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# Glycosylated carriers for cell-selective and nuclear delivery of nucleic acids

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## 1. ABSTRACT

Targeted gene delivery via selective cellular receptors has been realized as a crucial strategy for successful gene therapy by maximizing therapeutic efficiency in target cells and minimizing systemic toxicity. The membrane carbohydrate-binding proteins (membrane lectins) with different carbohydrate specificities are differentially expressed on the cellular and intracellular membranes of a number of cells. Their multiplicity, high affinity, and effective endocytosis after receptor binding as well as the biocompatibility of carbohydrate ligands endow them as potential ligands for glycosylated carriers in cell-selective delivery of nucleic acids. To achieve the *in vivo* application, glycosylated carriers/nucleic acid complexes have to fulfill certain conditions, including having a suitable size, minimal nonspecific interactions, low immunogenicity, and high uptake in target cells. Accordingly, the effective nuclear delivery of nucleic acids is the paramount important step for efficient gene transfer. This review summarizes the recent progress regarding application of glycosylated carriers for cell-selective and nuclear delivery of nucleic acids and their critical factors for efficient gene transfer. In addition, the development of new materials, such as carbon nanotubes, carbon nanospheres, and gold nanoparticles, as innovative carriers will be discussed with regards to glycosylation-mediated delivery of nucleic acids.

## 2. INTRODUCTION

Gene therapy represents a potentially important therapeutic advance for many refractory diseases. The evolution of treatment is based on the manipulation of specific genes encoding diseased-associated proteins by enhancing or inhibiting the expression of foreign or host genes, respectively. The drug-like genes or nucleic acids that have been investigated in various forms include plasmid DNA (pDNA), synthetic DNA, antisense or decoy oligonucleotide (ODN), aptamers, and siRNA. The clinical application of these nucleic acid drugs in the naked form faces many difficulties including enzyme degradation and low cellular uptake. In the past few decades, a vast number of attempts have been directly paid to develop carriers for nucleic acid drugs for sufficient therapeutic outcomes *in vivo*, which can be classified into viral and non-viral vectors. Although viral vectors can improve the therapeutic efficacy by high gene transfection in clinical trials, their safety concerns, including immunogenicity, host genome integration, and oncogenesis, are still major drawbacks. Alternatively, the non-viral vectors (i.e. cationic liposomes, polymers, and proteins) are potentially less immunogenic; however, their lower transfection efficiency and nonspecific interaction remain issues. To overcome these hurdles, a fabrication of non-viral vectors using cell-

selective ligands has been extensively developed to obtain effective targeted delivery of nucleic acids with high transfection efficiency comparable to viral vectors.

Among the possible targeting ligands, the membrane carbohydrate-binding proteins or membrane lectins (1) are of great interest. In mammalian cells, membrane lectins with different carbohydrate specificities are differentially expressed on the cellular membrane (2) as well as on the nuclear membrane (nuclear lectins) (3). The multiplicity, high affinity, and effective endocytosis after binding as well as biocompatibility of carbohydrate ligands endow them as a potential receptor-ligand pair for targeting delivery using glycosylated carriers (4, 5). Beyond the emerging applications of membrane lectins in targeted drug delivery reported by Brownlee and Monsigny groups in the 1980s (5, 6), their applications for delivering nucleic acids to specific cells has been recognized by many groups (7–9) and was later termed as "glycotargeting" (10, 11).

In this approach, carbohydrate or sugar moieties are either conjugated to nucleic acids or carriers by glycosylation. The latter involves a non-covalent complex with nucleic acids. Because glycosylated carriers are chemically flexible and can be tailored without changes in DNA function (12, 13), they provide a versatile application for various types of nucleic acids (14, 15). This glycosylation strategy exploits a highly selective delivery of nucleic acids to the target cells after receptor-mediated endocytosis. Recently, some sugar moieties in glycosylated carriers have been reported to be involved in the nuclear delivery of nucleic acids (16). In this review, recent applications and viewpoints of glycosylated carriers for cell-selective and nuclear delivery of nucleic acids are discussed.

## 3. NON-TARGETED DELIVERY OF NUCLEIC ACIDS

To achieve the *in vivo* gene transfer, nucleic acids must be delivered to the target cells, especially the nucleus, for them to be functional. This process has many reasons for concern including their stability in biological milieu, distribution or uptake in target cells, and nuclear delivery. Nucleic acids are negatively charged macromolecules which are biologically unstable due to nuclease degradation. Based on the electrostatic repulsion of nucleic acids to the cellular membrane, their cellular uptake is extremely low. Nucleic acids or pDNA are rapidly eliminated from the blood circulation with high clearance in the reticuloendothelial system after intravenous administration of ($^{32}$P)-labeled pDNA in mice (17, 18). The accumulated pDNA was gradually decreased over time in accordance with very low gene expression suggesting degradation by nuclease enzymes (18, 19).

Many delivery strategies have been developed to improve the bioavailability of nucleic acids. For practical use in the *in vivo* administration, nucleic acids can be simply condensed with cationic carriers via electrostatic interaction to protect themselves from nuclease degradation and increase cellular uptake. Due to the positive potentials of complexes, the nonspecific tissue accumulation of intravenously injected cationic carriers/pDNA complexes can be observed in the lung, liver, and spleen without tissue of preference (18, 20). In most cases, the highest gene expression is found in the lung where the first trap of complexes in pulmonary capillaries takes place. The uptake mechanisms of cationic carriers/pDNA complexes have been proposed to occur by endocytosis and non-endocytosis. In endocytotic pathways, especially receptor-mediated mechanisms, cationic carriers/pDNA complexes are routed to the endosomal and sometimes to lysosomal compartments where enzymatic degradation occurs. Therefore, the endosomal escape is an essential step to release pDNA for nuclear delivery. Finally, pDNA must transport across the nuclear membrane to gain access for transcription or function. In the quiescent state, the nuclear membrane excludes a diffusible nuclear access of macromolecules with MW > 45 kDa or 250–310 bp including pDNA unless they possess transporting signals (21). The nuclear import of pDNA is the most substantial step for successful gene transfer. With this premise, targeted gene delivery systems with effective nuclear import are of paramount importance to improve biodistribution to targeted cells and enhance nuclear translocation for gene expression.

