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Efficient preparation of carbohydrate–oligonucleotide conjugates (COCs) using oxime bond formation

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Abstract—Chemoselective ligation of carbohydrates to oligonucleotides was accomplished by oxime bond formation. The conjugation was performed by reacting an oxyamino sugar with an oligonucleotide containing an aldehyde moiety at the 5′-end. The carbohydrate–oligonucleotide conjugates (COCs) were obtained in good yield without the need of a protection strategy and under mild aqueous conditions. © 2001 Elsevier Science Ltd. All rights reserved.

Inhibition of gene expression by synthetic oligonucleotides offers an attractive therapeutic approach. Targeting oligonucleotides can be performed to complementary base sequences in mRNA (anti-sense strategy), to duplex DNA ('anti-gene' strategy) or to proteins (aptamere strategy). However, severe limitations, such as cellular uptake efficiency, cell-specific delivery, and stability against nucleases, have impeded the use of oligonucleotides in therapy. The design of delivery strategies that can improve the cellular targeting and uptake has emerged as a prerequisite for the therapeutic use of oligonucleotides. Oligonucleotides are generally internalised into cells by an endocytosis process. For this reason, a number of hydrophobic compounds, such as acridine, cholesterol, lipids and fusion peptides, have been anchored to oligonucleotides.[1,2] Although the intracellular concentration could be improved no specific targeting could be attained using this strategy. On the other hand, it is well known that cell surface sugar-binding receptors (lectins) specifically bind and internalise glycoconjugates bearing the appropriate sugar residue.[3,4] In addition to targeting, carbohydrate conjugation can also offer anti-aggregation properties.[5] Consequently, conjugation of a carbohydrate moiety with an oligonucleotide may represent a promising strategy for targeting cells or tissues with DNA with the ultimate aim to cure inherited and acquired diseases.

Incorporation of sugar residues inside the sequence has been recently achieved by using a disaccharide phosphoramidite[6] or by using a galactose-modified deoxyuridine phosphoramidite.[7] In the latter case, it has been shown that the oligonucleotides periodically directed, in space, a galactose array that was recognised cooperatively by the specific lectin RCA_{120}.[7] To the best of our knowledge, only two methods for conjugation at the 5′-extremity of oligonucleotides have been reported. The first uses a monomanoside phosphoramidite derivative, which is incorporated at the last stage of the automated DNA synthesis.[8] The second involves a direct solid-phase glycosylation using a trichloroacetimidate donor sugar in the presence of TMSOTf.[9] However, the direct glycosylation was incompatible with the presence of 2′-deoxyguanosine in the sequence due to the lability of it *N*-glycosidic bond under acidic conditions. Furthermore, in both cases the sugar moiety must be protected. Although these approaches have led to interesting applications, they suffer from limitations due to the chemical manipulations they require. Thus, development of a more efficient and selective ligation is of great interest. The conjugation reaction should take place ideally between unprotected oligonucleotide and carbohydrate with minimal chemical manipulation and under physiological conditions.

In this context, chemoselective ligation techniques are of key interest because the reactions proceed in aqueous solvent, and their high selectivity obviates the need for protection of other functional groups on the coupling partners. At the heart of this approach is the introduction of mutually and uniquely reactive functional groups (e.g. an aldehyde group and an aminooxy group) onto unprotected fragments and the coupling of these fragments in an aqueous environment. In particu-

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lar, chemoselective oxime bond formation has been used successfully for the assembly, in a user-defined way, of peptides,[10] glycopeptides,[11] as well as for the labelling of oligonucleotides and RNA.[12] In a recent paper, we have demonstrated that the oxime approach was also suitable for the preparation of peptide–oligonucleotide conjugates (POCs), namely oxime conjugation was performed by reacting an oxyamine and an aldehyde containing unprotected peptide and unprotected oligonucleotide together or vice versa.[13] We report here the extension of this strategy for the preparation of carbohydrate–oligonucleotide conjugates (COCs). The general strategy is depicted in Fig. 1. The aldehyde-containing DNA[13] is reacted with an unprotected oligosaccharide bearing an aminooxy group at the reducing end,[14,15] affording an oxime-linked glycoconjugate.

