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Gene therapy of cystic fibrosis: The glycofection approach

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Many cells express surface membrane lectins that selectively bind and carry glycoconjugates into intracellular endosomes; in addition, various intracellular membrane and soluble lectins act as shuttles between different compartments. On this basis, we developed glycosylated polycations, now called glycofectins (glycosylated polylysine and polyethyleneimine). Recently, we set up a simple way to transform oligosaccharides into glycosynthons suitable to substitute proteins or polymers. Glycofectins bind plasmid DNA leading to compact glycoplexes. Glycoplexes prepared with glycofectins were found to be much more active than naked plasmid to transfer genes to various types of cells including human airway epithelial and serous cells. The gene transfer efficiency was found to depend on the nature of the sugars borne by glycofectins. It appeared that the sugar-dependent efficiency was not only related to the uptake but also to the intracellular traffic of glycoplexes.

Keywords: **gene therapy, glycoconjugates, intracellular traffic, glycoplexes, polyplexes**

Abbreviations: **CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; Glp, pyroglutamate; Os_x, oligosaccaride containing x sugars; PEI, polyethyleneimine; Man, α-D-Mannose; Lac, β-D-lactose.**

Introduction

Gene therapy represents a potentially important advance in the treatment of cystic fibrosis (CF) which is the most common lethal autosomal recessive disorder in the Caucasian population. Since CF morbidity and mortality are mainly due to disseminated bronchiectasis leading to progressive respiratory failure, the delivery of a normal copy of the CF gene should target airway epithelial cells. Airway surface ciliated cells and airway gland serous cells are presumably the primary candidates to target, because they predominantly express the CFTR protein (cystic fibrosis transmembrane conductance regulator) [1,2].

Viral and non-viral gene delivery systems have been proposed for delivering the CFTR gene to the airways. Early *in vitro* studies using poorly differentiated airway epithelial cells showed that adenoviral vectors allowed a high gene transfer efficiency [3,4]. However, additional studies have shown that differentiated airway epithelial cells were poorly transduced by adenoviral vectors [5–7], mainly because the apical surface of differentiated airway epithelial cells lack the receptors required for efficient infection by adenovirus [8,9]. Nonviral gene delivery systems have been investigated as an alternative approach to viral vectors (for a review, see [10]). Cationic lipids deliver genes quite efficiently into some cell lines and some cells in primary culture, but their efficiency to transfer genes into well differentiated airway cells is low [11–14]. It was suggested that to enhance gene transfer efficiency in mature airway epithelial cells, vectors should be modified in order to target a receptor expressed in mature airway epithelial cells [15].

Synthetic cationic polymers have been used to introduce exogenous genes into cells (for a review, see [16]). For instance, poly-L-lysine that cooperatively interacts with nucleic acids is used to compact plasmid DNA containing a gene of interest and the resulting plasmid DNA/cationic polymer complexes are called polyplexes [17]. To achieve a cell-specific gene transfer, cell surface receptors that could mediate the uptake of polyplexes were targeted: polylysine which contains a free amino group per unit has been substituted with proteins such as transferrin to target airway cells through the transferrin receptor [18]. However, transferrin-polylysine conjugates are difficult to prepare and may cause immune responses. Alternatively, polylysine may be substituted by any small ligand that is recognized by a receptor expressed at the cell surface. Cells usually express at their plasma membrane, lectins that

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selectively recognize glycoconjugates containing oligosaccharide structures [19–21]. Therefore we have developed synthetic vectors containing carbohydrate ligands and we came up with polymers called glycofectins.

