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COMPREHENSIVE REVIEW

Glycotargeting to improve cellular delivery efficiency of nucleic acids

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Abstract Nucleic acids bearing glycans of various structures have been under vigorous investigation in the past decade. The carbohydrate moieties of such complexes can serve as recognition sites for carbohydrate-binding proteins—lectins—and initiate receptor-mediated endocytosis. Therefore, carbohydrates can enhance cell targeting and internalization of nucleic acids that are associated with them and thus improve the bioavailability of nucleic acids as therapeutic agents. This review summarizes nucleic acid glycosylation in nature and approaches for the preparation of both non-covalently associated and covalently-linked carbohydrate-nucleic acid complexes.

Keywords Carbohydrate · Nucleic acid · Drug delivery · Glycoconjugate · Lectin

Introduction

In the past two decades, nucleic acids have been investigated as potential therapeutic agents in various forms, including gene therapy, antisense oligonucleotides, antigene oligonucleotides, aptamers, and RNA interference (RNAi) [1]. Yet, their effectiveness has so far been hampered by a number of limitations. As nucleic acids are relatively large molecules with multiple negative charges, their cellular uptake is inefficient, resulting in poor bioavailability. In addition, cell targeting by these molecules is not specific. Research in nucleic acid chemistry has so far resulted in many structural modifications. These analogues included backbone, base residue, and internucleotide linkage modified nucleic acids. These modifications confer a wide variety of novel properties to nucleic acids, such as enhanced resistance to nucleases, increased ability to hybridize with complementary sequences, and higher efficiency in penetration through cell membranes.

In order to improve the cell uptake efficiency of nucleic acids, and consequently their bioavailability, a recent development in this area focuses on receptor-mediated endocytosis. In this approach, nucleic acids are either associated with a carbohydrate moiety non-covalently or directly linked to carbohydrate residues covalently. These carbohydrate residues can be recognized by cell surface carbohydrate-binding proteins–lectins. The interaction between lectins and carbohydrates then mediates the internalization of nucleic acids through receptor-mediated endocytosis. This strategy was termed glycotargeting [2] and has been demonstrated in many examples where carbohydrates served as the "magic bullet" and enhanced cellular uptake of nucleic acids that are associated with the glycans. In this review, the two major strategies that utilize the interactions between glycans and lectins to tackle the drug delivery problem of nucleic acids are summarized. In these strategies, a carbohydrate moiety can be non-covalently associated with nucleic acids, either directly or through a carrier molecule. Alternatively, the carbohydrate moiety can be directly linked to nucleic acids through covalent bond formation. Some of the chemistries involved in carbohydrate-oligonucleotide conjugate preparation were summarized in a recent review [3].

Natural occurrence and possible roles of sugar modified nucleosides

The occurrence of glycosylated nucleic acids is not as widespread as that of glycoproteins and glycolipids; howev-

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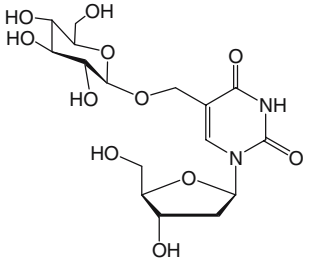
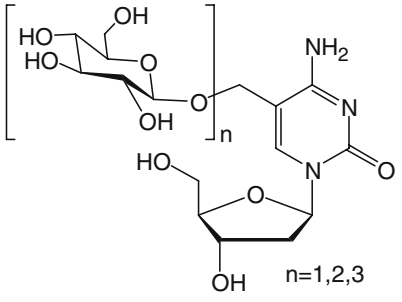
er, they do occur naturally, especially in bacteriophages and simple eukaryotes. One of the most common occurrences of glycosylation involves β-D-glucosylhydroxymethyluracil (as in **1**), or base J as it is known in the literature [4]. This base modification is found in some simple eukaryotes, such as *Euglena gracilis* [5] and *Trypanosoma brucei* which is the species causing African Sleeping disease [6]. In fact, base J has been detected in all species of the trypanozoon group [7]. In *T. brucei*, nucleoside J is found mostly in repetitive sequences such as the telomeric GGGTTA repeats and in the telomeric variant surface glycoprotein gene (VSG) expression sites involved in antigenic variation [6]. Although the functions of this base modification are not fully understood, it is hypothesized that J-modification may be involved in the translational repression of VSG gene expression and as a consequence antigenic variation [6, 8–11]. Proteins that bind duplex DNA containing J nucleoside (J-DNA) have been identified [9, 12, 13], and these J-binding proteins (JBP) may be involved in antigenic variation. The JBPs have been shown to interact with the major and minor grooves of J-DNA helixes at the J modification site and its immediate vicinity [14, 15]. In *E. gracilis*, the presence of nucleoside J is more uniform and is not localized in the telomeric repeats [5].



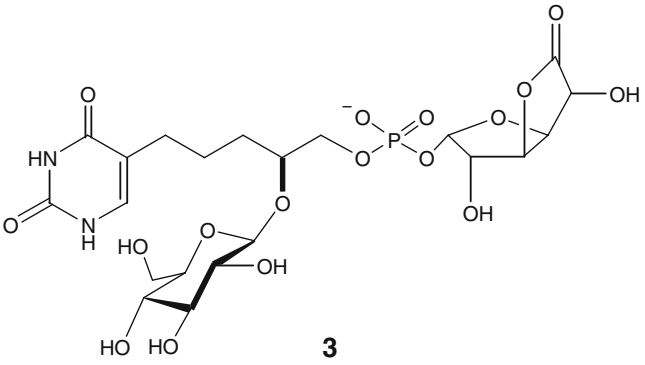
1

Another major naturally-occurring glycosylated nucleoside is glucosylated hydroxymethylcytidine. This modification was first identified in T-even bacteriophages [16, 17]. 5-*O*-Mono-, di-, and tri-glucosylated hydroxylmethylcytidines **2** were identified in *E. coli* phages T2, T4, and T6. In these phages, the occurrence of glucosylated DNA sequences accounts for about 1% of the total sequences [18, 19]. Glycosylation in these species increases the resistance of the phage DNA towards deoxyribonucleases [20].



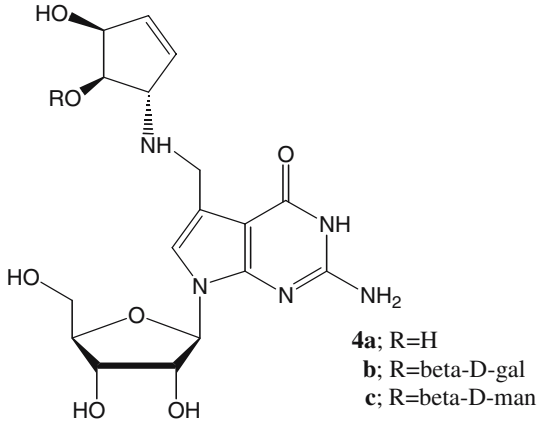
2

In *Rhizobium phage* RL38JI, deoxycytidine is replaced by as many as three hexosylated modified base residues [21]. In *B. subtilis* phage SP15, a more complex sugar residue **3** was identified to be covalently linked to uracil [22, 23]. In these phages, 62% of the thymine residues are replaced by 5-(4′,5′-dihydroxypentyl)uracil (as in **3**) with attachment to a phosphoglucuroniate via a phosphodiester linkage to one of the hydroxyl groups of the pentyl side chain (as in **3**).



3

In the RNA series, nucleoside queuosine (Q) derivative **4a**, which is a 7-deazaguanosine derivative with a cyclopentenediol side chain at C7 [24], was isolated from various organisms, including several mammals, starfish, linguals, hagfish, and wheat germ [10, 25]. These Q ribonucleosides can be glycosylated by either D-galactose (as in **4b**) or D-mannose (as in **4c**) in the β configuration [26].



4a; R=H
b; R=beta-D-gal
c; R=beta-D-man

Members of the Q nucleoside family are located at the first position of the anticodon (position 34) in bacterial and eukaryotic tRNAs that are specific for only four amino acids (Tyr, His, Asp, and Asn) [27, 28]. However, galactosyl (4b) and mannosylqueuosines 4c are only found in animal tRNAs.

The biological functions of glycosylated queuosines are not clear. However, based on the crystal structure of tRNA (asp) and a tRNA-tRNA-mRNA complex model, it was speculated that queuosine modification may serve as a structurally restrictive base for tRNA anticodon loop flexibility [29].

