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# Size Reduction of Galactosylated PEI/DNA Complexes Improves Lectin-Mediated Gene Transfer into Hepatocytes

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Hepatocytes are interesting targets for gene therapy applications. Several hepatocyte-directed gene delivery vectors have been described. For example, simple galactosyl residues coupled to polyethylenimine (PEI) gave an efficient vector which selectively transfected hepatocytes via the asialoglycoprotein receptor-mediated endocytosis [Zanta, M. A., et al. (1997) *Bioconjugate Chem*. *8*, 839−844]. However, the large size of these galactosylated PEI/DNA complexes prevented their use in vivo. We have investigated the role of the saccharide length on the size of glycosylated-PEI/DNA particles. When 5% of the PEI nitrogens were grafted with a linear tetragalactose structure (*l*Gal4), small and stable particles were formed upon complexation with plasmid DNA. These particles were essentially toroids having a size of 50−80 nm and a $\zeta$-potential close to neutrality. Moreover, these slightly charged PEI−*l*Gal4/DNA complexes were as selective as the previously described galactosylated−PEI vector to transfect hepatocytes, but in addition, they were more efficient. It is expected that the properties of the PEI−*l*Gal4/DNA complexes may increase their diffusion into the liver and their efficiency to transfect hepatocytes.

In the nonviral gene delivery field, the cationic polyethylenimine and its derivatives are efficient gene transfer vectors, both in vitro and in vivo (*1*−*5*). Recently, a hepatocyte-directed vector has been developed, consisting of a 5% galactose-bearing polyethylenimine (PEI−Gal)[1] (*6*). The PEI−Gal conjugate was prepared by reductive amination between PEI and lactose in the presence of sodium cyanoborohydride. This vector possesses a DNA-binding domain, an intrinsic endosomal-disrupting activity ("proton sponge" effect), and a galactose specific lectin-binding moiety. The transfection of hepatocytes expressing a galactose-specific membrane lectin was selective and highly efficient. Unfortunately, transmission electron microscopy pictures showed aggregated PEI−Gal/DNA complexes in physiological saline conditions. So, we have investigated the size and the morphology of glycosylated-PEI/DNA complexes (*7*), in relation with the length of the osidyl structure (1−9 units) and the glycosylation extent of the polymer (2.5−25% of grafted nitrogens). Only when 5% of the PEI nitrogens were modified with a linear tetragalactose structure (*l*Gal4), small and stable particles were formed with plasmid DNA.

The 25 kDa PEI (branched polymer, Aldrich) was glycosylated by the reductive amination procedure. Linear tetragalactose (Gal α3 Gal $\beta$4 Gal α3 Gal, *l*Gal4, Dextra Laboratories Ltd.) or tetraglucose (Glc α4 Glc α4 Glc α6 Glc, *l*Glc4, Sigma) (5 $\mu$mol) and sodium cyanoborohydride (25 $\mu$mol, Aldrich) were added to PEI (100 $\mu$mol of monomer, 100 $\mu$L of a 1 M, pH 7.0, stock solution) in 0.9 mL of borate buffer 0.2 M, pH 8.2, and left for 96 h at rt under continuous stirring. Low molecular weight compounds were removed by gel filtration on a Sephadex G-25 fine column (12.5 × 450 mm, Fluka) in 0.15 M NaCl. The grafting extent of the *l*Gal4 motif per PEI molecule, expressed as a percentage of glycosyl structures per nitrogen, was determined by using the resorcinol/sulfuric acid micromethod (*8*) and was 5%. The polymer amine concentration was determined by the TNBS assay (*9*). PEI−Gal (Figure 1A) and PEI-Glc (data not shown) were prepared as previously described (*6*), except that PEI conjugates were purified by gel filtration (see above). The monosaccharide was unable to prevent particle aggregation in physiological saline conditions (Figure 1A) at PEI nitrogen to DNA phosphate ratios (*N*/*P*) of 3 and 5. Increasing the length of the grafted saccharide to 4 units (*l*Gal4 or *l*Glc4) led to the formation of smaller particles (Figure 1A), which were only slightly charged, as shown by $\zeta$-potential measurements (Figure 1B). In these conditions, the particles were stable, indicating that the linear tetraglycosyl structure (*l*Gal4 or *l*Glc4) was able to prevent aggregation and masked the surface charge (Figure 1, panels A and B). This phenomenon occurred even at high *N*/*P* ratios (≥10). The *N*/*P* ratio slightly influenced the size of tetraglycosyl-PEI/DNA complexes in physiological saline conditions, in contrast to the size of DNA complexed with unmodified PEI, PEI−Gal or other PEI derivatives bearing peptides or proteins (*5*, *10*). The size and morphology of the PEI−*l*Gal4/DNA complexes were determined by negative staining transmission electron microscopy. A homogeneous population of essentially toroidal particles, with a size of 50−80 nm, was observed for *N*/*P* of 5 and 10 (Figure 1C).

