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Preparation of a Multitopic Glycopeptide–Oligonucleotide Conjugate

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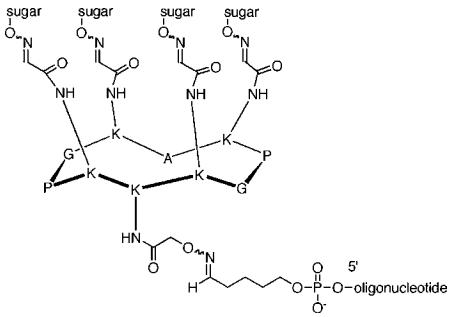
ABSTRACT



A novel strategy to prepare glycopeptide–oligonucleotide conjugates bearing a glycocluster is reported. The strategy utilizes a cyclodecapeptide scaffold as a key intermediate to anchor the carbohydrate cluster and the oligonucleotide through sequential oxime bond formation. The oligonucleotide glycocluster retains the binding affinity and recognition specificity for the target sequence. Furthermore, the conjugate shows enhanced binding to the specific lectins due to the cooperative effect produced by the carbohydrate cluster.

The use of antisense oligonucleotides for specific inhibition of gene expression represents an attractive therapeutic approach for the treatment of cancer and other viral diseases.[1] However, the poor cellular targeting and uptake of these agents seriously limit their applications in either cell culture or in vivo.[2] The problem of inefficient cell- or tissue-specific delivery of the oligonucleotides could possibly be overcome by the use of oligonucleotide conjugates containing ligands that could be recognized by the receptors on the surface of the target cells. For instance, there are a number of sugar binding proteins (lectins) that have been characterized at various cell surfaces.[3] These lectins are known to recognize and internalize different glycoproteins/neoglycoproteins bearing specific sugar residues through endocytosis. Such carbohydrate recognition events play a key role in natural processes such as signal transduction and cell adhesion. Since the individual carbohydrate−protein binding is weak, the natural processes employ multivalent interaction,[4] where carbohydrates adopt an oligomeric form assembled on a natural scaffold (the glycocluster effect).[5]

The glycocluster approach should be useful for the receptor-mediated delivery of the oligonucleotide. Earlier work has shown that the enrichment of oligonucleotide−glycoconjugate or oligonucleotide−carbohydrate complexes on the surface of certain cell types leads to the enhanced internalization of the conjugate by endocytosis.[6] However, due to the need for multivalent interactions, recent work has

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focused on the preparation of oligonucleotide conjugates[7] carrying multivalent carbohydrates. For instance, the preparation of oligonucleotide conjugates with a cholane scaffold bearing trivalent galactoside motif[8] or lectin recognizable DNA−carbohydrate conjugates with multiple lactose or cellobiose has been reported.[9] The solid-phase synthesis of oligonucleotide conjugates with a multiantennary carbohydrate construct has also been described recently.[10,11] Similarly, in another elegant report, specifically galactosylated oligonucleotides were used to design a periodic glycocluster by utilizing the half-sliding oligonucleotide strategy.[12] The oligonucleotide−glycoclusters were shown to cooperatively recognize the specific lectins. Thus, access to well-defined scaffolds that offer better control over the interval and spatial arrangement of carbohydrate motifs has assumed significance.

Recently, we reported a multimeric system for $\alpha_v\beta_3$ integrin targeting[13] by assembling multiple copies of c[-RGDfK-] peptide (a well-known ligand for integrins) on a "regioselectively addressable functionalized cyclodecapeptide template" (RAFT).[14] The advantage associated with the use of this template is that it permits the functional recognition units to be assembled and directed in well-defined and controlled spatial orientations. Moreover, the appropriately spaced lysine residues in the template prevent the overcrowding of the recognition units. We therefore decided to utilize the RAFT template for the preparation of oligonucleotide conjugated with the glycocluster. In the present work, we report on the synthesis of a new oligonucleotide conjugate bearing the carbohydrate cluster **1** (Figure 1). The binding affinity and the recognition specificity of the conjugate **1** for the target sequence is investigated. In addition, the ability of the glycocluster to recognize and bind with the specific lectins is also evaluated.

The RAFT template **4** with two addressable functional domains forms the key intermediate for the preparation of the oligonucleotide glycocluster **1** (Scheme 1). Multiple copies of the carbohydrate units can be attached on one face of the template, whereas the other face can be utilized for attachment to the oligonucleotide. The multiple lactose residues and the oligonucleotide were introduced on the template by sequential oxime bond formation. The oxime bond formation was selected for the carbohydrate−peptide and peptide−oligonucleotide conjugation because this linkage has shown to be highly efficient, as well as compatible with the use of unprotected fragments, and because reactions

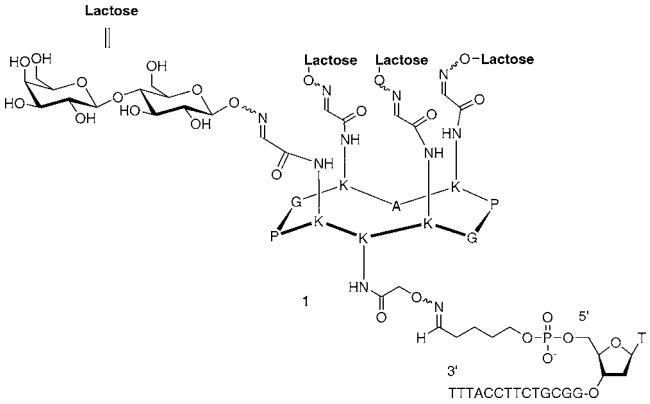

Figure 1. Glycopeptide−oligonucleotide conjugate **1** presenting the lactose cluster.