Nucleic acid delivery techniques

The phospholipid bilayer, which is the major component of cell surfaces, poses a significant barrier to the movement of ions. When designing a delivery methodology for nucleic acid therapeutics, three considerations need to be addressed: total uptake, uptake kinetics, and subcellular distribution. Nucleic acids are multiply-charged macromolecules. Because of the electrostatic repulsion between these negative charges and the negative charges on cell surfaces, cellular uptake of nucleic acids in general is inadequate and inefficient. In addition, from the pharmocokinetic point of view, uptake of nucleic acids tends to be slow, which extends exposure of nucleic acids to physiological conditions before they reach the target intracellular compartments. This slow uptake represents another difficulty for nucleic acids to be useful as therapeutic agents, i.e. a higher possibility to be degraded by nucleases. The resulting low bioavailability represents one of the major obstacles in nucleic acid-based therapies. Several mechanisms have been identified for nucleic acids to penetrate through cell membranes, including receptor-mediated endocytosis, adsorptive endocytosis, fluid-phase pinocytosis, and trafficking through cell membrane nucleic acid channels [30–33]. Depending on the mechanism of entry into cells, the subcellular fate of nucleic acids varies. In receptor-mediated endocytosis, for example, nucleic acids are encapsulated in endosomes after they are internalized. Most endosomes fuse with membrane-bound organelles, known as lysosomes. These nucleic acid molecules must now escape endosomes or lysosome membranes to reach their target compartments. It is speculated that nucleic acid leakage from endosomes or lysosomes can be facilitated by proteins present in these organelles. In addition, when endosomes fuse with other subcellular compartments, nucleic acids can also leak from endosomes. So far, a number of delivery vehicles have been investigated to improve the internalization efficiency of nucleic acids, including the use of carriers such as liposomes, peptides, and viral vectors. These techniques have been extensively reviewed elsewhere [1, 34–43]. In this section, nucleic acid delivery using carbohydrates as mediators will be discussed.

Lectins are proteins that recognize and bind to carbohydrates with high specificity. These carbohydrates can be a sugar itself, but they can also be the glycan moiety of glycosylated proteins or lipids. Lectins were found actively to direct their specific ligands to intracellular compartments, including endosomes, lysosomes and the Golgi apparatus. The idea of using lectin-glycan interactions to mediate cell targeting and cellular uptake of molecules has been under intense investigation during the past two decades [44, 45]. Two distinct strategies can be used for lectin-mediated drug delivery. In the first approach, drug molecules can be associated with a lectin, and the complexes formed can target the glycoprotein or glycolipids on the cell surface. Subsequent interactions between the lectin-glycoprotein (or glycolipid) facilitate the internalization of the drug molecules. Conversely, the drug molecules can be associated with a glycan moiety that is recognized by lectins on the surface of the target cells. The second approach utilizes endogenous cell surface lectins and would be more attractive because expensive lectins are not involved in the formulation of the delivery system. When a specific ligand comes in contact with the cell surface lectins, lectins may or may not be endocytozed depending on their structure and sugar-binding specificity. Because lectins recognize the terminal sugar residues of glycosylated ligands, endocytosis is not substantially influenced by the size and composition of the aglycone moiety of the ligand. These features allow for controls on the cellular uptake and intracellular trafficking. One important consideration in designing a glycotargeting strategy is the binding affinity because the drug delivery system must compete with other endogenous ligands existing *in vivo*. In general, in order for glycotargeting to work efficiently, multivalent glycans are normally required. Generally, mono- and bivalent ligands do not have high enough binding affinity to achieve appreciable glycotargeting [46].

A nucleic acid delivery system that exploits the interactions between glycans and lectins is a relatively recent technique. The first such approach towards receptor-mediated endocytosis of nucleic acids was implemented by Wu and Wu [47–49]. In their pioneering study, asialoorosomucoid was coupled to poly-L-lysine to form an asialoorosomucoid-poly-L-lysine conjugate. Then plasmid pSV2 CAT was complexed to the conjugate through electrostatic interactions between polylysine and the plasmid. This complex was then added to cultures of hepatoma cells which do or do not express asialoglycoprotein receptors. It was found that in cultured hepatoma cells devoid of the receptors, no transformation was detected; however, in the presence of the receptors, incubation of the cells with the complex led to transformation of the cells. Furthermore, when the individual component of the complex was incubated with cells containing the receptor, no detectable transformation was observed. This finding led to the establishment of the potential of lectin-ligand interactions in nucleic acid delivery. Following these observations, many different variations have been developed. These variations can be broadly categorized into either non-covalent carbohydrate-nucleic acid complexes or carbohydrate-nucleic acid conjugates.

Non-covalent carbohydrate-nucleic acid complexes

The first approach that has been under intense research for lectin-mediated nucleic acid delivery is through the forma-

tion of carbohydrate-nucleic acid complexes non-covalently. A number of methods have been developed that take advantage of electrostatic interactions, protein-ligand interactions, and DNA intercalation. The name "glycoplexes" has been given to these complexes brought together through non-covalent interactions.

Complex formation through ionic interactions

Work in this area is largely based on association of negatively-charged nucleic acids with glycosylated carriers bearing cations. The principle for this methodology is that positively charged carriers interact with the negatively charged nucleic acids electrostatically to form compact particles called toroids. When the polycationic carriers are linked to glycan ligands of the receptor, it is then possible to achieve cell specific targeting and enhance the uptake of nucleic acids through receptor-mediated endocytosis.

Polylysine

One such approach involves the preparation of glycosylated polypeptides that bear multiple positive charges [50]. In a commonly used strategy, polylysines are glycosylated at the ε-amino groups of some side chains with specific glycans (Fig. 1). These glycosylated polylysines were shown to help with the internalization of nucleic acids in cell cultures. The efficiency can be further increased by using a helper agent such as chloroquine [51] or a helper peptide, for example a peptide whose sequence resembles the N-terminal influenza hemagglutinin HA2 [52]. These helper agents presumably help the exit of the glycoplexes from the endosomes [52]. In order for the glycoplexes to work efficiently, the size of the polylysine and the number of glycosylation sites are critical. Smaller polylysines having about 200 lysine residues, with one third of the ε-amino groups glycosylated, were shown to have higher nucleic acid transfection efficiency.

A number of different versions of glycosylated polylysine-nucleic acid complexes have been reported [53, 54]. For example, an ethylene glycol spacer can be inserted in between the polylysine backbone and glycans (Fig. 2). Addition of the polyethylene glycol (PEG) spacer was shown to increase the solubility of the complexes. Transfection experiments showed that the lactose-PEG-polylysine complexes efficiently delivered DNA to a hepatoma cell line *in vitro*. Additionally, as the lactose-PEG substitution content increased up to 30%, the transfection efficiency increased. This observation clearly demonstrated that lactose served as the targeting moiety. In the meantime, this delivery system showed lower cytotoxicity compared to that of poly-L-lysine. The use of chloroquine again increased transfection efficiency, which indicates the involvement of hydrolytic degradation of the system in lysosomes [53].

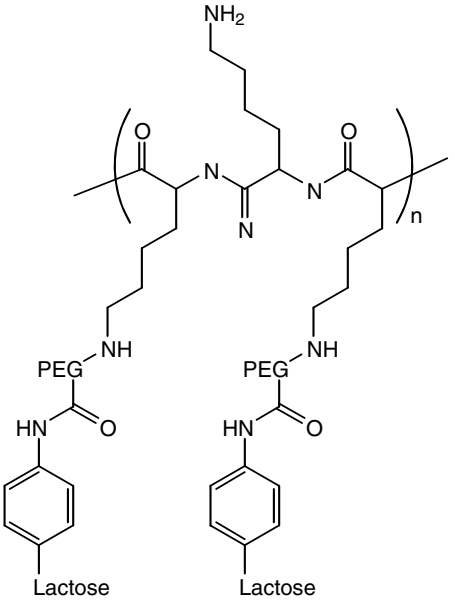


Fig. 2 An ethylene glycol spacer inserted in between a polylysine backbone and glycan

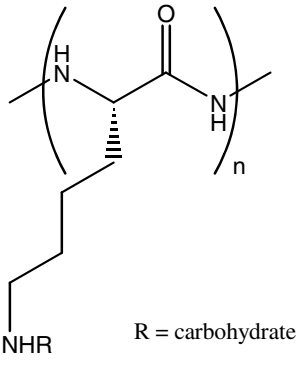


Fig. 1 Glycosylated polylysine in ε-amino groups of some side chains with specific glycans

Complexation of nucleic acids with glycosylated polylysines was also shown to increase the stability of nucleic acids towards nucleases. In a controlled experiment using plasmids that are either complexed or not-complexed with asialoorosomucoid-polylysine [55], the complexed DNA was protected from serum nucleases. Degradation of single-stranded nucleic acids was inhibited 3- to 6-fold in serum during 5 h of incubation. For complexed plasmids, greater than 90% of the plasmids remained full-length during 1.5 and 3 h incubations in serum or culture medium containing 10% serum, respectively. Plasmids that are not complexed with glycosylated polylysines were completely degraded after 15 min in serum or 60 min in medium.