## 4. THE RATIONALE OF GLYCOSYLATION FOR TARGETED DELIVERY OF NUCLEIC ACIDS

The membrane lectins are differentially expressed in normal and malignant cells and classified into cellular and intracellular lectins. Table 1 shows the membrane lectins related to the targeted delivery of nucleic acids. The cellular membrane lectins are well-characterized in liver and monocytic lineage cells and partly function in the host defense mechanism (22), for example, asialoglycoprotein receptors on hepatocytes (23), mannose receptors on macrophages (24), and fucose receptors on Kupffer cells (25). Generally, the membrane lectins recognize their corresponding carbohydrate ligands with high affinity. Thereafter, these bound structures are taken up into cells via endocytosis depending on the type of ligand-receptor and ligand binding specificity (2, 26, 27). The lectin receptor-mediated endocytosis induces endosome-lysosome trafficking of various glycosylated carriers, such as mannosylated emulsions (28, 29), mannosylated liposomes (30, 31), mannosylated chitosan (Man–chitosan) (32, 33), and mannosylated polyethyleneimine (Man–PEI) (34). This suggests that the lectin-binding and endocytosis may be less affected by the conjugated carriers or cargoes. The high targeting efficiency can be achieved when a multivalency or high density called "cluster effect" of carbohydrated ligands is applied (35, 36). It is noteworthy that carbohydrate ligands, such as galactose residues, can be recognized by several lectin receptors; asialoglycoprotein receptors on hepatocytes and putative galactose-particle receptors on Kupffer cells. However, the latter receptors seem to be predominant for uptake of larger-size (> 12 nm) galactosylated carriers but with somewhat less selectivity and affinity (37, 38). On the other hand, carbohydrate ligands with structural similarity, such as L-fucose and D-mannose, may be accommodated in the same binding site as shown by the uptake inhibition of each other (28, 39).

**Table 1.** The known and putative cellular membrane lectins and their ligands

| Lectin receptors | Ligands | Expression | References |
|---|---|---|---|
| Asialoglycoprotein | Galactose, asialoglycoprotein, *N*-acetylgalactosamine | Hepatocytes | (23) |
| Mannose | Mannose, fucose, *N*-acetylglucosamine | Macrophages, monocytes, Kupffer cells, hepatic endothelial cells, dendritic cells | (24, 27, 144) |
| Cationic-independent mannose-6-phosphate/insulin like growth factor II (CI-M6P/IGF-II) | Mannose-6-phosphate | Macrophages, hepatic stellate cells, cancer cells | (78, 145, 146) |
| Fucose | Fucose | Kupffer cells | (25) |
| Galactose particle | Galactose, fucose, *N*-acetylgalactosamine | Kupffer cells | (37, 38) |
| Selectin | Sialyl Lewis x | Inflamed or activated endothelial cells, cancer cells | (147) |
| Galectin | Galactose | Cancer cells | (95) |

The nuclear lectins play an essential role in regulation of nuclear transport of glycoproteins in cell homeostasis (Table 1). The nuclear transport of glycoproteins via nuclear lectins has been characterized to be involve a) sugar-specific binding, b) energy-dependent, c) unsynchronized cell-dependent, d) cell type-dependent nuclear lectin expression, and e) nuclear localization signal (NLS) peptide-independent pathway (40–42). Although there is a slight disparity in nuclear lectin expression and their nuclear transporting capacity in various cells, their potential for nuclear import of nucleic acids has been proposed using glycosylated carriers as will be discussed later. The selection of suitable carbohydrate ligands in glycosylated carriers may allow selective cellular uptake and effective intracellular trafficking of nucleic acids for efficient delivery in target cells.

## 5. GLYCOSYLATED CARRIERS FOR CELL-SELECTIVE DELIVERY OF NUCLEIC ACIDS

Glycosylated carriers have been extensively developed based on glycosylation with carbohydrate residues on the structure of cationic polymers, liposomes, chitosan and nanoparticles. The summary of carbohydrate ligands and various glycosylated carriers used for cell-selective delivery of nucleic acids is shown in Figures 1 and 2, respectively.

### 5.1. Hepatocytes

Hepatocytes associate with the pathology of many diseases including chronic hepatitis and hepatobiliary disease. The early attempts for hepatocyte-targeted gene delivery were reported using natural ligands of asialoorosomucoid-linked poly-L-lysine (PLL) (7) and asialofetuin-modified liposomes (43). The synthetic galactose ligands were extensively applied in galactosylated lipopolyamine (44), galactose residues-modified PLL (Gal–PLL) (8, 45, 46), and galactosylated PEI (Gal–PEI) (47, 48) due to better stability, reproducibility and low immunogenicity. The stable complexation, transfection efficiency, and cytotoxicity of these Gal–polymers seemed to be affected by the molecular weight of polymer (45, 48).

As for targeted lipid nanoparticles, we synthesized a galactose-modified cholesterol, cholesten-5-yloxy-*N*- (4- ( (1-imino-2-β-d-thiogalactosylethyl)amino)alkyl)formamide (Gal–C4–Chol) as a ligand of asialoglycoprotein receptors (14). Gal–C4–Chol can be stably anchored in the lipid structure of cationic liposomes to form galactosylated cationic liposomes (Gal-cationic liposomes) which were relatively low cytotoxic (14). By optimizing galactose density and physicochemical properties, Gal-cationic liposomes were extensively and selectively accumulated in the hepatocytes after intravenous and intraperitoneal administration in mice (49). The high gene expression was generated via an asialoglycoprotein receptor-mediated fashion. These Gal-cationic liposomes were used to deliver siRNA with a corresponding sequence to the endogenous *Ubc13* hepatic gene by intravenous administration (15). The silencing effect of these hepatocytes-targeted Ubc13 siRNA occurred in a concentration-dependent manner. An Ubc13 siRNA at a dose of 0.29 nmol/g suppressed more than 80% of *Ubc13* mRNA expression. These findings are coincident with a report by Park *et al.* for galactosylated chitosan with a polyethylene glycol (PEG) modification (Gal–chitosan–*graft*–PEG). Gal–chitosan–*graft*–PEG was able to enhance the solubility of chitosan and stability of its complex with a decoy ODN against the *YB-1* transcription factor (50). The significant inhibition of *YB-1* function was observed in HepG2 hepatoma cells, despite a lower effect than the control cationic liposomes.

Recently, hepatocyte-targeted gene transfer has been reported using a variety of galactose-containing carriers including galactosylated water soluble chitosan derivatives (51), DNA-encapsulating galactosylated lipid nanocapsules (52), and Gal–chitosan–*graft*–PEI (53). However, galactosylated polyamidoamine (PAMAM) dendrimer/α-cyclodextrin conjugates (Gal–α-CDE) exhibited high gene expression without cell-selective delivery to hepatocytes which was suggested by a shorter spacer of phenyl isothiocyanate (54). The efficient delivery of nucleic acids to hepatocytes was accomplished by the successful binding of galactose residues to asialoglycoprotein receptors using galactosylated carriers with a proper spacer. For instance, using four carbon atoms or higher between the galactose residues and carrier backbone in Gal–PEI (47) and Gal–C4–Chol (14) were required for high gene transfection. For this reason, Arima *et al.* developed lactosylated PAMAM dendrimer/α-cyclodextrin conjugates (Lac–α-CDE) containing galactose residues with a longer spacer and demonstrated a successful delivery of pDNA to HepG2 hepatoma cells in a cell-selective manner through asialoglycoprotein receptors (55). It is noteworthy to emphasize the importance of the spacer in addition to the design of the carbohydrate ligands themselves for cell-selective delivery of nucleic acids.