Glycofectins and glycoplexes

Glycosylated polylysine

One of the first glycoconjugate-based transfections was achieved *in vitro* and *in vivo* by Wu and coworkers by using DNA complexed with polylysine-asialoorosomucoid conjugates in order to target the galactose-specific membrane lectin of hepatocytes (for a review, see [22]). Problems related to the preparation and solubility of polylysine coupled to asialoorosomucoid on the one hand and to the various clinical risks in using a natural glycoprotein—including immune responses and viral contaminants—on the other hand, led to the use of glycosylated cationic polymers made with small sugar units as ligands [23]. Plasmid DNA complexed with polylysine bearing lactosyl residues was found to efficiently transfect hepatocarcinoma cells (HepG2 cells) and hepatocytes in primary culture which both express a galactose-specific membrane lectin [24,25]. Similarly, plasmid DNA complexed with polylysine bearing mannosyl residues efficiently transfect human macrophages expressing a mannose/fucose specific membrane lectin [26].

Glycosylated poly-L-lysines with an average degree of polymerization of 190 (Figure 1) are readily prepared by adding activated glycosides, such as those presented in Figure 2 [27,28] and Figure 3 [29,30] to poly-L-lysine, *p*-toluene sulfonate [25,31]. Glycosylated polylysines can be kept as dry powders after freeze-drying. The average number of neutral sugar residues bound per poly-L-lysine molecule was calculated from the sugar content determined by the resorcinol sulfuric acid micromethod [32] or by nuclear magnetic resonance on the basis of the intensities of the signals corresponding to the protons in *delta* position of the non-substituted and of the substituted lysine residues [33,34]. Efficient glycosylated polylysines contain around one third of their *epsilon* amino-groups substituted by sugar moieties. For visualization purposes, fluorescein-labeled glycosylated polylysines are readily available by substitution

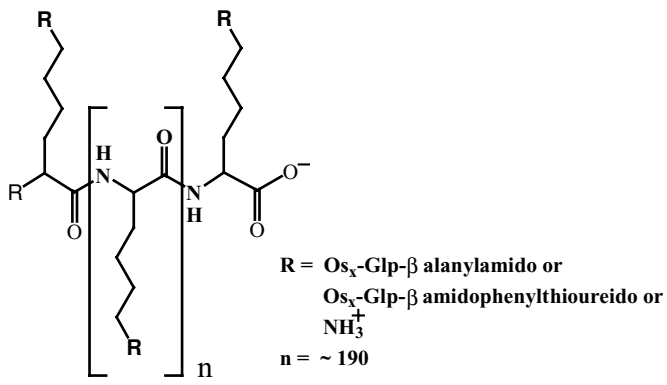

Figure 1. General structure of glycosylated polylysines.

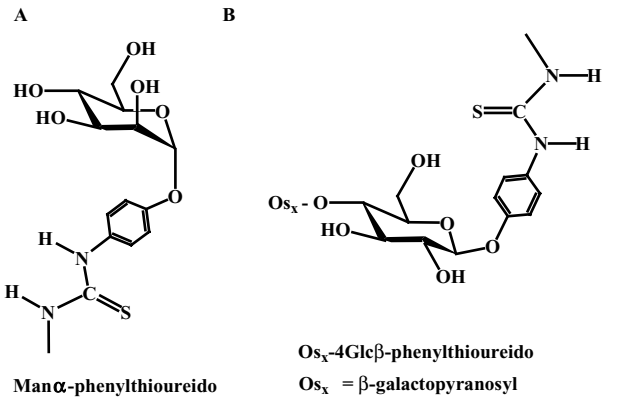

Figure 2. Glycosido-phenylthioureido moieties (A: mannopyranosido-α-, B: lactosyl-β-) present in polycations (polylysine or polyethyleneimine) upon reaction with glycosidophenylyisothiocyanates.