Despite the advantages polylysines have as nucleic acid carriers, a number of problems associated with the use of polylysine were noted, for example heterogeneity of the complex [56], tedious quality control procedures [57], poor solubility [49], and variability in the efficacy of complexes

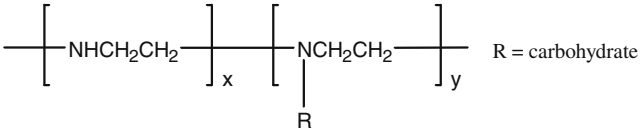


Fig. 3 The abilty of polyethylenimines to compact large nucleic acids into small particles

to effect delivery of nucleic acids to tissues bearing the receptors [58, 59].

Glycosylated polyamines

In addition to polylysines, other synthetic polycationic amines were developed as nucleic acid carriers. Among these, polyethylenimines (PEI) [60] (Fig. 3) have received a lot of attention due to their ability to compact large nucleic acids into small particles. In PEIs, because every third atom bears a positive charge, the molecules have a high capacity to interact with the negatively charged nucleic acids. In addition to this, PEIs are highly soluble in water. Use of PEIs as plamid carriers was shown efficiently to transfect a selection of cell types [61–70]. However, when PEIs were used as carriers for antisense oligonucleotides, the complexes were so stable that after their uptake by cells, the antisense oligonucleotides were not released from the complexes efficiently; therefore they were not able to hybridize and show antisense activity [71]. In addition to this drawback, PEIs are highly toxic. It was found that after internalization of the complexes, PEIs eventually were localized in the nuclei, where they may interfere adversely with the host genes [72].

Polycationic dendrimers and other polymers

Polycationic dendrimers were also considered as nucleic acid carriers for the preparation of glycoplexes [73, 74] because of the flexibility in the design of carrier structures. Starburst dendrimers (Fig. 4) were used as the "catch" for the negative charges of nucleic acids [75]. Glycans can be attached to the dendrimer, for example through disulfide bonds (as in Fig. 4). The transfection efficiency of this system seemed to vary depending on the cell types, from less than 1% transfection in EL-4 and Jurkat to up to 80% transfection in CV-1.

Glycosylated block copolymers containing both cationic polymer and PEG were also developed as nucleic acid carriers. The terminal carboxylic group of poly(2-(dimethylamino)ethyl methacrylate-*co*-*N*-vinyl-2-pyrrolidone) was first covalently linked to polyethylene glycol-bis(amine) (Fig. 5). The remaining free amino group then underwent reductive amination with lactose. The resulting glycosylated copolymer was used as a carrier to transfect HepG2 human hepatocarcinoma cells with luciferase plasmids. The terminal galactose residues were found greatly to enhance the transfection efficiency [76].

One important factor when designing cationic molecules that bind with oligonucleotides non-covalently is their binding affinity and ease to dissociate once they are at the target organelles. Thus, these cationic carriers must be able to bind nucleic acids with relatively high affinity in order to form stable complexes; however, once they reach their targeted subcellular compartment, dissociation must occur relatively readily in order for nucleic acids to function. A recent study examined the effect of oligonucleotide structures on the binding between cationic macromolecules and oligonucleotides. It was demonstrated that oligonucleotides, which are more structured, tend to bind cationic molecules more strongly [77]. Another consideration with the use of polycationic carriers is their cytotoxicity. On one hand, polycationic carriers should have sufficient number of positive charges to neutralize the negative charges of nucleic acids in order to facilitate their internalization; on the other hand, polycations are associated with cytotoxicity. Therefore, polycationic carrier-nucleic acid ratios need to be optimized.

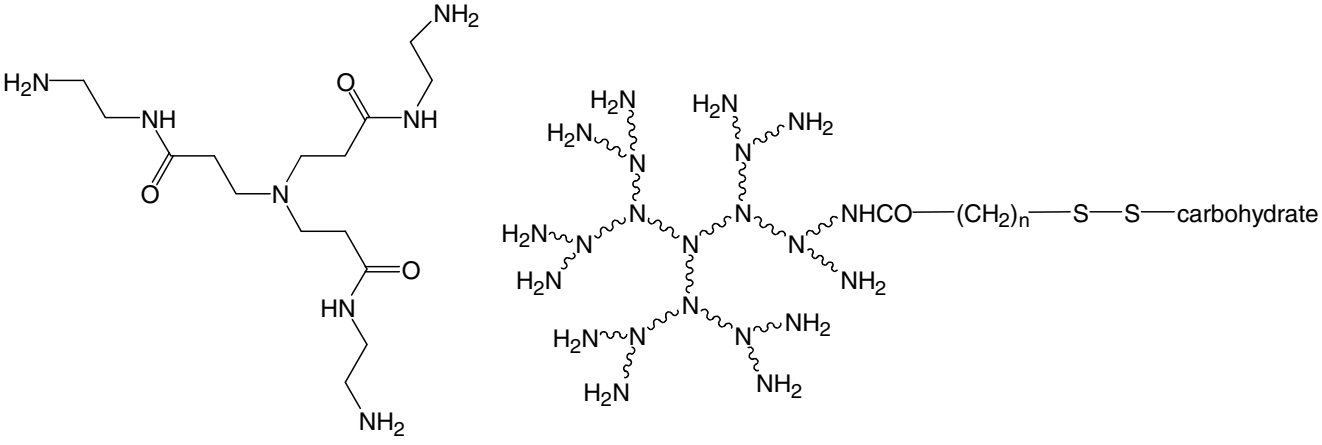


Fig. 4 Starburst dendrimers as the "catch" for the negative charges of nucleic acids. Glycans can be attached to the dendrimer, for example through disulfide bonds

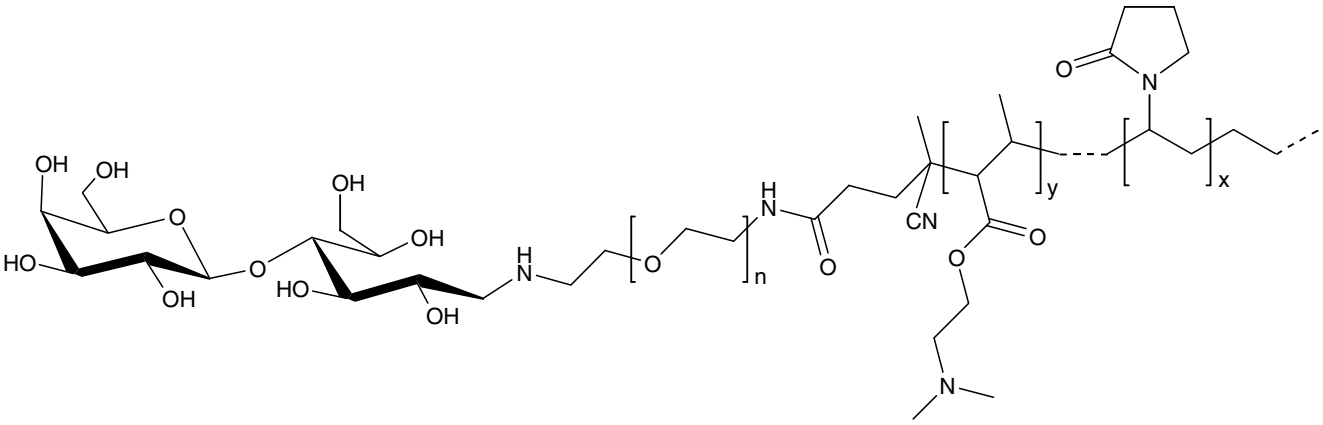


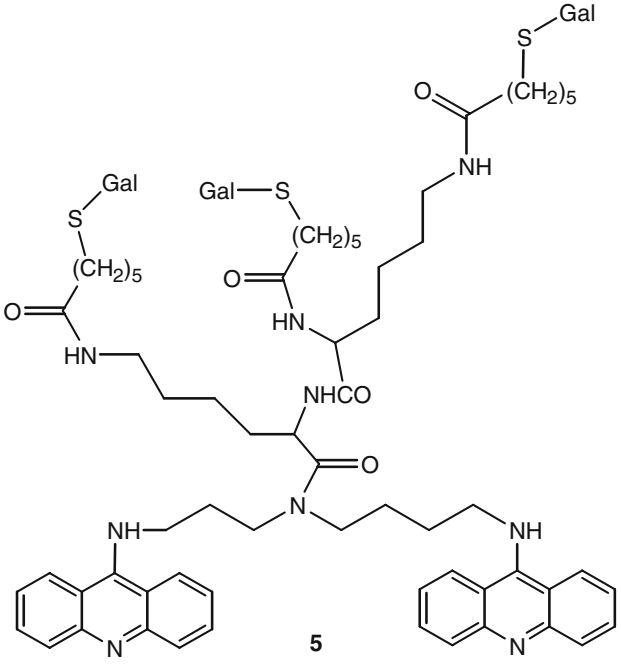
Fig. 5 The terminal carboxylic group of poly(2-(dimethylamino)ethyl methacrylate-*co*-*N*-vinyl-2-pyrrolidone) covalently linked to polyethylene glycol-bis(amine)

Complex formation through protein-ligand interactions

Alternatively, if nucleic acids bear a ligand that is recognized by a carrier molecule, then the nucleic acid can also associate with the carrier molecule through specific binding. In this approach, carrier proteins can be first glycosylated to form neoglycoproteins. These neoglycoproteins will then interact with their ligands that are covalently linked to nucleic acids to form complexes. One such example harnesses the interaction between streptavidin and biotin (Fig. 6) [78–80]. In this example, mannosylated streptavidin and biotinylated oligonucleotides are first prepared. Mixing of these two species then resulted in formation of glycoplexes associated through the tight interaction between streptavidin and biotin. When this glycoplex was used to deliver dodecakis (α-deoxythymidylate) [(α-dT)$_{12}$] to macrophages, a 20-fold increase in intracellular concentration of the oligonucleotide was observed compared with free oligonucleotide [78].