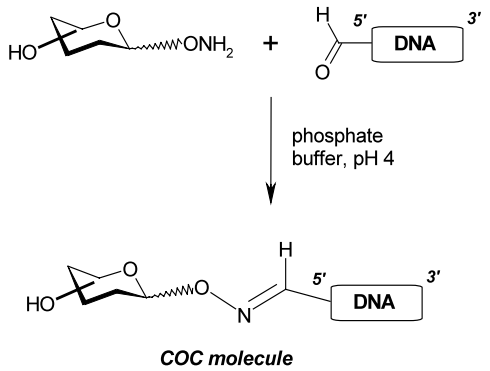
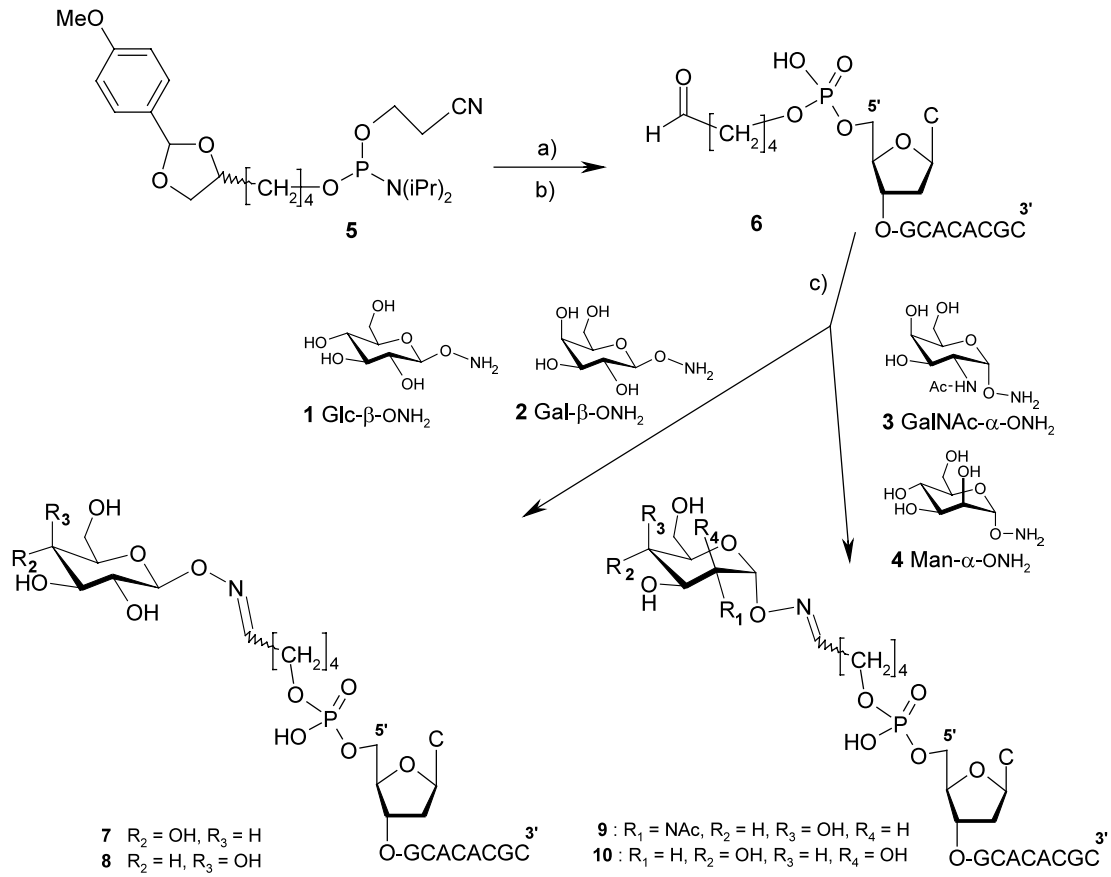


Figure 1. Chemical ligation of unprotected saccharide glycosylated with ONH_2 and DNA-containing aldehyde at its 5′-end via oxime bond formation.

For this purpose, an aminooxy moiety was introduced at the anomeric position of the sugar while the aldehyde function is incorporated into the oligonucleotide module at the 5′-extremity (Fig. 1). The four carbohydrates **1**–**4** (Scheme 1) containing the aminooxy moiety have been prepared by direct incorporation of *N*-hydroxyphthalimide as the nucleophilic moiety during the glycosylation step using the method we recently described (Scheme 1).[14] The β-aminooxy sugars Glc-β-ONH_2 **1** and Gal-β-ONH_2 **2** were thus obtained starting from penta-*O*-acetylated gluco- and galactobromide, respectively, by stereoselective β incorporation of *N*-hydroxyphthalimide followed by cleavage of the protecting groups by methylhydrazine. The α-aminooxy sugars GalNAc-α-ONH_2 **3** and Man-α-ONH_2 **4** were prepared by glycosylation of the corresponding glycosyl-fluoride with *N*-hydroxyphthalimide using boron trifluoride diethyl etherate as a promoter and subsequent controlled debenzylation and reductive acetylation followed by cleavage of phthalimide moiety by methylhydrazine.



