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In vitro gene delivery to HepG2 cells using galactosylated 6-amino-6-deoxychitosan as a DNA carrier

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Abstract—A chitosan derivative, 6-amino-6-deoxy chitosan (6ACT), was galactosylated and was investigated as a gene carrier. A series of galactose-modified 6ACT (Gal-6ACT) with degrees of substitution (d.s.) ranging from 3% to 50% per pyranose were prepared by reductive alkylation with lactose. DNA retardation assays showed that the electrostatic interaction between Gal-6ACT and plasmid DNA was not changed by galactose modification up to 50% per pyranose of 6ACT. Gal-6ACT with a d.s. of 38% was bound to galactose-recognizing lectin, RCA120. A significant increase in transfection efficiency for HepG2 cells was observed at degree of substitutions ranging from 18% to 50% and at N/P values ranging from 1.5 to 2.5. Under optimum conditions, Gal-6ACT showed about 10 times higher efficiency than 6ACT. However, a slight uptake by the galactose receptors on hepatocytes was observed by flow cytometric analysis. Moreover, Gal-6ACT with a d.s. of 38% mediated efficient gene transfer into both A549 and HeLa cells lacking the galactose receptor. These results suggest that the enhancement of transfection efficiency of Gal-6ACT was not due to the increase of receptor-mediated cellular uptake. In addition, the enhanced gene transfer efficiency was not specific to the galactose modification because the efficiency of glucose-modified 6ACT for HepG2 cells was similar as that of Gal-6ACT.

Keywords: Chitin; Chitosan; Drug delivery; Gene transfer; In vitro test

1. Introduction

Chitosan is a biocompatible, biodegradable polymer of low cytotoxicity that is obtained by deacetylation of chitin, a naturally abundant polysaccharide.[1,2] Chitosan has primary amino groups and spontaneously forms complexes with nucleic acids by electrostatic interaction. Therefore, chitosan has been investigated as a non-viral gene carrier[3–6] as well as other cationic polyelectrolytes, that is, polyethylenimine (PEI)[7,8] and poly-L-lysine (PLL).[9] Previous studies have suggested the potential of chitosan-based gene delivery, although its transfection efficiency was insufficient. To increase the efficiency, numerous chemical modifications of chitosan have been carried out.[10–19] For instance, trimethylation[10] of and hydrophilic polymer graft[11] on the chitosan backbone were carried out to provide solubility under physiological conditions. Glycosides,[12–14] folate,[15] and transferrin[16] were linked to bind with their receptors on cell surfaces. Urocanic acid-modified chitosan was reported to rupture endosomal vesicles through the proton sponge mechanism.[17] Furthermore, hydrophobic modifications with deoxycholic acid[18] or alkyl groups[19] enabled these derivatives to control the particle size by self-assembly of substituents in aqueous media.

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Among various modifications of chitosan, sugar modifications present an especially attractive course to efficient gene delivery. First, cell-specificity can be provided to the gene carrier through receptor-mediated recognition.[20,21] Second, recent studies indicate sugar-dependent intracellular trafficking[12,22] and nuclear import.[23] Third, sugar moieties are chemically stable compared with peptides and proteins.

We have recently reported 6-amino-6-deoxy chitosan (6ACT) as a novel chitosan-based gene carrier.[24,25] The transfection efficiency of 6ACT for COS-1 cells was 2.5-fold higher than that with chitosan, however, it was only 25% of that found with the PEI complex. In the present study, 6ACT was coupled with lactose by reductive alkylation, aiming to enhance gene transfer efficiency in hepatocytes expressing the galactose-recognizing asialoglycoprotein receptor (ASGP-R).

2. Results and discussion

Galactose modification of 6ACT was carried out by reductive alkylation with lactose according to the literature (Scheme 1).[26] To evaluate the binding of the galactose moieties on Gal-6ACT to galactose-recognizing lectins, aggregation of Gal38-6ACT induced by *Ricinus communis* agglutinin I (RCA120) was investigated (Fig. 1) by measurement of turbidity. In a mixture solution with RCA120, Gal38-6ACT showed markedly increased turbidity. The aggregation was rapidly dissociated with addition of excess lactose. On the other hand, unmodified 6ACT showed little interaction with lectin. These results indicated galactose-specific binding of Gal38-6ACT with RCA120.