We have shown that the terminal galactosyl residues on the PEI−*l*Gal4/DNA complexes were easily accessible and recognized by using a galactose-binding $RCA_{120}$ lectin-mediated agglutination (Figure 2). The recognition

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[1] Abbreviations: PEI, polyethylenimine; Gal, galactose; Glc, glucose; *l*Gal4, linear tetragalactose; *l*Glc4, linear tetraglucose; rt, room temperature.

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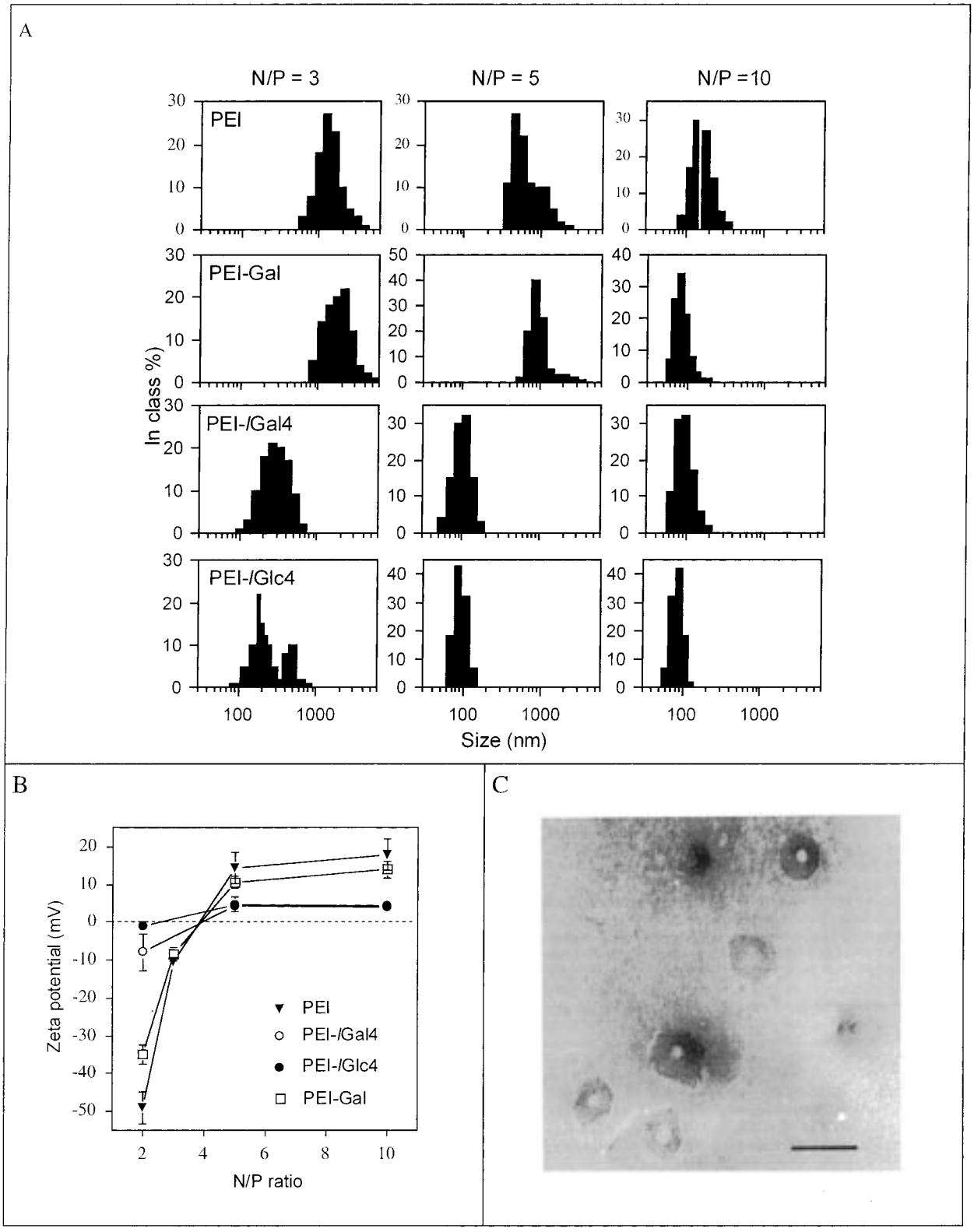