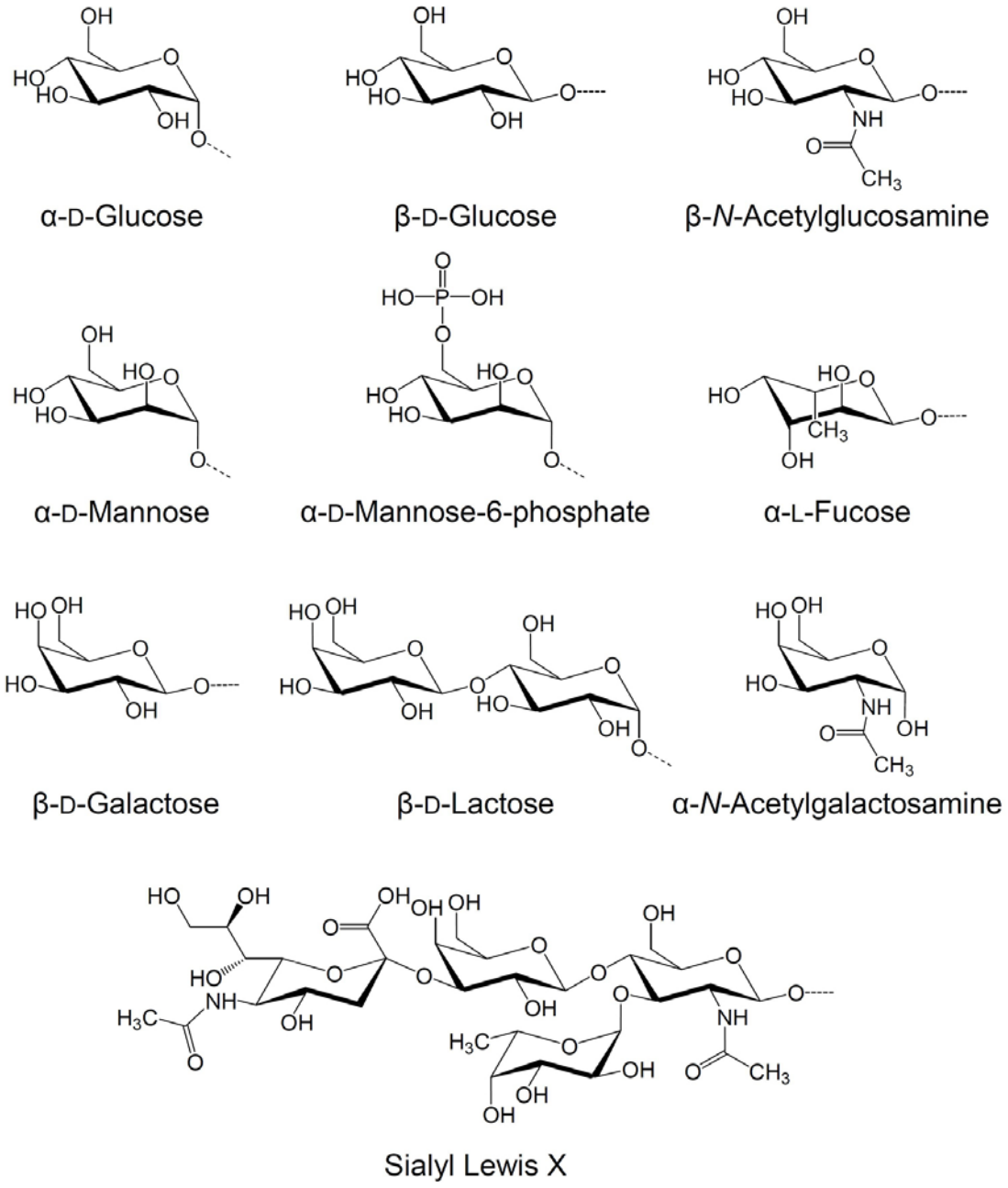


**Figure 1.** The carbohydrate ligands used in the targeted gene delivery.

**5.2. Macrophages**

There are many types of macrophages localized in various organs including the liver and lung, and their localization is related to their functions in host immune defense against infection, immune imbalance and inflammation. By employing a mannose receptor-targeting approach, mannosylated PLL (Man–PLL) was used to introduce pDNA and antisense ODN into human blood monocyte-derived macrophages (56) and alveolar macrophages (57), respectively. After intravenous administration in mice, these complexes exhibited a markedly high gene expression due to high affinity to mannose receptors on macrophages compared to other glycosylated counterparts (58, 59). We developed mannosylated cationic liposomes (Man-cationic liposomes) containing a mannosylated cholesterol derivative (Man–C4–Chol), cationic lipids, and helper lipids for complexation with pDNA (60) or CpG ODN (61). These complexes were selectively delivered to the liver nonparenchymal cells (NPC) consisting of Kupffer and hepatic endothelial cells with less nonspecific accumulation in the lung after intravenous and intraperitoneal administration (60–62). Subsequently, CpG triggered an immune response in the macrophages for suppression of a