The cytotoxicity of Gal38-6ACT and unmodified 6ACT for HepG2 cells was evaluated by a WST assay. Cell viabilities are represented as a percentage of living cells compared to the untreated control cells. The viabilities after exposure to Gal38-6ACT and unmodified 6ACT at 150 μg/mL were 114% and 92%, respectively (Fig. 2). The cytotoxicity of Gal38-6ACT was reduced in comparison with that of 6ACT, although none of the polyelectrolyes under examination showed high cytotoxicity.

An agarose gel retardation assay was carried out to estimate Gal-6ACT/pCMV-Luc complex formation. DNA migration disappeared when unmodified 6ACT was mixed at N/P ratios of 2 and above. This disappearance indicates that plasmid DNA completely forms the polyelectrolyte complex (Fig. 3). The same migration pattern was observed in mixing with Gal-6ACTs (d.s. of 18%, 38%, and 50%). These results indicated that interaction of Gal-6ACT with plasmid DNA was not affected by galactose modification with d.s. up to 50%. No inhibition of electrostatic interactions caused by the steric hindrance of modified-galactose moieties was found.

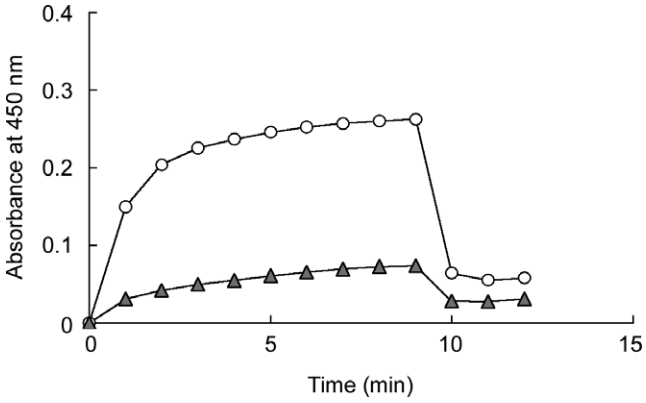
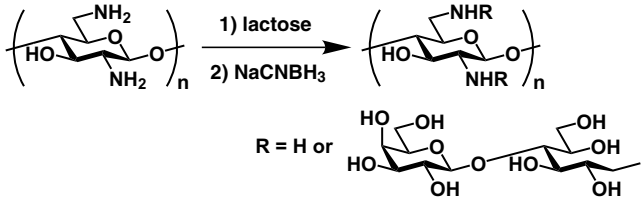


Figure 1. RCA120-induced aggregation of Gal38-ACT (open circle) and unmodified 6ACT (shaded triangle). The absorbance at 450 nm was continuously measured at every 9 min, then lactose was added to the mixture.



Product code	Lactose eq/pyranose	D.s./pyranose (%)
Gal03-6ACT	0.05	3
Gal18-6ACT	0.2	18
Gal25-6ACT	0.3	25
Gal38-6ACT	0.5	38
Gal50-6ACT	1.0	50

Scheme 1. Synthetic scheme of Gal-6ACTs.

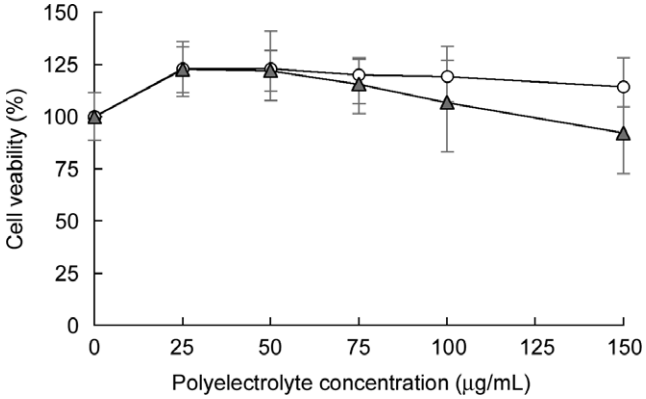


Figure 2. Evaluation of cytotoxicity of Gal38-6ACT (open circle) and unmodified 6ACT (shaded triangle) on HepG2 cells by WST assay. The cells were exposed with various concentrations of the cationic polyelectrolytes for 3 h and their viability was measured after 24 h.