Complex formation through nucleic acid-intercalator interaction

Another interesting approach towards the formation of glycoplexes takes advantage of the interactions between nucleic acids and DNA intercalators. In one such example, spermidine-bisacridine (as in **5**), a known DNA bis-intercalator, was derivatized with galactose residues [81]. This type of intercalation provides tight binding and a slow dissociation rate, which are two important considerations for the formation of stable noncovalent complexes. Interactions between bisacridine and nucleic acids resulted in the formation of a glycan-DNA complex. The trigalactosylated bisacridine carrier **5** was found to mediate the binding of DNA to both ricin lectin and to the asialoglycoprotein receptor on primary hepatocytes. However, on the basis of luciferase expression, a trigalactosylated bisacridine-plasmid complex did not induce transfection of the hepatocytes, which is probably due to lack of membrane destabilizing functions in the complex [81].



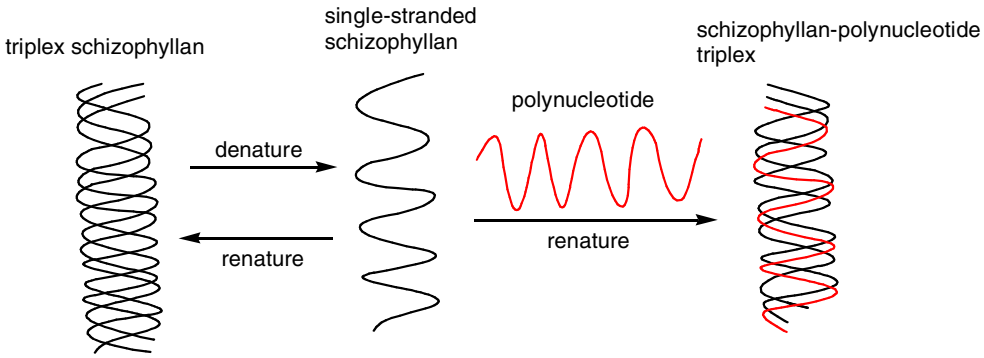
5

Complexation through nucleic acid-polysaccharide interaction

Another approach to the preparation of carbohydrate-nucleic acid complexes is to utilize the ability of some polysaccharides to interact with nucleic acids. In one of these examples, the polysaccharide schizophyllan, an extracellular polysaccharide secreted by the fungus *Schiz-*

glycan—streptavidin‖‖‖‖‖‖‖‖biotin—oligonucleotide

Fig. 6 Interaction between streptavidin and biotin



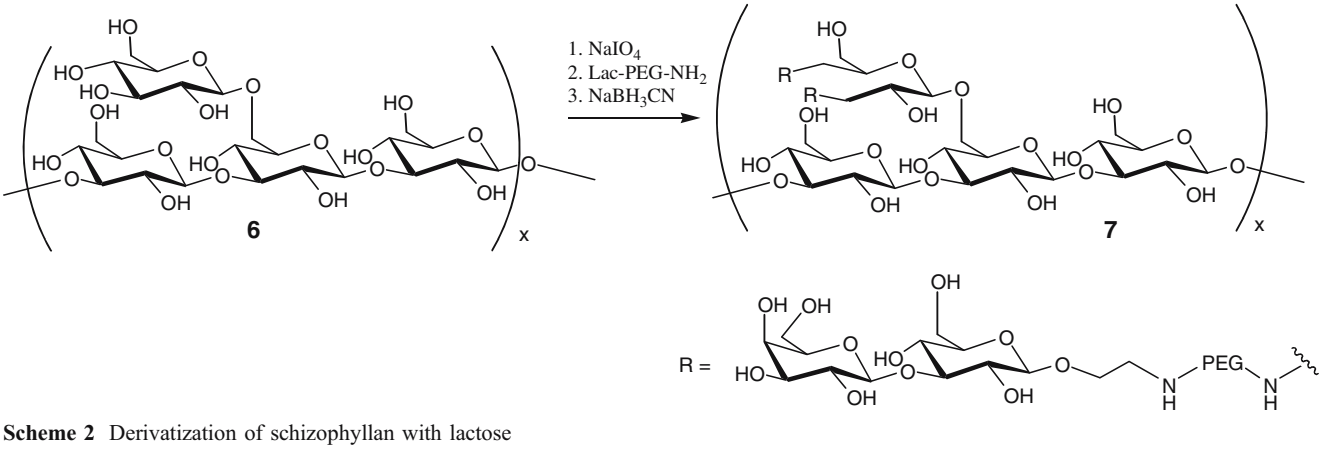
Scheme 1 Complexation of schizophyllan with DNA (Reprinted from *Biochim. Biophys. Acta*., **1670**, Matsumoto T, Numata M, Anada T, Mizu M, Koumoto K, Sakurai K, Nagasaki T, Shinkai S, Chemically modified polysaccharide schizophyllan for antisense oligonucleotides delivery to enhance the cellular uptake efficiency, 91–104, Copyright (2004), with permission from Elsevier [83])

ophyllan commune with β-(1→3)-D-glucan main chain and one β-(1→6)-D-glycosyl side chain **6** linked to the main chain at every three glucose residues, was used to bind oligonucleotides. In aqueous solution, schizophyllan exists in a triplex state; however, in dimethyl sulfoxide (DMSO), the triplex can be denatured into single-stranded "random coils". The denatured random coils can be renatured when water is added to the DMSO solution. Remarkably, the random coils of schizophyllan can interact with single-stranded DNA to form duplexes (Scheme 1). The complex protects the single-stranded DNA from being digested by nucleases. Due to the lack of β-1,3-glucanase in mammals, the complex has a long circulation time in blood. Another advantage of using schizophyllan to form complexes with single-stranded DNA is that the polysaccharide is a poor immunogen, and thus does not elicit immune response. Schizophyllan itself does not bind to the plasma membrane and induce endocytosis or other internalization processes. However, the polysaccharide can be modified by reductive amination (Scheme 2) to introduce a carbohydrate moiety that can be recognized by receptors. For instance, schizophyllan can be treated with periodate to generate aldehyde functions at the side chain glucose residues. Reductive amination is then carried out by first treating the oxidized schizophyllan with a bisamino(polyethyleneglycol) that has been derivatized with lactose, and then with sodium cyanoborohydride. This sequence of reactions introduces lactose into schizophyllan (as in **7**), which on one hand can associate with DNA, and on the other hand can be recognized by asiaoglycoprotein receptors [82–89].

The efficiency of β-lactoside-appended schizophyllan as antisense oligonucleotide delivery carrier was demonstrated in human hepatocytes [89]. Because schizophyllan does not form complexes with short and hetero-oligonucleotides, an oligonucleotide phosphorothioate sequence that is known to bind to c-myb mRNA was first tagged to poly(A)$_{40}$ (5′-GT GCCGGGGTCTTCGGGC-(A)$_{40}$-3′). After hepatocytes cell culture was incubated with the lactosylated schizophyllan—c-myb antisense oligonucleotide-poly(A)$_{40}$ complex for 2 days, viable cell number counts were significantly smaller than when the culture was treated with control where no lactoside was appended to schizophyllan [89]. The increased antisense activity was attributed to (1) the protection of the oligonucleotides through complexation [90], and (2) interactions between hepatocytes and the complexes.