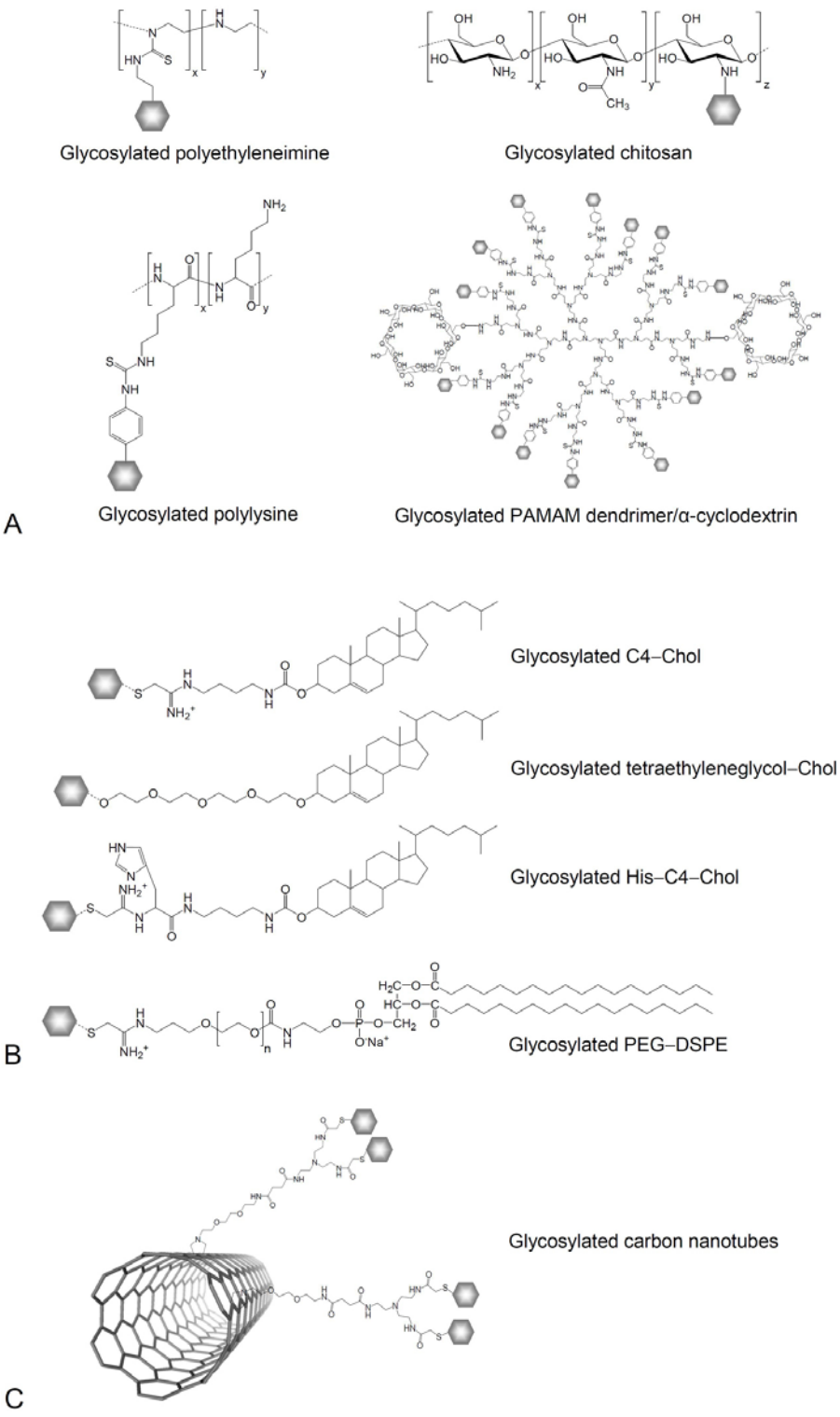


**Figure 2.** The structure of glycosylated carriers for targeted gene delivery. (A) Glycosylated polymers, (B) Glycosylated lipids in liposome formulations, and (C) Glycosylated carbon nanotubes. Hexagonal symbols represent carbohydrate ligands.

hepatic metastatic tumor (61). In addition, the more effective delivery of decoy ODN against NFκB (NFκB decoy) to Kupffer cells was observed in fucosylated cationic liposomes using Fuc–C4–Chol in a fucose receptor-mediated fashion (63). These led to the sufficient inhibition of cytokines and NFκB activity in a hepatitis

mouse model. Besides systemic application, pulmonary delivery of Man-cationic liposomes/NFκB decoy complexes exhibited low toxicity and good success in targeting to alveolar macrophages for sufficient anti-inflammatory therapy in a lung inflammation rat model (64). Recently, similar results were reported for high gene expression in alveolar macrophages using mannan-modified solid lipid nanoparticles after intratracheal instillation in rats (65). More recently, we have developed ultrasound responsive Man–PEG-cationic liposomes containing Man–PEG–DSPE lipid by incorporating perfluoropropane gas in the liposomes (66). A much higher amount of gene expression in liver NPC cells was triggered by external ultrasound exposure to the liver after intravenous injection of Man–PEG-cationic liposomes/pDNA complexes. The mechanism was proposed to be mannose receptor- and ultrasound-mediated.

The other macrophage-targeting systems were reported using Man–PEI (34), Man–chitosan derivatives (32, 33), mannosylated PAMAM dendrimer/α-cyclodextrin conjugates (Man–α-CDE) (67, 68) and cationized glucomannan-containing polysaccharides (69). Similar to Gal–α-CDE, Man–α-CDE with a short spacer was reported useful for high gene transfection in various cells without restriction to the expression of mannose receptors (67, 68). Based on the results of Lac–α-CDE (55), a longer spacer of Man–α-CDE may improve the recognition of mannose residues by mannose receptors for macrophage-selective delivery of nucleic acids. In the light of these findings, mannosylated carriers can deliver nucleic acids to resident macrophages by varied administration routes. Therefore, they are considered to be promising systems for macrophage-targeted gene delivery.

### 5.3. Dendritic cells

Dendritic cells (DC) are professional antigen-presenting cells that are localized in the spleen and peripheral organs, like skin, for capturing and processing antigens to T cells for immune response. Due to a high expression of mannose receptors on DC, Man–PEI was first employed by Diebold *et al.* for effective high gene expression in cultured-human DC derived from peripheral blood mononuclear cells (34). Based on these findings, a DNA vaccine has been extensively developed using mannosylated carriers for the treatment of cancer *in vivo*. We earlier demonstrated a DNA vaccine against ovalbumin (OVA)-expressing tumors by delivery of OVA-encoding pDNA (OVA-pDNA) using Man-cationic liposomes (70) and ultrasound responsive Man–PEG-cationic liposomes (71). The intravenously injected complexes significantly increased OVA expression in splenic $CD11^{+}c$ cells (71, 72). Thereafter, a MHC class I-restricted antigen presentation on these cells triggered Th1-cytokine release from CD8OVA1.3 T cells. Importantly, their more effective gene transfer in the liver, spleen, and peritoneal lymphoid tissues was observed after intraperitoneal administration compared to conventional vaccination routes (i.e. subcutaneous and intradermal) due to direct accessibility (62, 70). The intraperitoneally injected Man-cationic liposome/OVA-pDNA complexes exhibited high cytotoxic T cell activity against OVA-expressing tumors and prolonged survival of solid tumor-bearing mice (70). Similarly, Man-cationic liposomes were successfully extended to targeting of melanoma-associated antigen encoding pDNA for melanoma vaccination in mice (73).

The DC-targeting DNA vaccine using mannosylated carriers, including Man–PLL (74), Man–chitosan (75), mannosamine-coated poly (anhydride) nanoparticles (76), and mannose-mimicking quinic acid and shikimic acid-liposomes (77), have been reported for successful vaccination via conventional and oral routes. Man–chitosan/IL-12-encoding pDNA complexes efficiently suppressed tumor growth and promoted tumor apoptosis after intratumor injection in colon carcinoma-bearing mice (32). This effective immunotherapy was suggested by the sufficient gene transfer to DC in the tumor environment. Taking these findings into consideration, the efficient gene transfer to DC via local and systemic administrations of mannosylated carriers may allow their use for cancer immunotherapy and DNA vaccination.