Scheme 1. Preparation of aldehydic oligonucleotide **6** and conjugates **7**–**10**. *Reagents and conditions*: (a) automated DNA synthesis, then AcOH 80%; (b) $NaIO_4$; (c) phosphate buffer, pH 4.

The oligonucleotide **6** containing an aldehydic linker at the 5′-extremity was prepared by using phosphoramidite linker **5** bearing a methoxybenzylidene-protected diol. After introducing **5** by coupling at the last step of the automated DNA synthesis, the aldehyde was generated by oxidative cleavage of the intermediate diol with $NaIO_4$.[13] The conjugation reactions (Scheme 1) were then carried out in phosphate buffer at pH 4 as it is known that the optimal pH is around 4–5 for oxime bond formation.[16] A slight excess (2 equiv.) of the aminooxy sugars **1**–**4** was used. The course of the reaction was followed by reverse-phase HPLC and the reaction proceeded essentially to completion within 1 h to yield exclusively the corresponding oximes **7**–**10**. It is interesting to note that no major difference of reactivity was observed between the α and β anomer during the oxime-coupling reaction. As an example, HPLC analysis of crude mixture of conjugation with α- and β-sugar derivatives is depicted in Fig. 2A and 2B.

Subsequent RP-HPLC purification afforded the conjugates **7**–**10** in almost 50% isolated yield. The conjugates **7**–**10** were characterised by ES-MS. In all cases, the experimentally determined molecular weights were in excellent agreement with the calculated values.[17]

The chemical stability of the oxime linkage was then studied by incubating the conjugates in a phosphate buffer at pH 4 and 7. No significant hydrolysis or degradation products were observed even after 72 h of incubation at 37°C. This stability demonstrates the usefulness of the oxime bond for the conjugation of carbohydrate with DNA.

The hybridisation properties of conjugates **7**–**10** with their complementary strand d(GCGTGTGTGCG) were studied by melting temperature (T_m) measurements to assess the influence of the carbohydrate moiety on the stability of the duplex oligonucleotide. All the conjugates showed a similar melting temperature in comparison with the unmodified duplex ($T_m = 64$°C). From these results, it appears that the oxime conjugation with sugar does not alter the thermodynamic stability of the DNA duplex. This is important to note with respect to the recognition property that the conjugate must preserve in order to target a given mRNA or duplex-DNA. This encouraging preliminary result emphasises the usefulness to conjugate a DNA sequence with carbohydrate to confer cell-specific receptor internalisation properties to the resulting COC molecule while sustaining a priori the DNA recognition abilities.

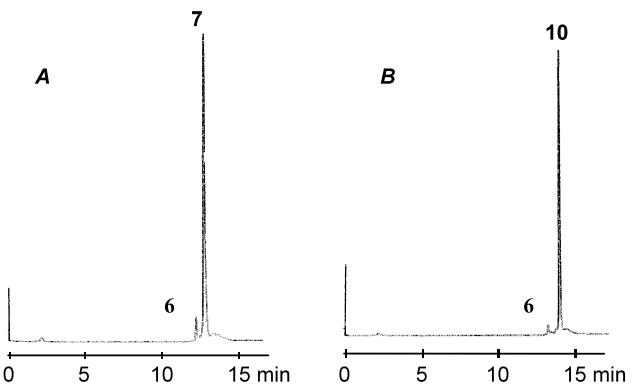


Figure 2. HPLC profiles (detection at 260 nm): (A) crude reaction mixture of aldehyde containing oligonucleotide **6** with Glc-β-ONH_2 **1**; (B) crude reaction mixture of aldehyde containing undecamer **6** with Man-α-ONH_2 **4**.

In conclusion, we have demonstrated that the oxime ligation between aminooxylated-carbohydrate and 5′-aldehyde-containing DNA represents a highly effective and rapid method for preparing COCs. This ligation proceeds in good yield, in aqueous conditions, without the need of protection either at the oligosaccharide or the oligonucleotide level. The DNA conjugated with GalNac, **9**, or Man, **10**, obtained in this study represent paradigm molecules to elaborate further COCs which may serve to target hepatic or dendritic cells via their corresponding asialoglycoprotein- or mannose-receptor. The simple and mild conditions are moreover compatible with a wide range of carbohydrates or DNA sequences which would open up numerous biological applications based on nucleic-acid manipulation in the near future.

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