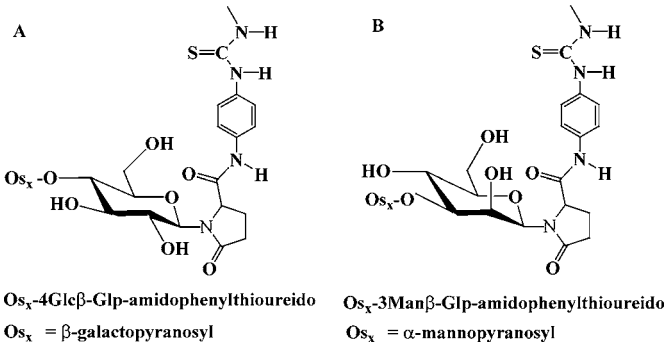

Figure 3. Glycosido-pyroglutamyl-amidophenylthioureido moieties A: lactosyl-β-, B: dimannosyl(mannopyranosyl-α-3mannopyranosyl-β-), present in polycations (polylysine or polyethyleneimine) upon reaction with glycosidopyroglutamyl-amidophenylyisothiocyanotes.

with fluorescein-isothiocyanate and contain an average of 3 fluorescein residues per poly-L-lysine molecule.

The preparation of plasmid DNA/glycosylated polylysine complexes (glycoplexes) takes about half an hour: solutions in serum-free medium of glycosylated polylysine and plasmid DNA are mixed and held for 30 min at 37°C. Then, the glycoplex suspension is supplemented with chloroquine (usually up to 100 μM), and added to each culture well. Chloroquine—which limits lysosomal degradation of the material taken up by a cell upon endocytosis, induces the release of the plasmid from the polyplex or the glycoplex and increases the transmembrane passage of DNA into the cytosol [24,35]—is required when polycations such as polylysine, glycosylated polylysine or gluconoylated polylysine [36] are used.

Glycosylated polyethyleneimine

Other polymers such as polyethyleneimine (PEI) do not require any endosomolytic agent [37,38]. Indeed, PEI contains a

high concentration of primary, secondary and tertiary amines (one nitrogen per M_r 43) which have pK from low to large values. PEI has been shown to induce endosomal swelling favoring the exit of polyplexes. PEI is among the most efficient synthetic vectors for gene transfer into various cell types, including airway epithelial cells *in vitro* [38–40] and *in vivo* in mice [41,42]. In order to allow cell-specific transfection, PEI has been substituted with galactose or mannose residues and these DNA/glycosylated PEI complexes were shown to efficiently transfect hepatocytes and dendritic cells, respectively [43,44].

We developed PEI substituted with the glycosides we were using to prepare glycosylated polylysines as well as with new glycosynthons. The branched PEI (M_r 25,000) (Figure 4) was glycosylated using glycosyl phenylisothiocyanate [27,28], (Figures 2 and 3) or with glycosyl-pyroglutamyl-succinimidyl esters [45] (Figure 5) in an ethanol/water solution (1/1, v/v) for 30 min at room temperature. Under such conditions, the free

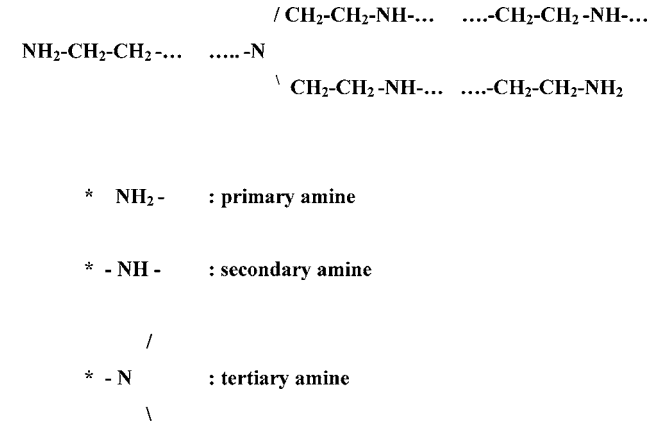

Figure 4. Scheme of branched polyethyleneimine. Primary and secondary amino groups may be substituted by reaction with an activated glycosides.