**Figure 1.** Particle size (A, C) and ζ-potential (B) measurements. DNA (40 $\mu$g of pCMVLuc) and the desired amount of glycosylated PEI or unmodified PEI were each diluted separately in 500 $\mu$L of a 150 mM NaCl solution. After 10 min, solutions were mixed, homogenized, and left for 10 min. Particle size (A) was determined by dynamic laser light scattering using a Zetamaster 3000 (Malvern Instrument, Orsay, France). ζ-Potential was measured electrophoretically using the same apparatus (B), as previously described (*15*). For transmission electron microscopy (C), a drop (5 $\mu$L) of a solution containing the PEI−*l*Gal4/DNA complexes ($N/P = 10$) was added to a carbon-covered grid and left for 1 min. Complexes were negatively stained with 30 $\mu$L of an aqueous uranyl acetate solution (1%, w/w), for 20 s. Observations were done at 80 kV on a Philips EM 410 transmission electron microscope, bar is 100 nm.

and agglutination of the PEI−*l*Gal4/DNA complexes by the $RCA_{120}$ lectin were totally reversible after the addition of a 1000-fold molar excess of galactose. Moreover, a nonspecific agglutination mediated by electrostatic interactions was unlikely, as shown by the results obtained with the PEI and PEI−*l*Glc4/DNA complexes, at $N/P = 10$ (Figure 2). These biophysical and recognition properties of PEI−*l*Gal4/DNA complexes confirmed that the

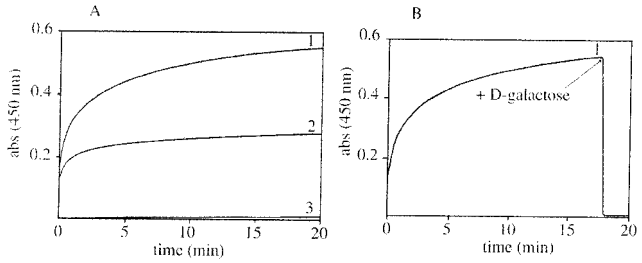


**Figure 2.** Accessibility of the galactosyl residues at the surface of the complexes. (A) PEI or PEI conjugates/pCMVLuc complexes (20 $\mu$g/mL DNA, protocol described in the legend of Figure 1) were incubated in the presence of *Ricinus communis* $RCA_{120}$ lectin (90 $\mu$g/mL, Sigma) in 300 $\mu$L (total volume) of 10 mM phosphate buffer pH 7, 150 mM NaCl. Agglutination was monitored by following the turbidity at 450 nm, during 20 min. (1) PEI−*l*Gal4/DNA complexes at $N/P = 10$, (2) PEI−*l*Gal4/DNA complexes at $N/P = 5$, and (3) PEI or PEI−*l*Glc4/DNA complexes at $N/P = 10$. (B) The RCA-mediated agglutination of PEI−*l*Gal4/DNA complexes ($N/P = 10$) was reversible when D-galactose was added to a final concentration of 1 M.

glycosyl structures controlled the particle behavior and were exposed at their surface.

Transfections were performed in the presence of 10% serum and without the plate centrifugation step that generally leads to higher efficacies (*6*, *11*). Gene transfer efficiencies were evaluated on the BNL CL.2 murine hepatocyte cell line which expresses a galactose specific membrane lectin (*12*, *13*) and on the HeLa human epitheloid carcinoma cell line which does not express a galactose specific receptor (*14*). In both cases, unmodified PEI-mediated transfection efficiency increased with the *N*/*P* ratio (Figure 3), according to the ionic cell binding mechanism. The PEI−*l*Gal4/DNA complexes showed improved efficiency over PEI−Gal and PEI-mediated gene transfer on BNL CL.2 cells (receptor +), at all *N*/*P* ratios. When *l*Gal4 was replaced by *l*Glc4 motif, transfection efficiencies were comparable to those obtained with the unmodified PEI. On HeLa cells (receptor −), none of the vectors showed any evidence of a selective gene delivery (Figure 3). The differences in expression levels are probably related to differences in the size of particles and to a lesser extent to their surface charge, both important parameters for cellular uptake. At the optimal *N*/*P* ratio, competition experiments with an excess of asialofetuin (20-fold molar excess over galactose), the natural ligand of the asialoglycoprotein receptor, decreased the transfection efficiency of the PEI−*l*Gal4/DNA complexes by 1 order of magnitude on BNL CL.2 cells. In contrast, the transfection of BNL CL.2 cells with PEI or PEI−*l*Glc4/DNA complexes was (only) affected by 2.5 or 5-fold in the presence of asialofetuin or albumin, respectively. The involvement of the asialoglycoprotein receptor was clearly demonstrated by both comparative and competitive experiments showing that transfection of hepatocytes with small and slightly charged PEI−*l*Gal4/DNA complexes was selective and efficient.