### 5.4. Hepatic stellate cells

Hepatic stellate cells (HSC) comprising 5–15% of the liver cell population are key effector cells used to release cytokines and collagen type I for intrahepatic connective tissue formation in liver fibrosis (78). In this pathological condition, mannose-6-phosphate/insulin-like growth factor II (M6P/IGF-II) receptors are up-regulated for high expression at the cellular membrane of these cells. HSC targeting using M6P ligands has been focused on the delivery of antifibrotic drugs but is rarely used for nucleic acids. Recently, Mahato and colleagues have developed HSC targeting of triplex-forming antisense ODN (TFO) using M6P-modified bovine serum albumin (M6P–BSA) via the use of reducible disulfide bonding (79, 80) and M6P–*N*- (2-hydroxypropyl)methacrylamide (HPMA) polymer via use of lysosomal enzyme-trigger cleavable GFLG peptide linker (81). While stable in rat serum, conjugated TFO was cleaved under certain conditions which allowed TFO to form a triplex with the target DNA in the rat α1 (I) collagen promoter resulting in inhibition of transcription of the collagen gene. M6P-targeting TFO conjugates exhibited a significant accumulation in hepatic stellate cells and liver NPC after intravenous administration in normal rats (79). Interestingly, the selective uptake in HSC became dominant in fibrotic rats (80). The uptake of M6P-targeting TFO conjugates was characterized by M6P density-dependent and M6P/IGF-II-mediated mechanisms (80, 81). In addition, the effective nuclear delivery of M6P-targeting TFO conjugates in HSC-T6 cells resulted in sufficient inhibition of collagen expression. These observations suggested that M6P-modified carriers are promising delivery systems of nucleic acid drugs to HSC for the treatment of liver fibrosis.

### 5.5. Lung epithelial cells

Gene delivery to epithelial cells has been mainly focused on gene therapy for cystic fibrosis (CF) and is ongoing in clinical trials. CF is the most common lethal autosomal recessive disorder for progressive respiratory failure in the Caucasian population. It is caused by the

mutations of the CF transmembrane conductance regulator (CFTR) gene in airway epithelial cells. The epithelial cell-targeted delivery of nucleic acids has been demonstrated by the Monsigny and Fajac groups. The lactosylated PLL (Lac–PLL) was an effective vector for pDNA delivery by offering high expression of a reporter gene in CF cell lines (82) and primary cultured CF and non-CF airway epithelial cells (83). In addition, the gene expression was increased in the presence of chloroquine indicating the endosomal escape of trapped Lac–PLL/pDNA complexes for enhanced gene transcription. The high transfection efficiency was also obtained by the use of lactosylated polyethyleneimine (Lac–PEI) (82, 84), cationic-lactosylated liposomes (85), β-D-acetylglucosamine substituted β-gluconoylated PLL (GlcNAc–GlcA–PLL) (82), and GlcNAc–GlcNAc–Man trisaccharide–*graft*–chitosan (86) compared to other glycosylated or unmodified counterparts. The function of lactose residues in Lac–PLL on transfection efficiency was demonstrated by the increased cellular uptake via a lactose binding receptor-mediated mechanism and the enhanced nuclear localization of pDNA (87). The successful gene delivery of lactosylated carriers indicates their potential for development of targeted gene therapy in airway epithelial-related diseases such as CF.

### 5.6. Inflamed or activated endothelial cells

Endothelial cells in various organs possess anatomically unique structures and different lectin expression depending upon their physiological function and pathological conditions (Table 1). For example, selectin receptors are greatly expressed on inflamed or activated vascular endothelial cells in the inflammation and tumor regions (88). The sugar chains called "sialyl Lewis X" ($sLe^x$) on the surface of leukocytes are recognized and bound to these endothelial cells for leukocyte attachment in a "rolling" manner. They have been suggested as potential target molecules for drug and gene delivery for the treatment of cardiovascular, inflammatory and cancer diseases. Recently, Yamazaki and Tano groups demonstrated that $sLe^x$-modified liposomes were highly accumulated in inflamed and tumor tissues after adhesion to blood vessels via selectin-mediated fashion (89, 90). These findings correspond to the ability of $sLe^x$-modified liposomes to inhibit the accumulation of leukocytes at inflamed endothelial cells in ischemic-reperfusion mice (91). Stahn *et al.* employed this approach to deliver antisense ODN against the adhesion molecule ICAM-1 to the IL-1β-activated endothelial HUVEC cells (92). The targeted antisense ICAM-1 ODN inhibited ICAM-1 protein expression, not mRNA, in a concentration-dependent manner. The inhibition mechanism was assumed to be resulted from the hybridization of antisense DNA to their corresponding mRNA to interrupt protein synthesis. These findings indicate the feasibility of $sLe^x$-modified liposomes for targeted delivery of nucleic acid drugs to activated endothelial cells in several diseases as aforementioned.

### 5.7. Cancer cells

Various membrane lectins or glycans are significantly expressed on a number of cancer cells associated with poor prognosis and tumor progression via adhesion, proliferation, migration, inhibition of apoptosis, and metastasis. These membrane lectins and glycans have been extensively studied for cancer targeting of drugs but few have been used to target nucleic acids. Recent studies have been reported using galactose-binding lectins (Galectins) (93) and β1-6 branching *N*-GlcNAc (94) as targeted receptors.

Galectins have been characterized into 15 subtypes expressed on the surface of many cancer cells, including prostate, lung and breast carcinomas (95). Based on high specific binding to galectins via galactose residues, modified citrus pectin (MCP) was used as a ligand and derivatized with different amine groups for pDNA complexation (93). The quaternized MCP/pDNA complexes exhibited high transfection efficiency in HEK293 cells through galactose residue-mediated targeting. The modified pectin was suggested for targeted gene delivery to cancer cells expressing galectins on their surface.

The overexpression of GlcNAc is closely related to the activity of N-acetylglucosaminyltransferase V (GnT-V) in metastatic cancer cells of colon, breast, melanoma, and kidney carcinomas (96). The kidney bean-derived lectin, *Phaseolus vulgaris* agglutinin-$L_4$ ($L_4$-PHA), was used for cancer diagnosis of GlcNAc-expressing malignant cancer cells (97). Kuroda and colleagues reported that $L_4$-PHA-modified bionanocapsules ($L_4$-PHA–BNC) were effectively accumulated in GlcNAc-expressing malignant tumors after intravenous injection in solid tumor-bearing mice model (94). The $L_4$-PHA–BNCs were able to deliver pDNA to these targeted cancer cells for gene expression, suggesting their potential use as a targeting gene vector for GlcNAc-expressing cancers.

## 6.GLYCOSYLATED CARRIERS FOR ENDOSOMAL ESCAPE AND NUCLEAR DELIVERY OF NUCLEIC ACIDS

The cellular uptake and intracellular trafficking of nucleic acid-containing glycosylated carriers is shown in Figure 3. Glycosylated carrier/nucleic acid complexes are taken up in the cells via endocytosis pathway and trapped in the endosome/lysosome vesicles. As stated, the release of nucleic acids from endosomes is a crucial step for trafficking to the intracellular site of action, such as pDNA-based vectors to the nucleus and siRNA, antisense and decoy ODN to the cytosol. Many polymers, including PEI and PAMAM dendrimers, have high endosomal escape capacity via proton-mediated osmolysis of the endosomal membrane called the "proton-sponge effect" (98, 99). As for targeting gene delivery to airway epithelial cells, Lac–PEI still retained a proton-sponge effect, albeit somewhat reduced, and was minimally toxic, thus, a high gene expression was observed (84, 100). In addition, the endosomolytic property of glycosylated PLL seems to be sugar-dependent since Lac–PLL/pDNA complexes are more efficient endosomal escape than Man–PLL/pDNA complexes (82, 101). The delayed endosomal escape is believed to be a rate limiting step resulting in low gene transfer.