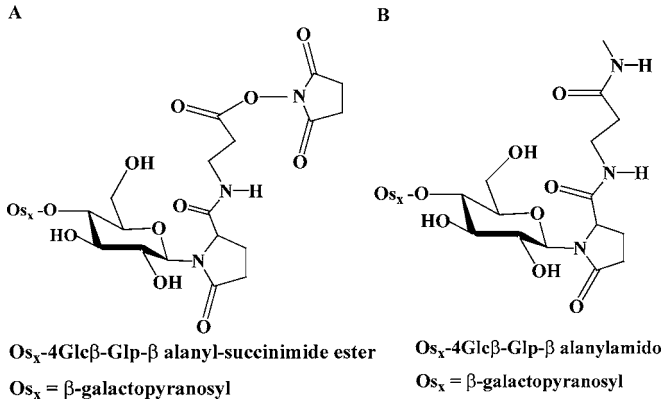

Figure 5. A: lactosyl-β-pyroglutamyl-βalanyl succinimide; B: lactosyl-β-pyroglutamyl-βalanyl amido, present in polycations (polylysine or polyethyleneimine) upon reaction with lactosyl-β-pyroglutamyl-βalanyl succinimide ester.

activated glycosides are not any more detectable upon thin layer chromatography on silica gel plates (in a 100/30/10/10/3 (per volume) ethanol/water/*n*-butanol/pyridine/acetic acid mixture). Upon removal of the ethanol by evaporation under reduced pressure, the water solution was freeze-dried. The more efficient glycosylated PEI contained between 5 and 10% of their amino groups substituted with a glycoside moiety. Glycosylated PEI were readily made fluorescent by adding fluorescein-isothiocyanate (dissolved in a (1/1, v/v) ethanol/water mixture) to a solution of the polymer in water. The solution was stirred for 30 min at room temperature and the fluorescein-labeled polymer solution was made free of ethanol and finally freeze-dried.

Glycoplexes were readily prepared by mixing a 150 mM NaCl solution of plasmid and a 150 mM NaCl solution of glycosylated PEI; the required amount of glycosylated PEI is that which gives a N/P ratio in the 5 to 10 range, N being the concentration of PEI amino groups that have not been substituted by the activated glycoside and P being the nucleotide phosphate concentration.

Gene transfer into airway epithelial cells using glycosylated polymers as vectors

Expression of membrane lectins by airway epithelial cells

The presence of lectins on the surface of airway epithelial cells and their involvement in the uptake of glycoplexes have been assessed by using either fluorescent neoglycoproteins [27,46] or fluorescent glycoplexes. Fluorescein-labeled neoglycoproteins were prepared by coupling 4-isothiocyanatophenyl-glycosides to bovine serum albumin (BSA) and fluorescein isothiocyanate. They were then purified by gel filtration and any traces of free fluorescein derivatives were removed by selective precipitation of the fluorescent neoglycoprotein in a mixture of ethanol (9 vol) and water (1 vol) [27,28]. The average number of sugar moieties per BSA molecule was 25 ± 3 and the number of fluorescein molecules per neoglycoprotein was 3 ± 1. The fluorescence intensity of 1 μg/ml of fluorescein-labeled neoglycoproteins was determined by spectrofluorimetry upon digestion with pronase [47]. To ascertain that the fluorescent material has been taken up by a cell and that it is localized in acidic (endosome and lysosome) compartments, the cell-associated fluorescence intensity was analyzed before and after a final incubation for 30 min at 4°C in the presence of 50 μM monensin [47,48]. Monensin which neutralizes acidic compartments, allows the recovery of the fluorescein fluorescence which is quenched in an acidic environment; thus, a higher fluorescence intensity of the monensin-treated cells demonstrates the presence of the fluorescein-labeled neoglycoproteins or glycoplexes in endosomal/lysosomal compartments. The amount of cellular neoglycoproteins or complexes was determined after standardization of the flow cytometer by using calibrated fluorescein-labeled beads [47].