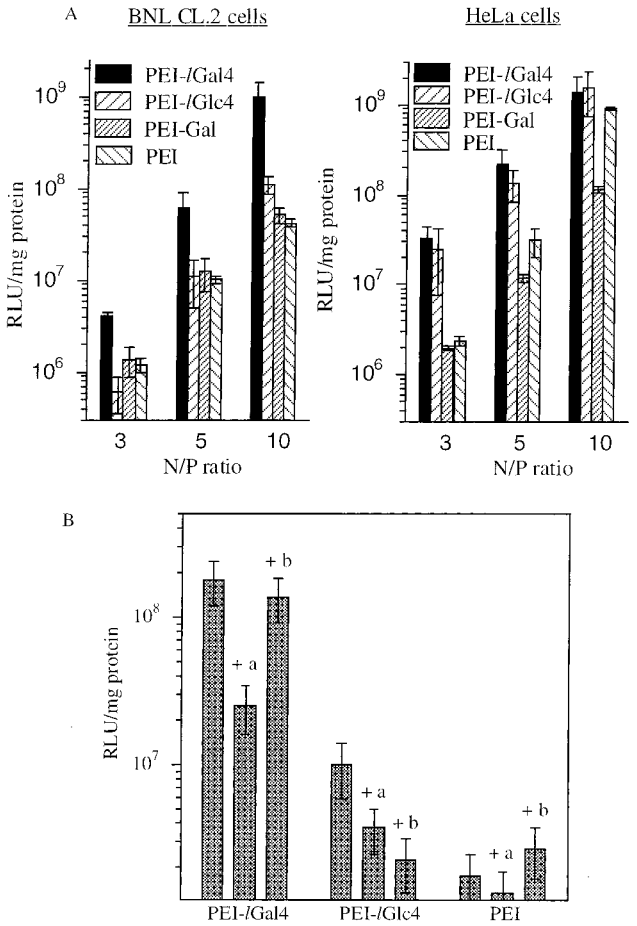


**Figure 3.** Transfection efficiency of the glycosylated PEI/DNA complexes on the BNL CL.2 murine hepatocyte and HeLa human epitheloid carcinoma cell lines. A day prior to transfection, (5−6) × $10^4$ cells/well were seeded in 24 well tissue culture plates and were incubated at 37 °C in a 5% $CO_2$ humidified atmosphere. Before transfection, cells were supplemented with 1 mL of fresh complete medium containing 10% FCS (DMEM with high glucose (4.5 g/L) for BNL CL.2 and MEM with Earle's salt for HeLa cells). DNA (6 $\mu$g of pCMVLuc, for a triplicate experiment) and the desired amount of PEI or PEI conjugates were diluted separately in 150 $\mu$L of 0.15 M NaCl. After 10 min, the polycation was added to the DNA, the solution was homogenized and left for 10 min. The polycation/DNA complexes (100 $\mu$L/well) were added onto the cells. For the competition experiments, BNL CL.2 cells were transfected with pCMVLuc (1 $\mu$g) complexed with PEI−*l*Gal4, PEI−*l*Glc4 or PEI at $N/P = 10$. Before the addition of the polycation/DNA complexes onto the cells, 1 mg of asialofetuin (+a) or bovin serum albumin (+b) was added per well. After 24 h of incubation, cells were lyzed and luciferase gene expression was quantitated using a commercial kit (Promega) and a luminometer (Biolumat LB 9500, Berthold). Results were expressed as light units integrated over 10 s, per mg of cell protein using the BCA assay (Pierce). Values are the mean ± sd of three independent experiments made in triplicate.

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