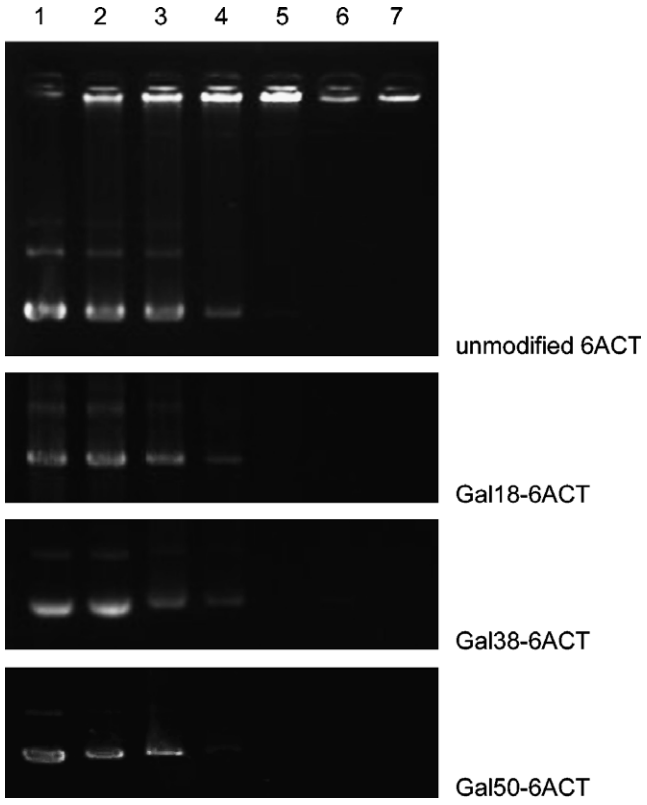


Figure 3. Agarose gel electrophoresis of a mixture of pCMV-Luc and 6ACT and Gal-6ACTs with d.s. of 18%, 38%, and 50% (top to bottom). Lanes 1–7: The mixtures of polyelectrolyte and pCMV-Luc in the N/P ratios 0, 1, 1.5, 2, 2.5, 3, and 3.5. Each lane contained 0.5 μg of pCMV-Luc.

The in vitro transfection efficiency of Gal-6ACTs (d.s. of 3%, 18%, 25%, 38%, and 50%) for HepG2 cells was investigated by the luciferase reporter gene assay (Fig. 4). The luciferase expression level was represented as relative light unit (RLU) normalized to a percentage of control PEI's value. PEI and unmodified 6ACT showed similar gene transfer efficiencies for HepG2 cells. A remarkable increase in efficiency was observed in transfection mediated by Gal-6ACTs with d.s. ranging from 18% to 50%. The maximum RLU value of Gal38-6ACT was 10-fold higher than that of PEI. Likewise, transfection efficiencies of Gal-6ACT with d. s. of 18%, 25%, and 50% increased 5- to 7-fold over PEI. Only a slight increase was observed for the Gal03-6ACT derivative (data not shown). Optimum N/P ratios for gene transfer with these Gal-6ACTs were found to be 1.5–2.5 with some differences between each of the optimal conditions. The gene transfer efficiencies decreased with increasing N/P ratios in a range up to 20 (data not shown).

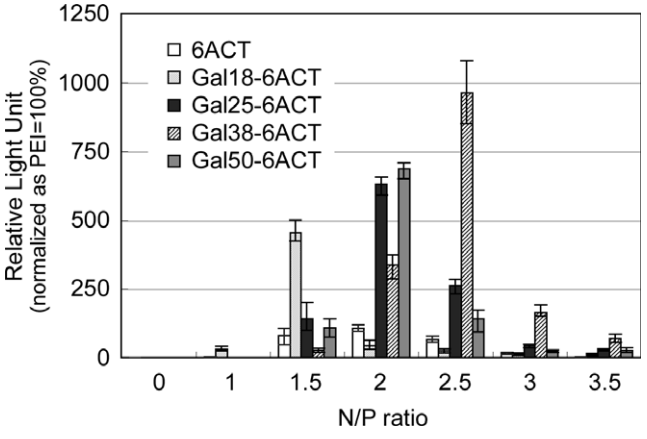


Figure 4. Relative transfection efficiency of Gal-6ACT/pCMV-Luc for HepG2 cells. Luciferase activity level is represented as relative light unit (RLU) and was normalized against PEI.