We investigated the nature of membrane lectins expressed by airway cells. We studied immortalized, normal human bronchial

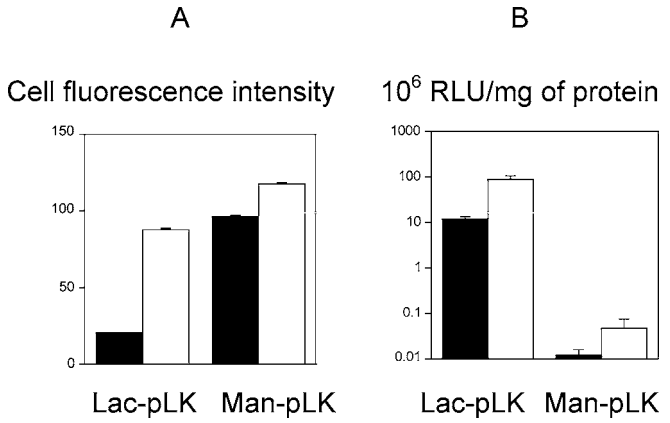

Figure 6. (A) Uptake of glycoplexes by immortalized CF airway epithelial cells (ΣCFTE29o-cells; ■) and CF airway gland serous cells (CF-KM4 cells; □). Twenty-four h after seeding, cells were incubated for 2 h in the presence of plasmid (pCMV*Luc*, 5 μg) complexed to lactosylated polylysine (Lac-pLK) or mannosylated polylysine (Man-pLK). The cell fluorescence intensity was measured by flow cytometry. (B) Gene transfer with glycosylated polylysines into immortalized CF airway epithelial cells (ΣCFTE29o-cells; ■) and CF airway gland serous cells (CF-KM4 cells; □). Twenty-four h after seeding, cells were incubated for 4 h in the presence of plasmid (pCMV*Luc*, 5 μg) complexed to lactosylated polylysine (Lac-pLK) or mannosylated polylysine (Man-pLK). Chloroquine (100 μM) was added to complexes made with polylysine. Fourty-eight h later, luciferase activity was measured by chemiluminescence.

16HBE cells, CF human tracheal ΣCFTE20o-cells, normal airway gland serous MM-39 cells and CF airway gland serous CF-KM4 cells. A membrane lectin recognizing neoglycoproteins bearing α-D-mannopyranosides was evidenced at the surface of all these cells; this cell surface lectin was shown to induce the cell uptake of its ligands on the basis of the enhancement of the cell fluorescence intensity upon a monensin incubation at 4°C [39,40]. In agreement with this finding, the most efficient uptake of glycoplexes by these cells was observed with glycoplexes bearing α-D-mannosyl residues (Figure 6A). A membrane lectin recognizing α-D-mannopyranosides was also evidenced in airway epithelial cells in primary culture; in addition, these cells also recognize—but with a lower efficiency—lactosyl, α-L-rhamnosyl, α-L-fucosyl and α-D-glucosyl residues. Among glycoplexes, primary-culture cells took up more efficiently those bearing α-D-mannosyl, lactosyl or α-D-glucosyl residues [49].

Airway cell glycofection using glycosylated polylysines as vectors

Using various glycofectins, we have shown that some sugars enhanced the vector capability of polylysine and yielded high expression of luciferase, used as a reporter gene, in immortalized, normal and CF airway surface epithelial cells, normal and CF airway gland serous cells (Figure 6B) as well as in primary cultures of normal and CF airway surface epithelial cells [39,40,49,50–52]. Some of the most efficient glycosylated polylysines: lactosylated and α-glucosylated polylysines, were as efficient or more efficient than commercially available non-viral vectors such as Lipofectamine or PEI [39,40]. Moreover, an efficient CFTR gene transfer was obtained with α-glucosylated polylysine leading to the expression of a normal CFTR protein at the cell surface of immortalized CF airway gland serous cells [40].