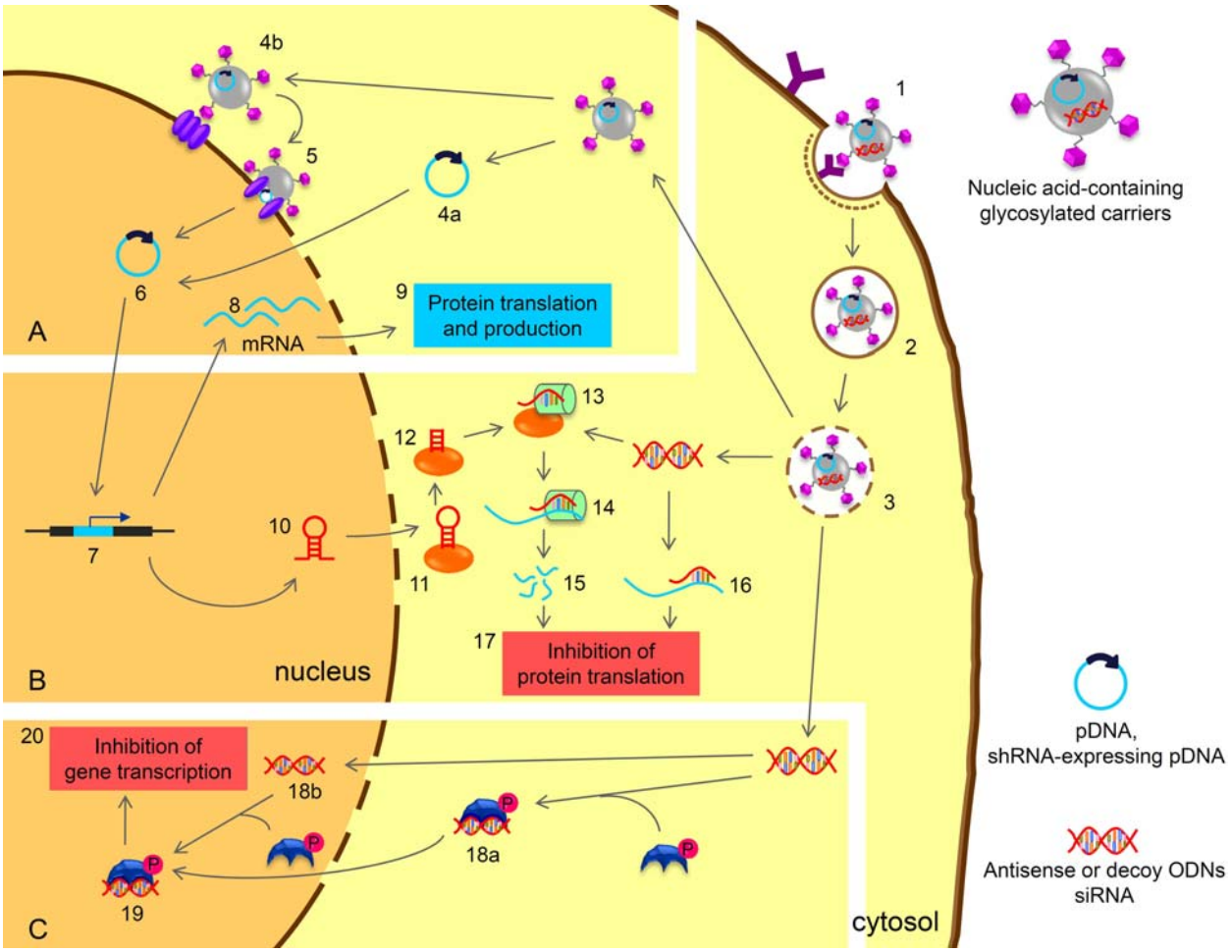


**Figure 3.** Schematic representation of cellular uptake and intracellular trafficking pathway of nucleic acids using glycosylated carriers. Nucleic acid-containing glycosylated carriers bind to their corresponding lectins for cellular uptake (1). These carries are sorted to the endosomes (2). The endosomal escaping of glycosylated carriers (3) triggers the release of nucleic acids, including (A) pDNA-based vector, (B) siRNA and antisense ODN, and (C) decoy ODN for their intracellular site of action. (A) The pDNA can enter the nucleus (6) by passive (4a) or nuclear protein-mediated mechanisms (4b, 5) for gene transcription (7, 8) and protein production (9). (B) The short hairpin RNA (shRNA)-expressing pDNA follows the same step as pDNA to produce shRNA (10). After cytosolic translocation, shRNA is processed to be siRNA (11, 12). The siRNA is then loaded into Ago/RISC protein complex (13). The binding of siRNA (14) or antisense ODN (16) mediates mRNA cleavage (15) or inactivation (16) leading to inhibition of protein translation (17). (C) Decoy ODN binds to activated transcription factor protein (18a) or directly enters the nucleus before binding (18b). The transcription factor protein-bound decoy ODN in the nucleus (19) inhibits gene transcription (20).

To increase the endosomolytic property, a histidine moiety that has a pKa value for protonation at the endolysosomal pH, has been attached to glycosylated carriers using histidylated conjugates, such as Lac–His–PLL (102), Gal–His–C4–Chol (103) and Man–His–C4–Chol (104). Similar to Lac–His–PLL/pDNA complexes, Gal– and Man–His–liposomes/pDNA complexes exhibited a more efficient gene transfer than their unmodified counterparts via asialoglycoprotein receptor- and mannose receptor-mediated mechanisms in HepG2 hepatoma cells and peritoneal macrophages, respectively. The enhanced gene transfection was triggered by the sufficient pH-buffering capacity of these histidylated carriers leading to efficient endosomal escape. Fusogenic peptides having endosomolytic properties, such as influenza HA-2 (8, 57, 105), influenza E5 derivatives (87, 106), GALA (106) as well as pH-sensitive dioleoylphosphatidylethanolamine (DOPE) lipid (107), have been reported for endosomal escape of glycosylated PLL and liposomes.

Based on the emerging knowledge of the carbohydrates in nuclear transport using glycoproteins, glycosylation strategies have been employed for nuclear delivery of enhanced gene transfer. Fajac *et al.* demonstrated that Lac–PLL/pDNA complexes are the most efficient vectors for gene transfer in airway epithelial cells (82). While most complexes were trapped in the

endolysosomal compartments, 8% of the complexes were localized in the nucleus within 6 h as determined by dual labeling of the carriers and pDNA (101). However, the nuclear localized complexes remained intact with no dissociation of pDNA. They have also reported the nuclear import of Lac–PEI/pDNA complexes via NLS- and lactose-independent mechanisms in digitonin-permeabilized cells (108).