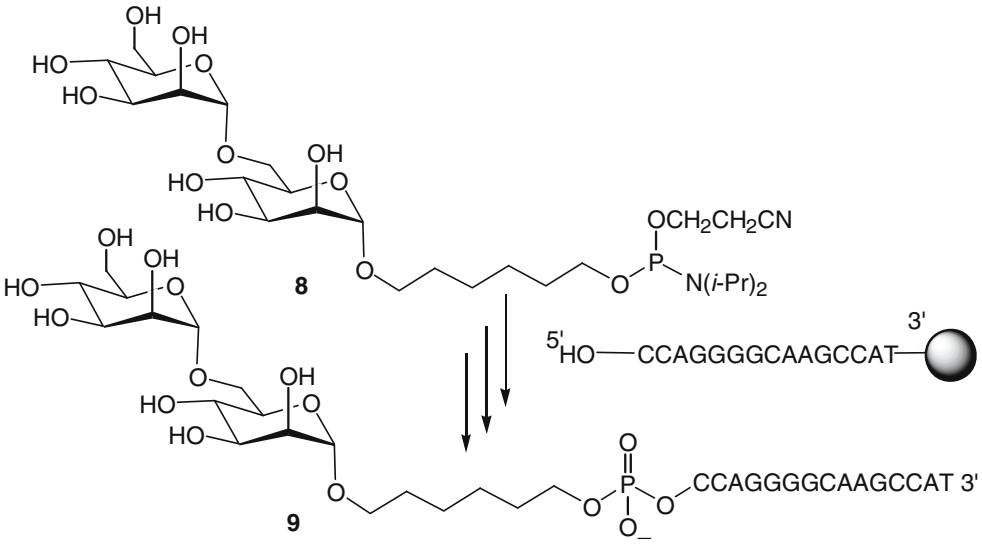
Carbohydrate-nucleic acid conjugates

The other general strategy that utilizes lectin-carbohydrate interactions for the delivery of nucleic acids is to introduce covalent linkages between carbohydrates and nucleic acids.



Scheme 2 Derivatization of schizophyllan with lactose

Scheme 3 Incorporation of dimannoside into a 15-mer oligonucleotide through phosphoramidite chemistry on solid support

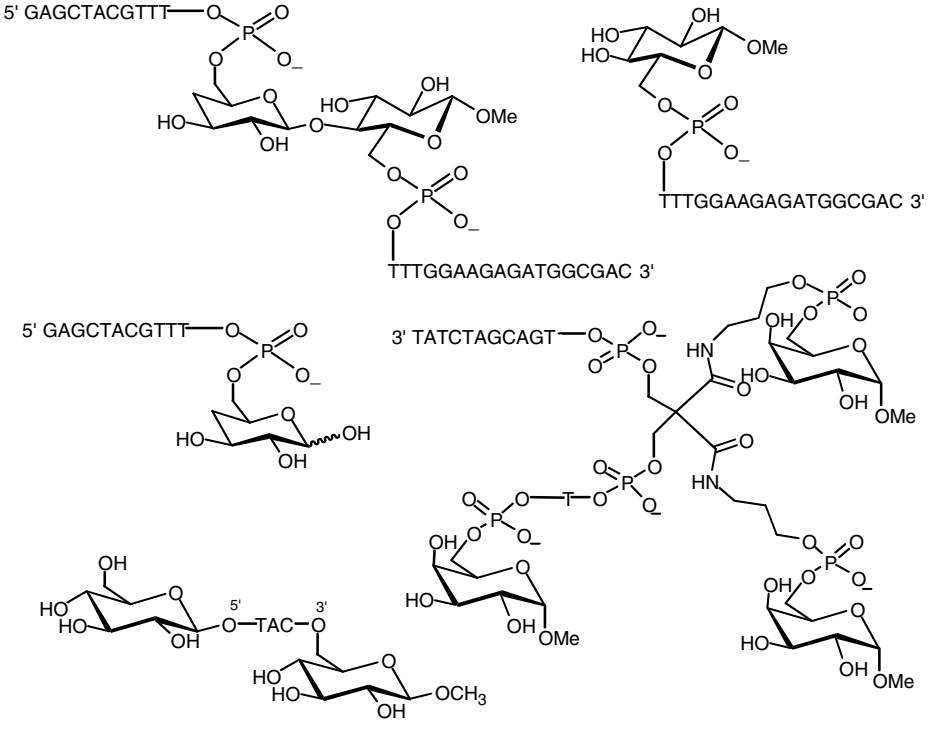


Compared with the non-covalent association, the carbohydrates and oligonucleotides are now linked by covalent bonds. Conjugates produced by this approach are likely to be more homogeneous and easier to characterize compared with the carbohydrate-nucleic acid complexes. However, the covalent linkages in some cases do present steric effects on the conformation of nucleic acids and can affect their binding properties with complementary sequences. Additionally, the preparation of covalent conjugates can sometimes present significant synthetic challenges. So far, a number of systems have been investigated in connection with the preparation of carbohydrate-nucleic acid conjugates.

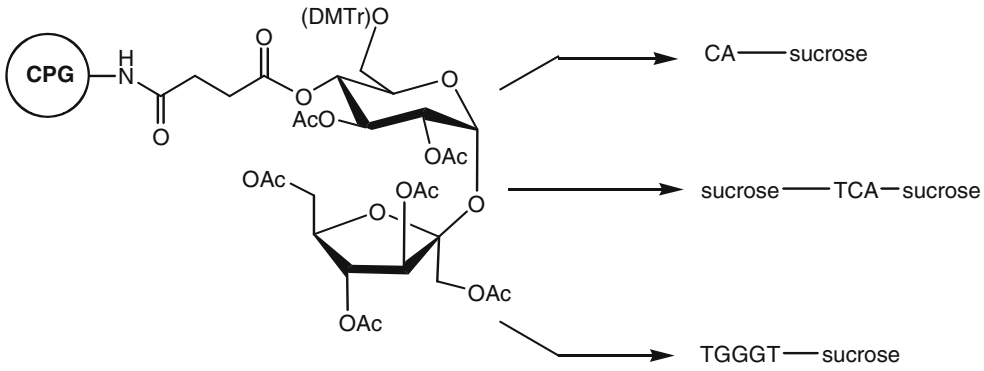
Coupling of sugar phosphoramidites with oligonucleotides

One of the most straightforward methods for the introduction of a carbohydrate moiety into an oligonucleotide sequence is through the coupling of a sugar phosphoramidite **8** with oligonucleotides on solid supports. This method was first demonstrated by Akhtar et al. in the preparation of mannose phosphoramidite and the subsequent incorporation of mannose into a 15-mer oligoribodeoxynucleotide **9** through phosphoramidite chemistry-based solid phase synthesis (Scheme 3) [91]. The study was undertaken in an attempt to enhance the uptake of antisense oligonucleotides by macrophages through

Fig. 7 Some carbohydrate-oligonucleotide conjugates prepared by phosphoramidite chemistry



Scheme 4 Oligonucleotide assembly on immobilized carbohydrate residues



receptor-mediated endocytosis due to the fact that macrophages express high levels of cell-surface mannose-binding lectins.

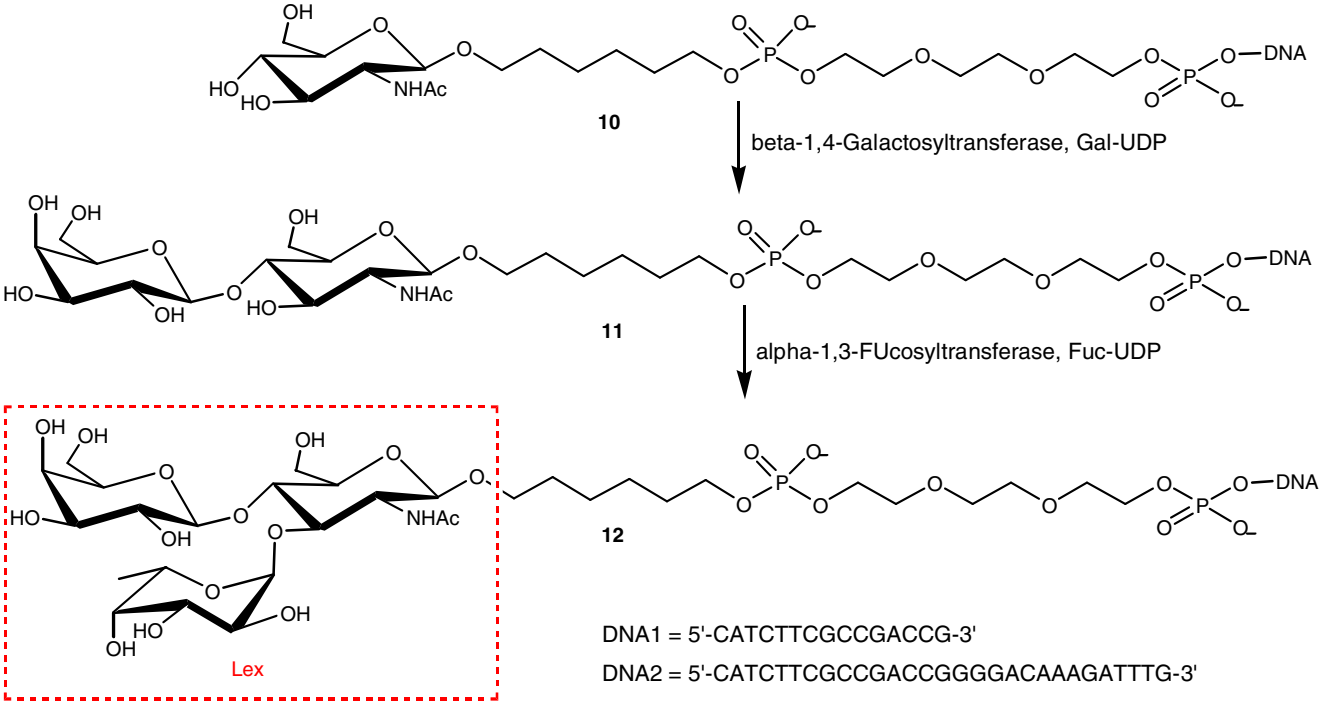
Subsequently, this approach was demonstrated in a number of other studies (Fig. 7) [92–94]. It provides a general and versatile means to introduce covalent linkages between glycans and oligonucleotides. However, due to the relative difficulty with complex carbohydrates, this approach is rather limited to conjugate formation between oligonucleotides and simple carbohydrates.