However, and in contrast with other glycosylated polylysines, glycoplexes made with mannosylated polylysine induced a very weak expression of the reporter gene in all the airway epithelial cells studied (Figure 6B) [39,40]. Similar discrepancies have been observed in the case of vascular smooth muscle cells which take up efficiently lactose-borne glycoplexes and poorly α-galactose-borne glycoplexes, while an efficient gene expression was obtained with poorly taken up glycoplexes [53]. Hence, the fact that complexes are taken up in large amounts by cells does not mean that these complexes are able to transfer genes efficiently. In the case of mannosylated complexes with airway epithelial cells, the mannose-specific uptake is jeopardized by an intracellular trafficking leading to an inefficient gene expression. In contrast, the entry of low amounts of lactosylated complexes is presumably balanced by a different intracellular trafficking leading to a very efficient gene expression. Indeed, the low gene transfer efficiency obtained with mannosylated complexes as compared with lactosylated complexes was found to be due in part to their inefficient exit from the endosomal compartments, their high accumulation in lysosomes and their inefficient nuclear import [54]. Moreover, in an *in vitro* transcription assay, we showed that the glycoplex obtained by compaction of a plasmid DNA with mannosylated polylysine prevented the initiation of transcription. This suggests that the presence of a plasmid DNA in the nucleus does not guarantee an efficient gene expression [54].

One common intracellular limiting step encountered when using polylysine as vector is a poor endosomal escape of the plasmid or of the glycoplex. It is why all the experiments need to be conducted in the presence of membrane disrupting agents, such as chloroquine or fusiogenic peptides which increase the transmembrane passage of the plasmid DNA into the cytosol and/or limit the lysosomal degradation of the endocytosed material [35,55]. Unfortunately, these membrane disruptive agents are cytotoxic and they are not suitable for *in vivo* applications. To circumvent this drawback, we have designed a polylysine partially substituted with histidyl residues which become cationic upon protonation of the imidazole groups at slightly acidic pH. This protonated polymer induces the leakage of acidic vesicles containing plasmid/histidylated polylysine complexes and hence favors the delivery of plasmid DNA into the cytosol [56]. This histidylated polylysine was shown to efficiently transfect various cell types including immortalized normal and CF airway surface and airway gland serous cells, and airway epithelial cells in primary culture [56,57]. Complexes

made with histidylated polylysine are likely to be taken up by these cells as in many other cells through a non-specific uptake. Attempts to enhance the specificity and the level of gene expression by adding sugar residues on histidylated polylysine did not succeed, so far.

Airway cell glycofection using glycosylated PEI as vectors

The data gathered with glycosylated polylysines underline that for a successful gene transfer with synthetic vectors, the aim is not so much to obtain a large cellular uptake of the complexes but rather to find a vector which favors both a specific uptake and an intracellular trafficking allowing plasmid DNA to escape from endosomes, to avoid accumulation in lysosomes, to readily diffuse in cytosol, to enter into the nucleus and finally to be efficiently expressed. In this prospect, we developed glycosylated polyethyleneimines which should allow a cell-specific uptake and an efficient exit from endosomes. Indeed, in immortalized airway gland serous cells, the gene transfer obtained with plasmid DNA/Lac-PEI complexes *in the absence of chloroquine* was more efficient than that obtained with plasmid DNA/unsubstituted PEI complexes *in the absence of chloroquine* and also more efficient than that obtained with plasmid DNA/Lac-polylysine complexes *in the presence of chloroquine* [49]. These data suggest that lactosylated PEI did retain the endosomal swelling property of the sugar-free polymer.

Future trends

One of the most important key issues regarding non-viral gene delivery is mastering of intracellular trafficking, especially the nuclear entry. In addition to allowing a cell-specific uptake, the sugar present on glycoplexes may also play a key role in the intracellular trafficking. Indeed, membrane lectins are present in several compartments including pre-lysosomes, Golgi apparatus and endoplasmic reticulum (for a review, see [58]) and soluble lectins are present in cytosol and nucleus [59]. Several data suggest that macromolecules can be imported into the nucleus by a sugar-dependent process which would not involve the classical, highly cationic peptide-dependent nuclear import signal [60–62]. To overcome the nuclear barrier, gene delivery systems may bear nuclear localization signals to help the plasmid DNA to get access into the nucleus by translocation through the nuclear pore as suggested by sever authors including [63]. We are currently working along this line and specially on the possibility to take advantage of the sugar-dependent nucleus import mechanism.

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