To estimate the internalization efficiency of the galactose-modified polyplexes, the complexes internalized into HepG2 cells were quantified by flow cytometry (Fig. 5). Gal38-6ACT complexes were prepared at N/P = 2.5 with a fluorescein-labeled pCMV-Luc as a fluorescent probe. HepG2 cells were exposed to the complexes in the same manner as transfection. The fluorescence of complexes adhered on the cellular surface was quenched by trypan blue to perform exact quantification of inter-

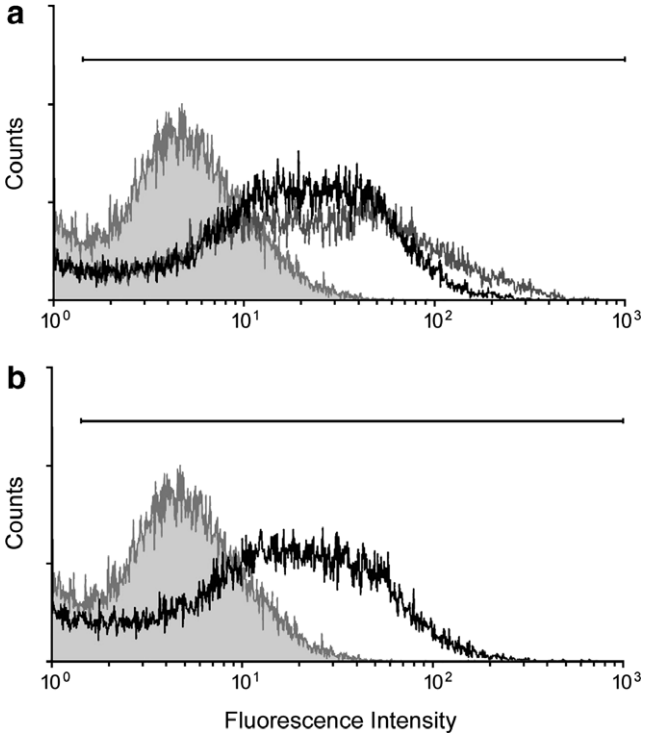


Figure 5. Flow-cytometric analysis of cellular uptake of complexes. The histograms show the proportion of fluorescein-labeled complexes internalized into HepG2 cells. A total of 20,000 cells in the gated area were counted. (a) Internalization of complexes prepared with Gal38-6ACT (black line) and unmodified 6ACT (gray line). Shaded histogram means a negative control group. (b) Internalization of Gal38-6ACT complexes in the presence of 16 mM lactose.

nalized complexes.[27] The cellular uptake level of Gal38-6ACT complexes was 81% of that of unmodified 6ACT complexes (Fig. 5a). Contrary to expectation, this result indicated that enhanced transfection efficiency of Gal-6ACTs was not due to the increase of cellular uptake. Moreover, inhibition assays revealed that the internalized amounts of the Gal38-6ACT complex decreased only 10% in the presence of 16 mM lactose (Fig. 5b). In previous reports, the transfection efficiency of galactose-modified carriers for hepatocytes significantly decreased in the presence of such inhibitors as lactobionic acid or galactose, indicating galactose receptor-mediated internalization.[28,13]