Alternatively, carbohydrate moieties can be first immobilized on solid supports, followed by oligonucleotide assembly using the standard phosphoramidite chemistry. A number of examples were demonstrated by Montesarchio et al. [95–98]. In one of these examples [97], a sucrose moiety was first attached to the solid support, and this was followed by oligonucleotide synthesis using the standard phosphoramidite chemistry (Scheme 4). It was found that the sucrose units at selected oligonucleotide sequences did not interfere with duplex formation or with the ability of G-rich sequences to adopt a quadruplex structure. In the meantime, the presence of the sucrose moiety increased the chemical and enzymatic stability of the oligonucleotides.

One obvious drawback to this approach is that immobilization of carbohydrates on solid supports tends to be less efficient and requires tedious protection strategies.

Enzymatic elaboration of carbohydrate moiety of a glycosylated oligonucleotide

Using phosphoramidite chemistry, simple carbohydrate residues can be readily incorporated into oligonucleotides. However, in order to introduce complex carbohydrate structures, laborious protection and deprotection strategies are required. Additionally, glycosylation can be very



Scheme 5 Enzymatic elaboration of glycan on glycosylated oligonucletides

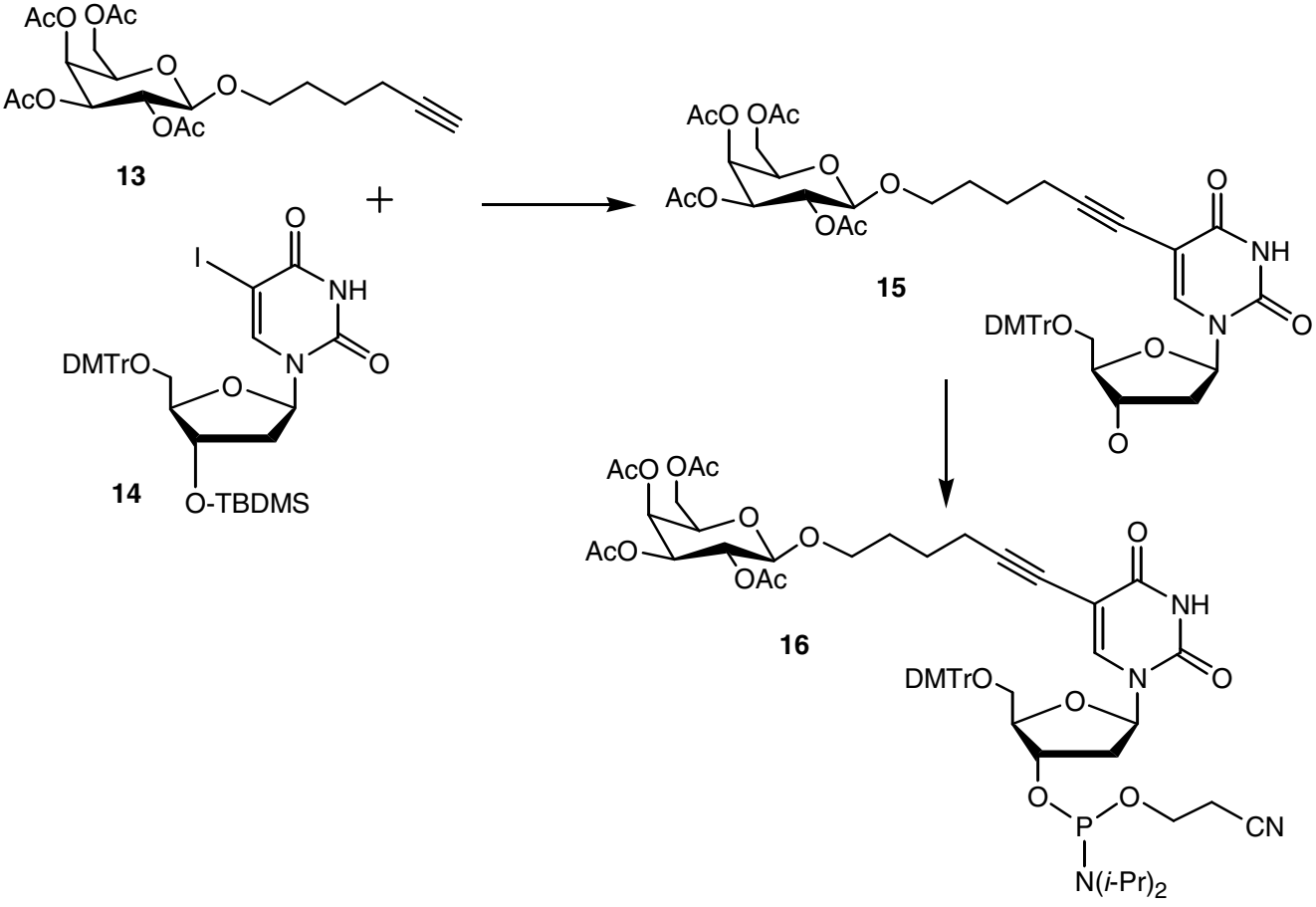
challenging in some cases. One way to bypass these difficulties is to utilize the glycosyltransferases that are becoming increasingly available. The first such approach was demonstrated by Wang and Shepperd in the chemoenzymatic synthesis of LeX-conjugated oligonucleotides (Scheme 5) [99]. In order to furnish a LeX trisaccharide (Galβ1-4(Fucα1-3)GlcNAc) structure on an oligonucleotide, the reducing end of GlcNAc was first attached to the oligonucleotide using standard phosphoramidite chemistry. The resulting conjugate **10** was then subjected to enzymatic glycosylation effected by *β*-1,4-galactosyltransferase and α-1,3-fucosyltransferase using the appropriate sugar nucleotides as donors to give lactosylated **11** and LeX-derivatized **12** conjugates. Modification of oligonucleotides by the introduction of a carbohydrate residue at the 5'-position did not affect their hybridization properties. Additionally, the LeX conjugated oligonucleotides were favourably recognized by antibody specific for LeX.

Derivatization from the nucleoside base residues

Sugar derivatization of oligonucleotides can also be achieved by covalently linking a sugar moiety to the purine or pyrimidine base residues through a linker. This method was demonstrated by Kobayashi et al. in the synthesis of galactose-derivatized oligonucleotides [100–102]. First, suitably protected galactose derivatized with an alkyne function **13** is reacted with suitably protected 5-iododeoxyuridine **14** through a palladium-mediated coupling reaction to introduce a covalent bond between the galactose and uridine moieties. Conversion of the product **15** into its phosphoramidite **16** is then followed by solid-phase synthesis using standard phosphoramidite chemistry (Scheme 6). The method allows for the convenient introduction of sugar residues at predefined locations of the oligonucleotides. The resulting glycoconjugate is readily recognizable by galactose-binding lectins.