Surprisingly, the nuclear localization of complex-dissociating Lac–PEI was evidenced in airway epithelial cells after nasal instillation in mice (109). In addition, Klink *et al*. reported that β-D-Lac–PLL/pDNA complexes effectively accumulated in the nucleus and perinuclear regions more efficiently in the presence of chloroquine, peptide, and glycerol (87, 110). The nuclear accumulation was markedly inhibited by wheat germ agglutinin. This suggested mechanism of nuclear entry was via the recognition of lactose residues of a putative galactose/lactose-specific lectin at the nuclear membrane. This hypothesis, nevertheless, is not in agreement with the observation that there was no nuclear import of lactosylated BSA after cytosolic microinjection in Hela cells (42). Instead, it might be explained by the difference in expression of nuclear lectins in various cells and unknown factors.

A more direct comparison has been demonstrated using glycosylation of a multifunctional envelope type nano device (MEND) with β-GlcNAc, α-mannose, and β-galactose residues for gene transfection in Hela cells (111). In this system, pDNA was condensed with polycations, followed by encapsulation in glycosylated MEND. Although the cellular uptakes were similar, glycosylated MEND showed higher transfection efficiency than unmodified MEND in normal (10–25-fold) and synchronized (14–58-fold) Hela cells. The confocal image analysis revealed larger nuclear localization of glycosylated MEND suggesting sugar residue-mediated nuclear delivery for improved gene transfer. This agrees with the enhancement of nuclear entry of Man–α-CDE in various cell lines (67, 68). Although the precise mechanism needs further investigation, evidence of glycosylated-mediated nuclear localization may make it a potential route for the nuclear delivery of nucleic acids.

## 7.FACTORS AFFECTING TARGETED DELIVERY OF NUCLEIC ACIDS USING GLYCOSYLATED CARRIERS

The processes of access to target cells, intracellular trafficking, and gene transcription, dictate the efficiency of *in vivo* gene transfer. To achieve therapeutic effects of nucleic acids using glycosylated carriers, many factors have to be optimized besides the design of the carbohydrate ligands. Under *in vivo* conditions, glycosylated carriers/nucleic acid complexes may nonspecifically interact with many biological molecules, such as proteins and blood cells, due to their positive charges. The accessibility to the target cells for binding is another factor to consider. After uptake, the endosomal escape for nuclear translocation of pDNA is an important step as described previously. The factors related to targeted delivery using glycosylated carriers are discussed in this section.

### 7.1.Size

Generally, pDNA is complexed with cationic carriers via electrostatic interactions in low ionic strength buffer such as 5% dextrose. The higher the amount of cationic carriers, the smaller the complex size and the more stable the pDNA condensation (112). The size of glycosylated complexes designates their accessibility to the target cells after administration. Under physiological or high salt conditions, complexes with positive charge have the tendency to aggregate by neutralization resulting in increased size or precipitation (113). These may limit their use *in vivo*, especially for hepatocyte targeting (114, 115).

Hepatocytes are anatomically localized beyond the fenestraes with 100–150 nm in size of hepatic endothelial cells. It is important to prepare complexes that are smaller than this cut-off. The reduced size of Gal–PLL (45) or Gal–PEI/pDNA (48, 112) complexes made them effective for accumulation in hepatocytes. More importantly, very large size complexes trended to be exclusively trapped in the lung capillary before reaching the target cells leading to failure of targeted gene delivery. For cancer gene therapy, complexes have to penetrate through leaky tumor vasculature which is permeable to liposomes of less than 400 nm in diameter (116). Hence, a controlled size of complex is one of the crucial factors for effective delivery of nucleic acids to target cells.

### 7.2.Surface potential

The neutralization of cationic complexes by biological milieu, such as proteins and blood cells, may affect not only complex size but also cellular uptake. Low cellular uptake due to neutralization by endogenous proteins (117) or PEG modification (118) leads to low transfection efficiency. However, increasing the cationic charge ratio of complexes does not alleviate this problem. Upon intravenous administration, cationic complexes immediately bind to blood cells and then are nonspecifically trapped in the lung (119). We demonstrated this factor on gene targeting using Gal- (120) and Man- (121) cationic liposomes. The efficient gene transfer at the optimal charge ratio of these complexes in the target cells was dramatically reduced at a higher cationic charge ratio due to high trapping in the lung. In order to obtain a better understanding, we evaluated the effects of blood components on asialoglycoprotein receptor-mediated gene transfer of Gal-cationic liposomes after intraportal administration in mice (122). Pre-incubation with serum increased the tissue binding affinity of these complexes and thus, the gene transfection in the liver was enhanced by reducing lung entrapment. On the other hand, blood cells could competitively bind to Gal-cationic liposomes for dissociation of pDNA leading to low transfection efficiency.

There are many methods, such as modification with PEG, for reducing the surface charge to lower the nonspecific interaction. Recet

ly, we developed a new vector system called surface charge regulated (SCR) liposomes which forms a more stable complex with pDNA for *in vivo* applications (123). Complexes were prepared in ionic buffer containing 5–10 mM sodium chloride which is a much lower concentration than isotonic saline (150 mM sodium chloride) as in physiological conditions. Gal-SCR liposome/pDNA complexes delayed aggregation in saline compared to Gal-cationic liposome/pDNA complexes. Overall, 10- to 20-fold higher gene expression was observed in HepG2 hepatoma cells. It was suggested by an accelerating release of pDNA from Gal-SCR liposomes. These findings emphasize the need to control the surface charge for sufficient cellular uptake, pDNA condensation, targeting, efficiency, and ultimately gene expression.

### 7.3. State of target cells

The expression of membrane lectins depends on whether the cells are in differentiated or activated states. This dynamic phenomenon is prominent in monocytic lineage cells which have high differentiating capacity (Table. 1). Monocytes and macrophages express mannose and M6P receptors while differentiated macrophages, Kupffer cells, have additional lectins of fucose and galactose-particle receptors on their cellular membrane. In addition, Lac–PEI using the proton-sponge effect for endosomal escape of pDNA was more efficient for gene transfer than unsubstituted PEI and Lac–PLL (100). However, the transfection efficiency was lower in differentiated airway epithelial cells compared to poorly-differentiated epithelial cells. The low uptake due to absence of expression of lactose-binding lectins was suggested for the low gene expression of Lac–PLL in differentiated airway epithelial cells. In contrast, this was unlikely in the case of Lac–PEI since similar uptake to undifferentiated cells was observed. An unknown factor was suggested for the results of Lac–PEI.

Furthermore, many molecules, such as dexamethasone (124) and interleukin-4 (125), have been reported for up-regulation of mannose receptors on macrophages which may augment the targeting efficiency using mannosylated carriers. On the other hand, the down-regulation of mannose receptors is mediated by various stimuli, including lipopolysaccharide/phorbol ester (126) and interferon-γ (127), as well as HIV-1 infection (128), all of which may hamper the success of macrophage-targeted delivery of nucleic acids using mannosylated carriers. It is noteworthy that the alteration in expression of membrane lectins and intracellular trafficking or function in different cell states are critically important to achieve cell-selective delivery of nucleic acids using glycosylated carriers.