The unexpected results in the present study can be explained based on the particle sizes of the Gal-6ACT complexes. For instance, Gal25-6ACT/pCMV-Luc formed large aggregates under the optimal N/P ratio for gene transfer, because the complexes were electrostatically neutralized and showed insufficient charge repulsions under the conditions (Fig. 6). In water, the average diameter of the complexes reached nearly 2 μm at N/P = 2, whereas it was 130–270 nm at the N/P ratios from 2.5 to 5. Furthermore, swelling of the complexes and secondary aggregation caused by an increase in ion strength were observed in PBS. Average particle sizes increased to around 1 μm at the N/P ratios ranging from 1.5 to 5. Because an upper size limit for the clathrin-mediated internalization has been reported,[29] the large aggregates of Gal-6ACT/pCMV-Luc were excluded from cellular uptake through the clathrin-mediated pathway, even though galactose moieties of Gal-6ACT were recognized by ASGP-R. Gal-6ACT complexes seemed to be internalized into cells via electrostatic adhesion to the cell surface as well as unmodified 6ACT. The complex surfaces are electrostatically heterogeneous and the partial positive charges are sufficient for interactions with cell membranes, although the zeta potential of the complexes prepared at N/P = 2 was −6.0 mV in PBS (Fig. 6b).

Moreover, the increase of transfection efficiency by Gal38-6ACT was also observed in cell lines lacking ASGP-R. Gal38-6ACT showed 3.1-fold higher luciferase expression levels than that of unmodified 6ACT for both A549 and HeLa cells, respectively (Fig. 7). The results supported that internalization through ASGP-R has not significantly contributed to the increase in transfection efficiency of Gal-6ACTs.

The increase in transfection efficiency of Gal-6ACT complexes therefore seems to be related to intracellular trafficking. Hashimoto et al. indicated that the galactose modification of chitosan facilitated intracellular trafficking.[12] It was also reported that the escape of lactose-conjugated PLL/DNA complexes from endosomes/lysosomes was rapid compared with mannose-conjugated complexes.[22] The increase of transfection efficiency of sugar-6ACTs was probably based on a similar mechanism. To examine whether galactose moieties of Gal-6ACT play a specific role in the trafficking, glucose-modified 6ACT (Glu32-6ACT) was prepared and its transfection efficiency for HepG2 cells was compared with that of unmodified 6ACT. As shown in Figure 8, the efficiency of Glu32-6ACT-mediated gene

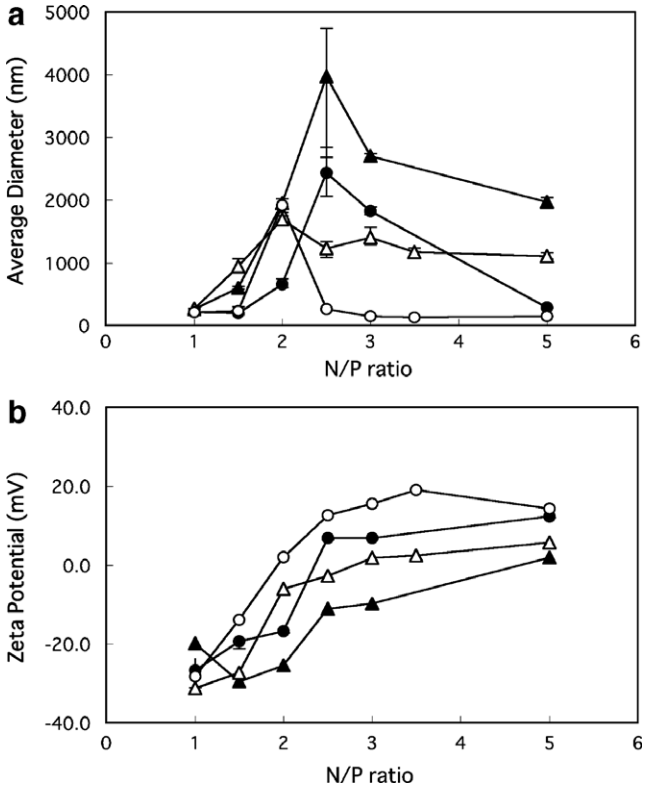


Figure 6. Average particle sizes (a) and zeta potential (b) of Gal25-6ACT (open symbol) and unmodified 6ACT (shaded symbol) complexes with pCMV-Luc in water (circle) and PBS (triangle) at 25 °C.

