems. This galactosylated polyornithine showed slightly weaker DNA-condensing ability than unmodified polyornithine in agarose gel electrophoresis assay. Nevertheless they showed highly enhanced transfection efficiency compared to unmodified polyornithine in HepG2 cell line, while showed similar or lower efficiency in NIH3T3 cell line. This result showed the possibility of galactosylated polyornithone as a receptor mediated gene delivery carrier to specific cell. Moreover, the galactosylation of polyornithine highly reduced its original cytotoxicity in both HepG2 and NIH3T3 cell lines. Therefore

galactosylated polyornithine could have great potential as a non-viral gene delivery carrier into hepatoma cells with high transfection efficiency and low cytotoxicity.

Experimental Section

The Synthesis of galactosuylated polyornithine. Polyornithine (8.018 mg; 0.5 μmol) and the prescribed amount of lactose were dissolved in 10 mL of 0.1 M sodium tetraborate buffer at pH 9.0. After the addition of sodium cyanoborohydride (5 equiv to lactose), the mixture was reacted for 2 days at 40 °C with stirring. The product was purified by dialyzing against distilled, deionized water (MW cutoff, 10000) and then freeze-dried.

Molecular weight measurement of polymers by MALLS. Each molecular weight was determined by MALLS in combination with size-exclusion chromatography (SEC).[16] The SEC system included a P680 HPLC pump from Dionex Corporation (USA). Polymer samples were detected by a three-angle laser-light-scattering detector (miniDAWN Tristar, 30 mW GaAs laser, 690 nm, K5 cell) and an interferometric refractometer (Optilab DSP, P10 cell) from Wyatt Technologies (USA). A 1% formic acid aqueous solution was used as an eluent after filtration through a 22-nm filter and degassing. The d*n*/d*c* value for each polymer was also measured by the same interferometric refractometer.

Transfection assay. HepG2 and NIH3T3 cells were seeded at a density of 3×10^4 cells/well in 24-well plates with 600 μL of Dulbecco's modified Eagle's medium (DMEM) (Cambrex Bio Science, USA) containing 10% Fatal Bovine Serum (FBS) (GIBCO, USA) and grown to 70-80% confluence for one day. Before transfection, the medium was exchanged with 480 μL of FBS free DMEM. The cells in each well was treated with 120 μL of PBS solution containing 1 μg of pCN-Luci plasmid DNA at various weight ratios for 4 h at 37 °C.[16] Following 4 h-treatment of polyplexes, the medium was replaced by 600 μL of fresh medium containing 10% FBS. After an additional incubation for 2 days, the growth medium was removed. The cells were rinsed with 240 μL of phosphate buffered saline (PBS) and lysed for 30 min at room temperature by using 120 μL of reporter lysis buffer (Promega, USA). The luciferase activity was measured using a LB 9507 luminometer (Berthold, Germany), and the protein content was measured by using a Micro BCA assay reagent kit (Pierce, Rockford, IL).

Agarose gel electrophoresis. Complexes were prepared at various weight ratios between the polymer and the pCN-Luci plasmid. The complexes were formed by incubation in HEPES buffer (25 mM HEPES, pH 7.4, 10 mM $MgCl_2$) at room temperature for 30 min. Each sample was then electrophoresed on a 0.7% (w/v) agarose gel and stained in a buffer containing ethidium bromide (EtBr, 0.5 μg/mL) at 37 °C for 1 h. The location of the DNA was analyzed on a UV illuminator.

Cytotoxicity assay. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was used to measure cytotoxicity. HepG2 and NIH3T3 cells were seeded in 96-well tissue culture plates at a density of 8×10^3 cells/well in 90 μL DMEM medium containing 10% FBS. The cells achieving 70-80% confluence after 24 h were exposed to 10 μL of various concentrations of the polymer solutions. After 2 days, 26 μL of the solution of MTT (Sigma, USA) (2 mg/mL in PBS) was added to each well. After an additional 4 h-incubation at 37 °C, the media was removed, and the resulting formazan was dissolved with 150 μL of dimethylsulfoxide (DMSO). The absorbance was measured at 570 nm by using a microplate reader (Molecular Devices Company, USA). The relative cell viability was calculated as a percent absorbance to untreated control cells.

Supporting Information. NMR data is available on request to the corresponding author.

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