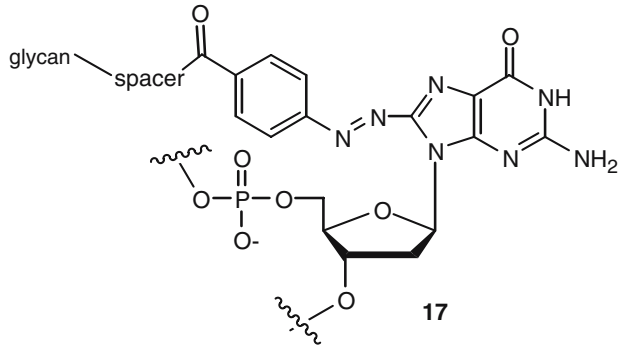
Diazocoupling

Linkages between glycans and nucleic acids can also be generated through azo functions. In the reported studies, the azo functional groups were introduced at the C8-position of guanosine (as in **17**) [103, 104]. This allows for incorporation of multiple glycosylation sites into plasmids (Scheme 7). In addition, this modification seemed to have little impact on the



Scheme 6 Introduction of a carbohydrate residue on the pyrimidine ring

complementarity of the base pairs because the C8-position of guanine faces the major groove of DNA duplexes [105].



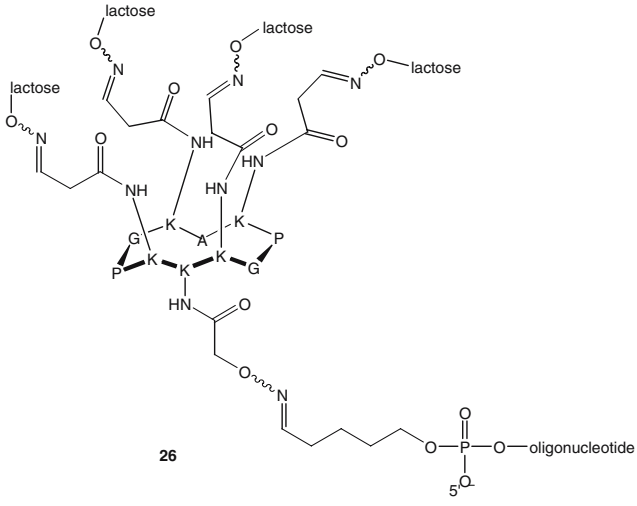
The conjugates formed showed higher melting temperature and stronger nuclease resistance both to exo- and endonucleases than native plasmids. They were also able to bind to galactose-specific lectin RCA120 specifically with relatively strong binding affinity.

Oximation

Another efficient conjugation method involving reactions between aminooxy sugars and activated carbonyl functions was first undertaken by DeFrancq et al. [106]. The aldehyde functions on oligonucleotides **19** are generated by periodate oxidation of terminal 1,2-diols in **18**. Treatment of the oligonucleotide aldehyde **19** with aminooxysugar **20** leads to the formation of oxime ethers (Scheme 8) very efficiently in aqueous solution at pH 4.6. The oxime linkages are stable at pH 7.0 for over 72 h. Using this approach, 3′,5′-bisconjugation through an oxime bond formation was achieved [107, 108].

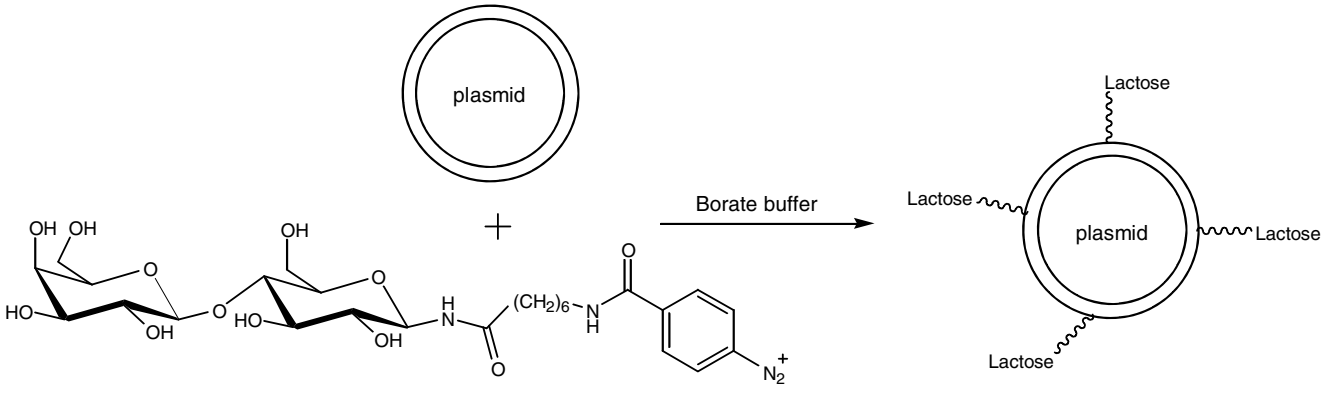
Using a modified version of this approach, it is also possible to carry out the conjugation on solid support. First, aminooxy functions can be incorporated onto a solid support in a protected form as in **22**. When a masked aminooxy function is treated with an aldehyde **23** in the presence of hydrazinium acetate, an oxime ether (as in **24**) formed in a site-specific manner. The product **24** can survive the ammonolytic conditions that are required for the cleavage of the olignucleotides from solid-support (Scheme 9). Melting point studies showed that the hybridization abilities of the conjugates **25** are comparable to those of the unmodified oligonucleotides [109].

This chemistry was recently used to prepare a carbohydrate cluster-oligonucleotide conjugate bearing a cyclodecapeptide anchor **26** [110]. The binding affinity of the conjugate with its complementary sequences was retained. In the meantime, the conjugate showed a greatly enhanced binding affinity towards concanavalin A lectin, due to the cluster glycoside effect [111].



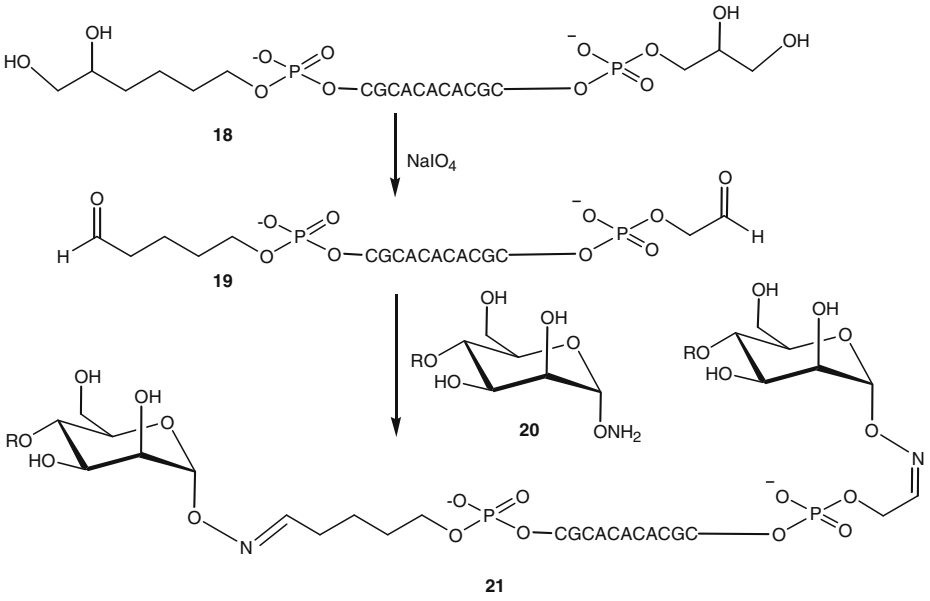
Reductive amination

Reductive amination, which is widely used in glycoconjugate preparation, also provides a facile way to form carbohydrate-oligonucleotide conjugates. In this approach, reactive carbonyl functions, usually aldehydes, are allowed to react with amino functional groups followed by treatment with sodium cyanoborohydride to produce a stable amine linkage. The reaction proceeds most effi-



Scheme 7 Lactose-plasmid conjugate formation by azo bond formation

Scheme 8 Formation of oximes between an aminooxy sugar and an oligonucleotide aldehyde



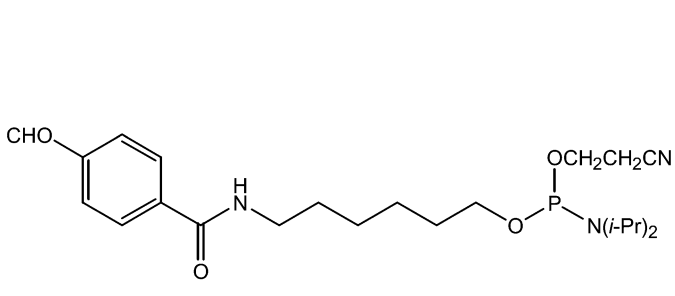
ciently under slightly basic conditions. This method provides a very mild way to form carbohydrate-oligonucleotide conjugates.