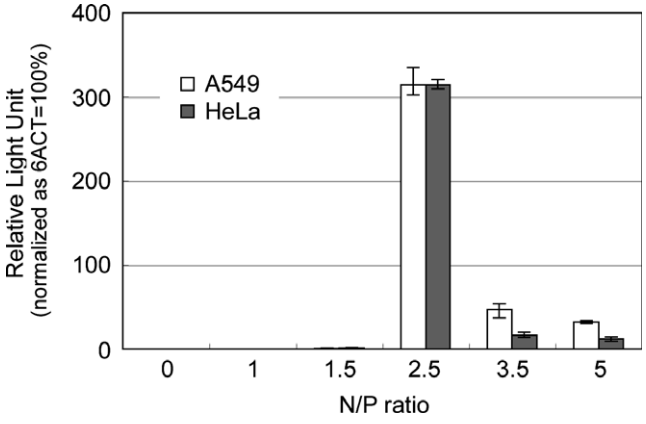


Figure 7. Relative transfection efficiency of Gal38-6ACT/pCMV-Luc for A549 and HeLa cells (open bar and shaded bar, respectively). Luciferase activity level was represented as relative light unit (RLU) and was normalized against unmodified 6ACT (N/P = 2.5).

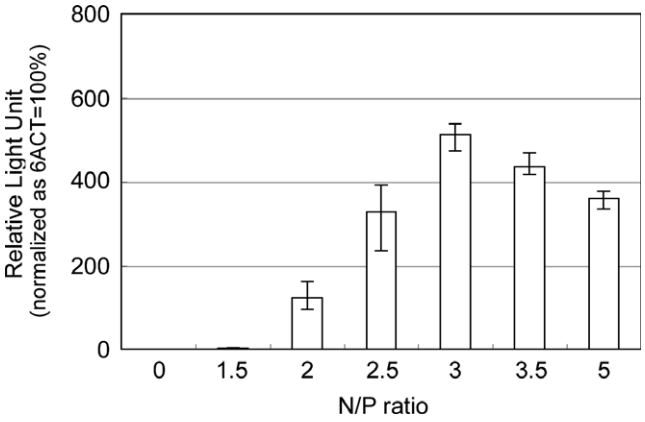


Figure 8. Relative transfection efficiency of Glu32-6ACT/pCMV-Luc for HepG2 cells. Luciferase activity level was represented as relative light unit (RLU) and was normalized against unmodified 6ACT (N/P = 2.5).

transfer was fivefold higher than the unmodified 6ACT. The results suggest that the increase of gene transfer by glycosylated 6ACT was not galactose dependent.

The influence of sugar modification on another process, nuclear import, is not clear. Roche et al. recently reported sugar-dependent and classical NLS-independent nuclear import using glycosylated BSA.[23] The nuclear import efficiency was found to depend on the structure of the sugar. In the transfection of airway epithelial cells, Klink et al. pointed out that lactose-modified PLL demonstrated efficient nuclear import.[30] Moreover, Fajac and co-workers revealed that gene expression efficiency was not directly related to sugar-dependent cellular uptake.[31] These reports suggest that the sugar modification affects intracellular trafficking. However, it is also reported that lactose moieties linked onto PEI could not function as a nuclear localization signal.[32] The role of glycosylation of polymeric gene carriers in the nuclear localization process has not been established.

In conclusion, Gal-6ACT with d.s. up to 50% was prepared and investigated as a gene carrier. Gal38-6ACT was bound to RCA120 in a galactose-specific manner. The transfection efficiency of Gal-6ACTs for HepG2 cells increased through the range of d.s. from 18% to 50%. However, ASGP-R on HepG2 cells hardly contributed to cellular uptake of Gal-6ACT complexes, and the amount of complexes internalized into cells did not increase. Aggregated Gal-6ACT complexes were likely excluded from clathrin-mediated endocytosis due to size limitations. The increase of transfection efficiency of Gal-6ACT was therefore likely due to improvements in intracellular trafficking. The increase of transfection efficiency by Gal-6ACT was observed in A549 and HeLa cells, supporting this hypothesis. Moreover, Glu-6ACT showed similar gene transfer efficiency as Gal-6ACT for HepG2 cells. These results suggest that the sugar moiety linked on 6ACT backbone affects the intercellular transport besides ligand–receptor interactions on the cell surface.