## 8. PERSPECTIVES

From advances in material technology, an emerging class of nanomaterials, including carbon nanotubes, carbon nanospheres, and gold nanoparticles are receiving enormous attention in many fields including biomedicine and nanotechnology. Although they possess unique characteristics of mechanical strength, electrical and cell penetrating properties, their application as biomaterials still faces many challenges due to their water insolubility, cytotoxicity, and nonspecific uptake (129, 130).

Recently, a modification with a cationic glycopolymer (glucose and lactose residues) at the surface structure of carbon nanotubes (131) has been reported for improving water dispersity and reducing the cytotoxicity. The cationic nature of carbon nanotubes (132) and gold nanoparticles (133, 134) exhibited a stable complex formation with fluorescent-labeled ODN and pDNA. On the other hand, glucose-derived carbon nanospheres with negative surface potential had to be coated with polycationic molecules before DNA complexation (135). These cationic glycosylated carbon nanotubes and gold nanoparticles effectively delivered fluorescent-labeled ODN to the cytosol and perinuclear region in Hela cells (132, 134) corresponding to the nuclear localization of glucose-derived carbon nanospheres (135). Their uptake mechanisms were speculated to occur via a lectin-mediated manner (136, 137). As a result, the high transfection efficiency was observed as an increased amount of these cationic glycosylated carbon nanotubes and gold nanoparticles was used. Interestingly, the intravenous injection of glucose-modified carbon nanotubes showed a high accumulation in the lung (138) while the intraperitoneal injection of glucose-derived carbon nanospheres showed a high accumulation in the spleen, liver and brain (135). However, data on their safety, targeting mechanisms, and *in vivo* studies need to be investigated although the early indications of these glycosylated biomaterials may be proposed cell-selective delivery of nucleic acids.

Although the efficient gene transfer can be improved by glycosylated carriers, a transient expression due to methylated silencing of the transgene may be one of limitations for clinical gene therapy. To overcome this hurdle, Roy-Chowdhury and colleages developed an excellent glycoproteo-liposome to deliver transposon pDNA for long-term reduction of jaundice in UGT1A1-deficient hyperbilirubinemic Gunn rats (139). The *Sleeping Beauty* (SB) transposon system, containing a donor plasmid of the therapeutic gene and a helper plasmid of a transposase, has been reported to promote transposition of the transgene through transposase activity into the host genome, thereby prolonging the expression of the transgene (140). Interestingly, a human *UGT1A1* therapeutic gene and transposase gene was constructed in the same *SB* plasmid (139). The glycoproteo-liposomes containing a fusogenic galactose-terminated F-glycoprotein selectively delivered the encapsulated transposon pDNA to hepatocytes via asialoglycoprotein receptor-mediated mechanism after intravenous administration. Thereafter, the fusogenic protein triggered cytosol deposition bypassing the endolysosomal pathway for sufficient gene expression. The therapeutic efficiency by reduction of serum bilirubin level was 30% in 2 weeks and remained throughout the 7-month period without immunogenic side-effects. The combination of efficient hepatocyte-targeted gene delivery and designed gene construct suggests a new platform for successful gene therapy with possible clinical applications.

**Table 2.** The known and putative nuclear lectins and their ligands

| Lectin receptors | Ligands | Expression | References |
|---|---|---|---|
| CBP35 | β-galactose, β-lactose, β-*N*-acetylgalactosamine | NIH/3T3 fibroblasts | (148) |
| CBP67 | α-glucose | Rat liver cells | (149) |
| CBP70 | α-glucose, β-*N*-acetylglucosamine | Hela cells | (150) |

Adapted with permission from (16)

While gene therapy using a viral vector holds much promise, it has been intensely criticized for clinical applications due to serious adverse effects including death and cancer development during clinical trials. Based on a 2007 survey, safety concerns lead to increases of trials using naked DNA and non-viral systems to be 18 and 8%, respectively (141). Most of the non-viral vectors used in clinical trials (Phase I and II) are lipid nanoparticles and cationic liposomes which incorporate or form a complex with antisense ODN (e.g. EGFR) and pDNA (e.g. CFTR, IL-2). These formulations have been suggested for administration by intravenous infusion or intratumor, and nasal instillation for the treatment of cancer and CF, respectively (142, 143). The targeted gene delivery using glycosylated carriers seems to show therapeutic advantages; however, many issues including lectin specificity (Table 1 and 2) and toxicity are needed to be carefully evaluated before clinical study (144-150). Although there is currently no targeted gene therapy using a non-viral system in clinical trials, the success of liposomal DNA formulations may guide the potential of targeted gene therapy for entering clinical trials in the near future.

## 9. CONCLUDING REMARKS

Glycosylated carriers represent an avenue for cell-selective and nuclear delivery of nucleic acids. This strategy allows the improved bioavailability of nucleic acids by increasing accumulation and nuclear import in the target cells. By employing a design approach in combination with functional molecules, such as fusogenic peptides or other endosomolytic agents, the targeting efficiency using these glycosylated derivatives can be augmented. According to the multiplicity of membrane lectins in a variety of mammalian cells and their ligand specificity, targeted delivery of nucleic acids using glycosylated carriers can be extended to wide applications in many diseases including chronic hepatic diseases, infectious diseases, Gaucher's disease, CF, and cancers. Nevertheless, it is ultimately important to define the specificity of glycosylated carriers to targeted cells under certain disease conditions as well as reduce the carrier-associated toxicity to ensure clinically therapeutic outcomes. Based on their relative safety, cell-selective delivery, and desired pharmacokinetic behavior, glycosylated carriers can be one of most promising delivery systems for nucleic acid drugs towards a large panel of refractory diseases.

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**Abbreviations:** siRNA: Small interfering RNA, Man–C4–Chol: Cholesten-5-yloxy-*N*- (4- ( (1-imino-2-β-D-thiomannosylethyl)amino)alkyl)formamide, NFκB: Nuclear factor kappa B, Fuc–C4–Chol: Cholesten-5-yloxy-*N*- (4- ( (1-imino-2-β-L-thiofucosylethyl)amino)alkyl)formamide, Man-PEG-DSPE: mannose-modified 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N- (poly (ethylene glycol)2000, IL-12: Interleukin 12, ICAM-1: Inter-cellular adhesion molecule 1, IL-1β, Interleukin-1 beta, Influenza HA-2: Influenza hemagglutinin 2, GALA: a synthetic, amphiphilic 30-amino acid peptide containing a glutamine-alanine-leucine-alanine repeat, HIV-1: Human immunodeficiency virus 1.

**Key Words:** Carbohydrate, Gene delivery, Glycosylated carrier, Glycotargeting, Lectin, Nuclear import, Nucleic acids, Review

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