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Synthesis of Glycosylated Polyethylenimine with Reduced Toxicity and High Transfecting Efficiency

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Abstract—A safe and efficient synthesis of glycosylated polyethylenimine using titanium (IV) isopropoxide and sodium borohydride has been carried out as a substitute for the highly toxic sodium cyanoborohydride method currently used. Polyplexes formed between DNA and the various glycosylated polyethylenimines appeared to be much less cytotoxic than polyethylenimine (PEI)/DNA polyplexes. © 2000 Elsevier Science Ltd. All rights reserved.

Glycosylated polyethylenimines have been shown to be efficient vectors for in vitro plasmid transfection into cells harboring the corresponding sugar receptor lectin.[1] Currently, they are synthesized by reductive amination of a disaccharide or of an oligosaccharide with PEI aminogroups, by using the expensive and highly toxic[2] sodium cyanoborohydride as reagent.[1a–3] As PEI is a strong polycation in which approximatively one on six amino nitrogen is protonated at pH7, the risks due to the potential presence of residual cyanide ions in the products[4] make this procedure unusable for clinical trials and the pharmaceutical industry. We now report a novel safe and efficient synthesis of this family of glycosylated polymers, and present the characteristics of the obtained products.

Sodium borohydride in aqueous sulfuric acid[5] and pyridine-borane[6] were not compatible with cationic polymers. As PEI is insoluble in apolar solvants, the use of sodium triacetoxyborohydride[7] was also unsuitable. On the other hand, Bhattacharyya[8] used the reducing system titanium (IV) isopropoxide/sodium borohydride for the synthesis of alkylamines and phenethylamines from the corresponding aldehydes or ketones. As these reagents are safe and inexpensive, and ethanol can be used as solvent, we decided to try to extend this method to the aminoreduction of glucides and especially to the synthesis of glycosylated polyethylenimines.

As shown in Scheme 1, polyethylenimine 25 kDa (Aldrich) and disaccharide (lactose or maltose) reacted cleanly with Ti $(OiPr)_4$ in EtOH. The complex formed was reduced with sodium borohydride. The reaction mixture was then purified by dialysis to afford the expected glycosylated PEI in good yield.[9] The number of glycosylic substitutions was determined by using the resorcinol/sulfuric acid method after the final dialysis.

In vitro transfection (Fig. 1) has been studied on three different cell lines using **3a**, **3b**, **4a** and **4b** as DNA-complexing vector and compared with the transfection efficiency of free PEI. For HepG2 cells, derived from a human hepatoblastoma, cell survival does not change by introduction of glycosyl residue but transfection efficiency was reduced. No beneficial effect of lactose substitution and galactose receptor targeting could be detected on gene transfer efficiency in these conditions. With ECV304 cells, obtained by transformation of a human endothelium, transfection level is higher with **3a** but toxicity is the same than with free PEI for low substitution percentages (**3a** and **4a**). On the other hand, **3b** is very interesting because, although the transfection slighly decreases with higher maltose level, cell survival increases a lot especially at high N/P values. For HeLa cells, derived from a human cervix epitheloid carcinoma, cellular survival is better than with free PEI for all the substituted compounds (LD_{50} >0.3 mM) but the transfection efficiency decreases for the highest substituted compounds **3b** and **4b**. On the other hand, maltose and lactose both confer an interesting protecting effect at

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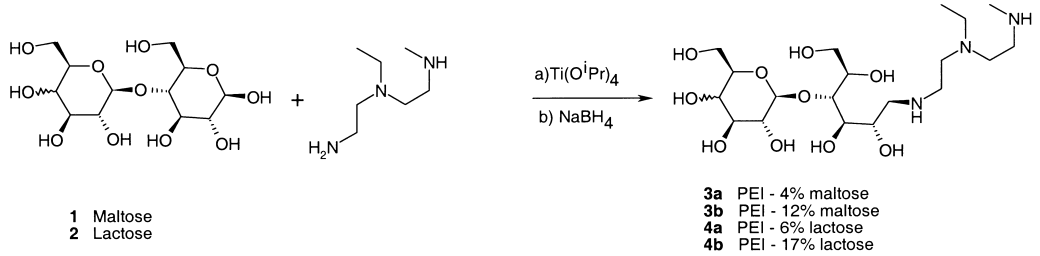

Scheme 1.