3. Experimental

3.1. General

The preparation of 6ACT was described in a previous paper.[24] The average molecular weight (MW) of 6ACT, as determined by static light scattering, was 36 and 42 kDa. The later 6ACT was only used to prepare glucosylated derivatives. PEI with an average molecular weight of 25 kDa was purchased from Aldrich (St. Louis, MO). Dulbecco's modified Eagle medium (DMEM) was purchased from Nissui Pharmaceutical (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Gibco BRL. *R. communis* agglutinin I (RCA120) was purchased from Vector Laboratories (Burlingame, CA). Other reagents and solvents were purchased from Aldrich or Wako (Osaka, Japan) and used as received, except as noted. Elemental analysis was performed at The Faculty of Science, Osaka City University. pCMV-Luc plasmid DNA encoding firefly luciferase was kindly provided by Professor Hashida from Kyoto University. Fluorescein-labeled pCMV-Luc was prepared using Label IT fluorescein Labeling Kit (Mirus Bio, Madison, WI) according to the manufacturer's instructions. HepG2 (human hepatocellular carcinoma), A549 (human lung carcinoma), and HeLa (human cervix epithelial carcinoma) cells were cultured in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 mg/L streptomycin in an incubator supplied with 5% CO_2 at 37 °C.

3.2. Preparation of sugar-modified 6ACTs

The typical procedure used for the preparation of sugar-modified 6ACTs was as follows: The acetate salt of 6ACT (10 mg, 0.031 mmol of pyranose unit) and the prescribed amount of lactose were mixed in 1% lactic acid (1 mL). The reaction mixture was stirred for 1 h at room temperature (rt). After addition of sodium cyanoborohydride (1.2 equiv to lactose), the mixture was further stirred for 24 h at rt. The desired product was separated by ultrafiltration with a Centricon (5-kDa cut-off), and washed with 1% sodium hydroxide, water, 2% acetic acid, and then water again. Lyophilization provided Gal-6ACT as a pale-yellow amorphous solid. The degree of substitution in the products was estimated by elemental analysis.

Gal03-6ACT: 79% yield; Anal. Calcd for $(C_6H_{12}N_2O_3)_{0.97}(C_{18}H_{34}N_2O_{13})_{0.03}\cdot 1.0H_2O\cdot 1.0\ C_2H_4O_2$: C, 40.48; H, 7.58; N, 11.29. Found: C, 40.43; H, 7.23; N, 11.50.

Gal18-6ACT: 89% yield; Anal. Calcd for $(C_6H_{12}N_2O_3)_{0.82}(C_{18}H_{34}N_2O_{13})_{0.18}\cdot 2.2H_2O\cdot 1.0C_2H_4O_2$: C, 38.30; H, 7.71; N, 8.79. Found: C, 38.38; H, 7.70; N, 8.76.

Gal25-6ACT: 74% yield; Anal. Calcd for $(C_6H_{12}N_2O_3)_{0.75}(C_{18}H_{34}N_2O_{13})_{0.25}\cdot 1.4H_2O\cdot 1.0C_2H_4O_2$: C, 40.18; H, 7.51; N, 8.52. Found: C, 40.40; H, 7.49; N, 8.57.

Gal38-6ACT: 78% yield; Anal. Calcd for $(C_6H_{12}N_2O_3)_{0.62}(C_{18}H_{34}N_2O_{13})_{0.38}\cdot 1.5H_2O\cdot 1.0C_2H_4O_2$: C, 40.64; H, 7.43; N, 7.55. Found: C, 40.55; H, 7.29; N, 7.77.

Gal50-6ACT: 85% yield; Anal. Calcd for $(C_6H_{12}N_2O_3)_{0.50}(C_{18}H_{34}N_2O_{13})_{0.50}\cdot 1.7H_2O$: C, 40.72; H, 7.52; N, 7.91. Found: C, 40.55; H, 7.29; N, 7.77.