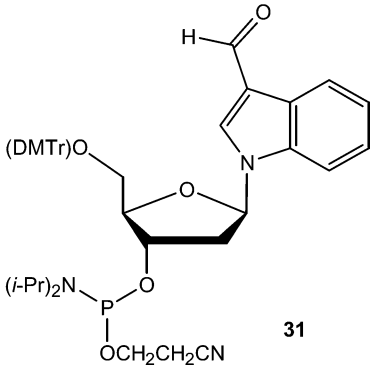
In an example demonstrated by Sando et al. [112], unprotected carbohydrates **27** (lactose, maltose, cellubiose, and maltoheptose) were covalently linked to aminoalkylated oligonucleotides **28** under mild conditions (aqueous borate buffer, pH 8.0 at 60°C) followed by reduction using sodium cyanoborohydride to give stable conjugates **29** (Scheme 10). No notable side reactions were detected.

With the development of methods to introduce aldehyde groups into nucleic acids, as reviewed elsewhere [113], and the availability of commercial phosphoramidite bearing aldehyde functions (**30** from SoluLink Biosciences and **31** from Link Technologies and Trilink Biotechnologies) [114, 115], it should also be possible to form carbohydrate-oligonucleotide conjugates by reacting amino sugars with oligonucleotides bearing aldehyde modifications.

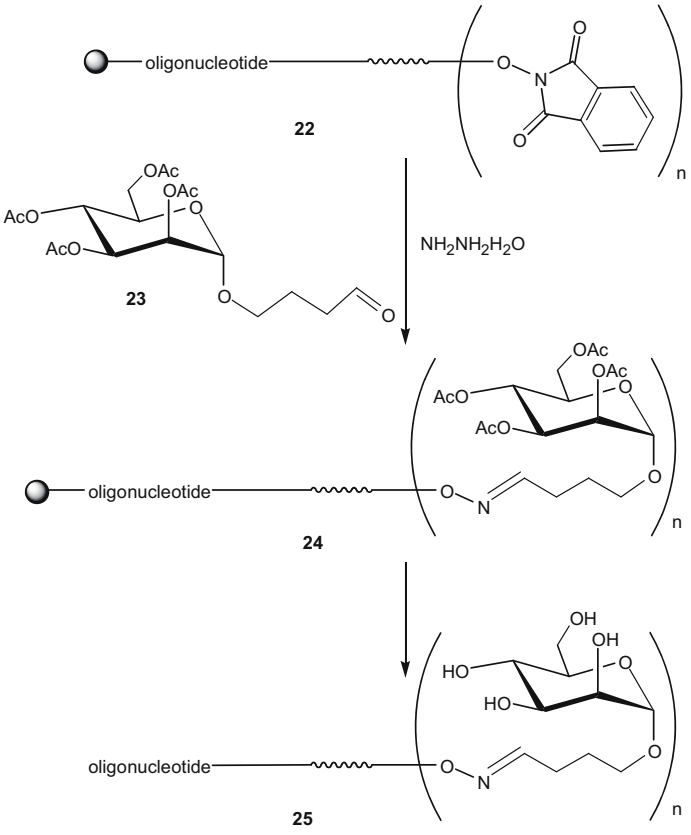
DNA/oligonucleotide delivery in the form of covalent conjugates with carbohydrates

DNA/oligonucleotide delivery in the form of covalent conjugates with carbohydrates is not as well explored as in the form of non-covalent complexes. Compared with non-covalent complexes, it is possible to prepare chemically and structurally uniform covalent DNA/oligonucleotide-carbohydrate conjugates. Because the interaction between individual carbohydrate and lectin is rather weak, it is often necessary to construct conjugates bearing multivalent carbohydrate moieties in order to enhance the affinity. Ideally these carbohydrate moieties are separated by 4 to 20 Å in order to optimize the binding affinity [116]. Most system investigated so far target at hepatocytes, but it should also be possible to extend this strategy to other cell types, such as macrophages where mannose receptors are expressed.

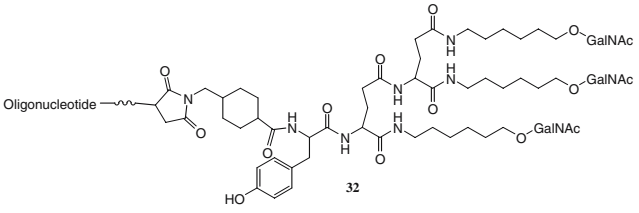




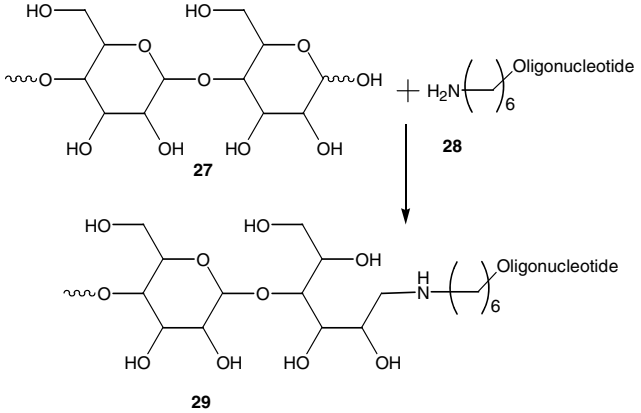
Scheme 9 Solid phase oximation



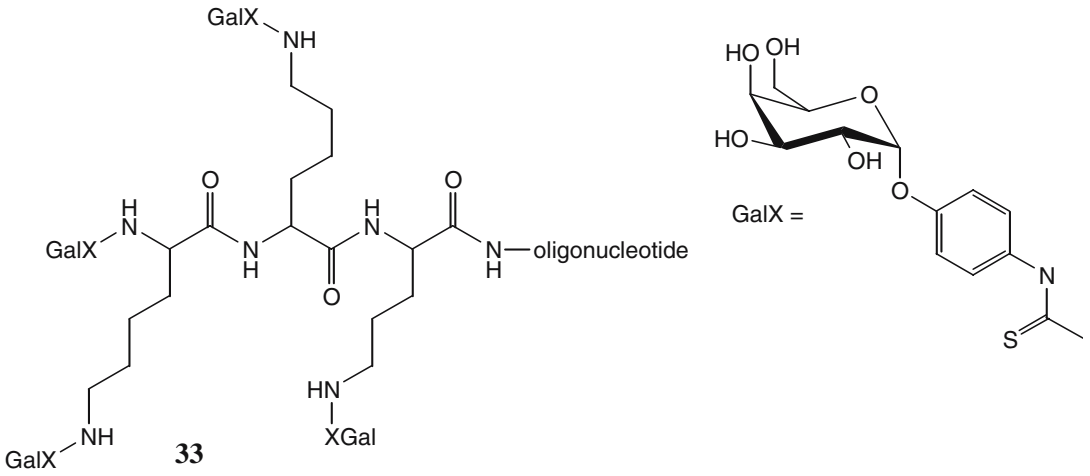
When methylphosphonate oligonucleotides conjugated to trivalent *N*-acetylgalactosamine (as in **32**) are targeted to human hepatocellular carcinoma (HepG2) cells, a 20- to 40-fold increase in cell uptake was observed [117]. Binding affinity between the conjugates and Gal/GalNAc receptors was tight, with a K_d of 7 nM. The same trivalent GalNAc construct was also used to form conjugate with antisense oligonucleotide that target HBV in hepatoma cells. At 1 μM concentration, an increase of antisense activity by 20 fold was observed. When injected to mice from tail vain, rapid and high uptake of the conjugate was observed in liver [118].



Oligonucleotides conjugated to tetravalent galactosides (as in **33**) were also used to target the asiaologlycoprotein receptors on parenchymal liver cells. Compared with non-conjugated oligonucleotides, hepatic uptake of conjugated oligonucleotides was far more efficient [119]. The affinity was in the low nanomolar range. In comparison with non-glycosylated oligonucleotides, *in vivo* experiments showed that hepatic uptake was enhanced from 19 to 77% when oligonucleotide bearing tetravalent GalNAc moieties is injected in rats. Additionally, specific oligonucleotide accumulation in parenchymal liver cells was improved by 60-fold. Meanwhile, the carbohydrate moieties were determined to be essentially non-toxic at mg/kg concentrations.



Scheme 10 Reductive amination to form carbohydrate—oligonucleotide conjugates



Perspectives

Glycotargeting for nucleic acid delivery represents an avenue for the incorporation of structural determinants into nucleic acids. This concept allows for potential cell-specific targeting and augments the bioavailability of nucleic acids as therapeutic agents. Because of the specificity the lectins have towards their carbohydrate ligands, it is possible to develop nucleic acid delivery platforms based on a structure-function relationship. This method can be extended to a wide range of cell types where surface lectins are identified. So far, most studies have focused on the roles of extracellular lectins. In addition to the extracellular lectins, intracellular lectins could also play an important role in enhancing the bioavailability of nucleic acid therapeutics. These soluble intracellular lectins can help route nucleic acids to individual cellular compartments. Further work is also necessary in order to identify the intracellular fate of the carbohydrate moieties of the glycoconjugates/complexes.

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