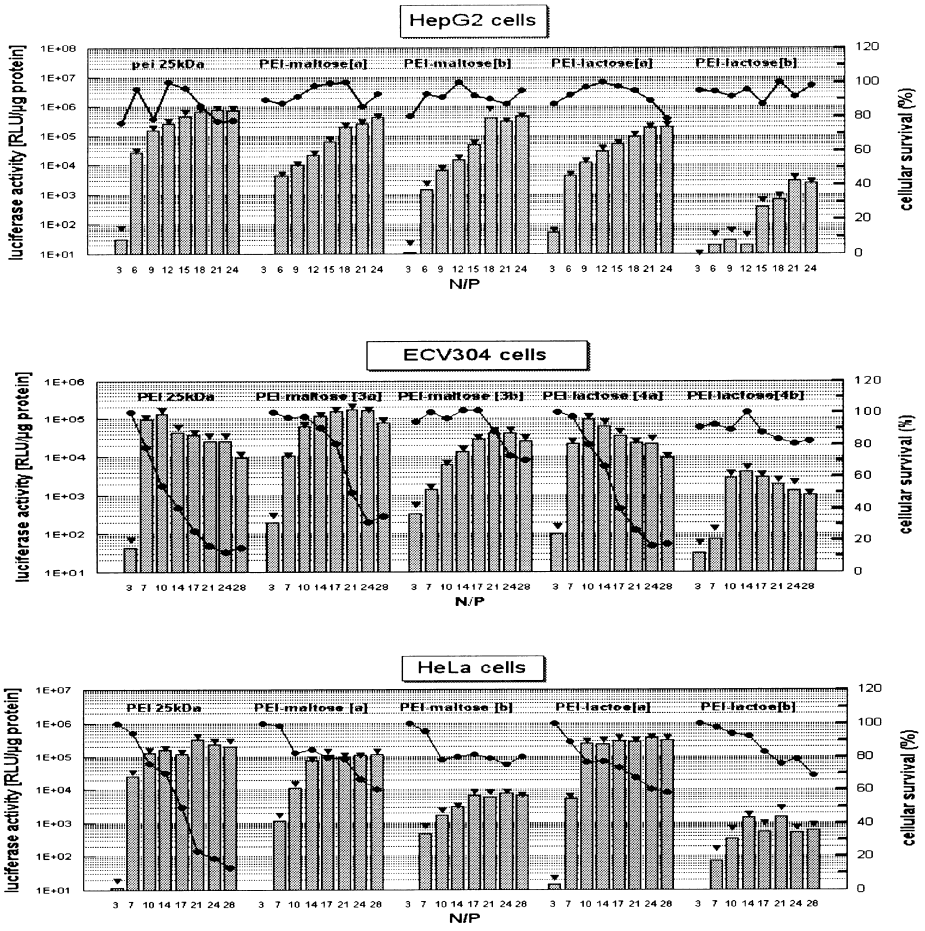

Figure 1. Transfection with 2 mg DNA/well during 2 h in serum free medium. A plamid encoding for the firefly luciferase gene under the human-cytomegalovirus promoter was used. Luciferase expression was measured 48 h post transfection (bars represent the means of results for three wells). Cellular survival was assessed by the determination of cellular proteins in the transfection well at the end of the experiment (black line).[10]

low substitution percentage (**3a**, **4a**) especially at high N/P level.

For in vivo use, much higher DNA concentration have to be injected and physico-chemical properties of the complexes DNA/polymer might notably change.[11] Polyplexes of the same N/P ratios than for in vitro experiments have been studied.[12] The size of the glycosylated PEI–DNA complexes formed[13] have been determined by dynamic light scattering and found around 200 nm whatever the percentage of glycosylation.

In conclusion, the glycosylated PEI obtained by this safe and inexpensive method can be considered as good candidates gene transfection vectors both for in vitro and in vivo use.

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9. A typical experimental process for PEI glycosylation is illustrated for **3a** as follow: PEI 25 KDa from Aldrich (500 mg, 0.02 mmol) was dissolved in 20 mL of anhydrous ethanol. Maltose (252 mg, 0.7 mmol) was added and the resulting solution was stirred under nitogen atmosphere for 15 min. Titanium isopropoxide (0.295 mL; 1 mmol) was slowly added and the resulting mixture was kept stirring overnight. Sodium borohydride (28.5 mg; 0.75 mmol) was then added and stirring was carried on for 8 h. The reaction mixture was filtered and concentrated to 10 mL. The resulting solution was finally dialyzed for 12 h (exclusion size membrane 12000).Yield 80%.
10. DNA (plasmid vector pXL2774) is dissolved in NaCl 150 mM at 80 mg/mL. PEI polymers were diluted in water at double used concentration and mixed volume to volume with DNA solution. Immediately prior to transfection, cells were washed twice with 500 mL of serum free-medium. 50 mL of polymer/DNA complexes solution were added dropwise to the cells (three wells per ratio condition). When the cells were transfected in "absence of FCS", the medium was supplemented by fetal calf serum 2 h after transfection. After transfection, cells were incubated at 37 °C in humidified air with 5% CO2 for an additional 44 h, up to the evaluation of the efficiency of gene transduction.The gene expression was monitored using the Luciferase Assay System (promega) according to the manufactuer protocols; the toxicity of the polymer/DNA mixture was assassed by cell lysat concentrations. Results were reported as enzymatic activity per mg of lysate protein.
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12. Polyplexes of different, N/P ratios (expressed as moles of amines per mol of phosphate) were obtained by mixing equal volumes of various concentrations of PEI with plasmid DNA at the final concentration of 250 mg DNA/mL in 5% glucose and 20 mM NaCl.
13. Size determinations of the PEI/plasmid DNA complexes were performed as previously described (11[a,b]) using a Coulter N4 Plus particle analyzer (Coulter). The means particle diameter was obtained from the unimodal fit analysis following dynamic light scattering done at a 90° angle at 20 °C. All samples were measured at a DNA concentration of 10 μg/mL in 5% glucose and 20 mM NaCl.