Glucose modification of 6ACT was carried out with maltose in a similar manner as the preparation of Gal-6ACT to provide Glu32-6ACT: Anal. Calcd for $(C_6H_{12}N_2O_3)_{0.68}(C_{18}H_{34}N_2O_{13})_{0.32}\cdot 1.0H_2O\cdot 1.3C_2H_4O_2$: C, 40.86; H, 7.43; N, 8.05. Found: C, 40.55; C, 40.57; H, 7.07; N, 8.09.

3.3. Lectin-induced aggregation

Gal38-6ACT and unmodified 6ACT (20 μg/mL) were separately incubated with RCA120 (0.5 mg/mL) in PBS (90 μL). Then, galactose (10 μL of a 100 mM solution) was added. The turbidity at 450 nm was continuously monitored with a UV–vis recording spectrophotometer (UV-2500PC, Shimadzu, Kyoto, Japan).

3.4. Cytotoxicity

The cytotoxicity of Gal38-6ACT and unmodified 6ACT for HepG2 cells was evaluated using a Cell Counting Kit-8® (Dojindo, Kumamoto, Japan) as described in a previous paper.[24] Briefly, the cells were seeded at a density of 10^4 cells/well in a 96-well microtiter plate. Following 20 h of incubation the medium was replaced with fresh medium containing the cationic polyelectrolytes in a range up to 150 μg/mL. The polyelectrolytes were removed after 3 h of exposure time. The cells were further incubated for 22 h and then treated with a Cell Counting Kit-8® for 2 h. Cell viability was estimated from the absorbance of medium at 620 and 450 nm. The percent of cell viability in the absence of a polyelectrolyte was normalized to 100%. The measurements were carried out in octaplicate.

3.5. Agarose gel retardation assay

Unmodified 6ACT and Gal-6ACTs (d.s. of 18%, 38%, and 50%) were separately mixed with pCMV-Luc at the prescribed N/P ratio, which was represented as the ratio of amino groups in the 6ACT skeleton to the phosphate groups in pCMV-Luc. The mixtures were left to stand for 15 min at rt prior to agarose gel electrophoresis (1% w/v agarose). The migration pattern was visualized by UV transillumination after ethidium bromide staining and photographed using an ATTO Printgraph (ATTO, Tokyo, Japan).

3.6. In vitro transfection for cultured cells

Cells were seeded at a density of 5×10^4 cells/well in 1 mL of the growth medium in 24-well plates and incubated for 20 h prior to transfection. At the time of transfection, the culture medium was replaced with 200 μL/well of fresh medium and either 50 μL of complexes or naked pCMV-Luc solutions were added into each well (1 μg pCMV-Luc/well). The complexes were prepared by mixing of cationic polymeric gene carrier with pCMV-Luc and then left for 15 min at rt. After 3 h of exposure the medium containing the polyplexes was removed and 1 mL/well of fresh medium was supplied. The cells were further incubated for 48 h and the luciferase activity in cells was measured by the Steady-Glo® Luciferase Assay System (Promega) according to the manufacturer's instructions using a Fluoroskan Ascent FL microplate luminometer (Thermo Labsystems). The measurements were carried out in triplicate.

3.7. Flow cytometry

Cells pre-cultured for 20 h were incubated with the complexes prepared with fluorescein-labeled pCMV-Luc in the same manner as the transfection. After 3 h of exposure of the medium, the cells were treated with 0.25% trypsin/EDTA solution containing trypan blue (1.2 mg/mL). The resultant cell suspensions were collected and washed in PBS twice. Re-suspended cells were analyzed by a FACS Calibur with the CellQuest program (both BD Bioscience, Tokyo, Japan). A total of 20,000 cells in the gated area were counted. For the inhibition assay, 16 mM lactose was supplied during incubation with complexes.

3.8. Measurement of particle size and zeta potential

Gal-6ACT was mixed with pCMV-Luc and then left to stand for 15 min at rt. Particle size and zeta potential were measured with a Zetasizer Nano ZS (Malvern Instruments, Southborough, UK) at 25 °C. The measurements were carried out in triplicate. In measurements in PBS, the solution of the complexes prepared by the same manner was mixed with $10\times$ PBS to a final salt concentration of $1\times$ PBS and then left to stand for 15 min at rt. The zeta potential in PBS was measured only once.

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