can be carried out in the absence of coupling reagents with minimal chemical manipulation.[15,16] The preparation of the RAFT template with tetravalent carbohydrate units and their ability to recognize specific lectins in solution has been reported earlier.[17]

The aminooxylated glycopeptide conjugate **2** reported herein was prepared by a similar procedure (Scheme 1). The protected linear peptide **3** was assembled on an acid-labile resin using the standard Fmoc/*t*Bu protocol. The presence of the C-terminal glycine unit was essential to prevent epimerization during the cyclization step. The head to tail cyclization was carried out in DMF at high dilution with PyBOP/DIEA to give the cyclodecapeptide scaffold **4**. The Boc protecting groups were removed under acidic conditions, and the four BocSer(O*t*Bu) moieties were coupled to the free amino group on the side chain of the lysine residues. Finally, the Alloc group was removed by using the standard procedure,[18] and the serine moieties were deprotected under acidic conditions to obtain **5** containing four free serine residues on the same face of the template. The serine oxidation of **5** with $NaIO_4$ generated the four glyoxylic aldehyde groups that were subsequently reacted with the aminooxylated lactose **6**[19] to obtain the glycopeptide conjugate **7** with a tetravalent lactose cluster. The conjugation reaction was carried out in 70% aqueous AcOH solution[17] and monitored by RP HPLC. The aminooxy linker was then generated on the free amino group of the lysine on the other

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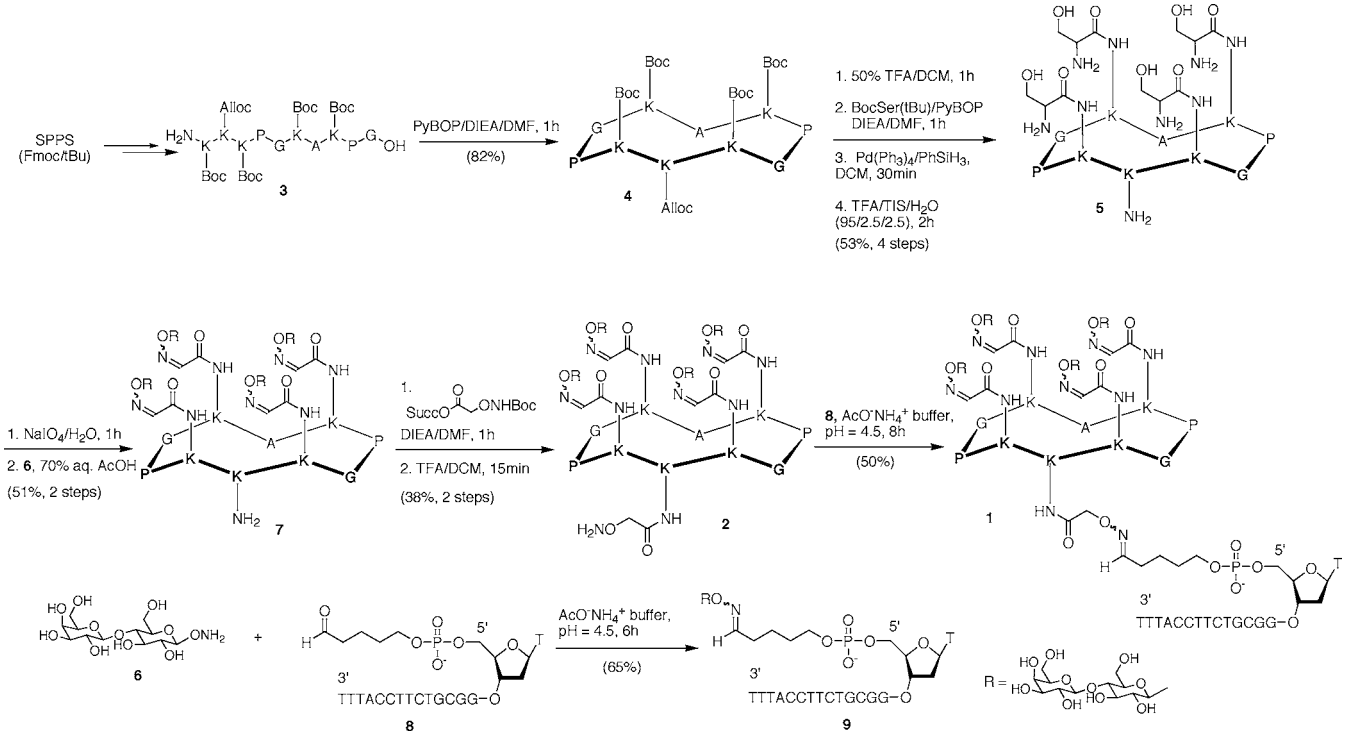
Scheme 1. Preparation of Glycopeptide−Oligonucleotide Conjugate **1**



face of the template by reaction with *N*-hydroxysuccinimide ester of *N*-Boc-*O*-(carboxymethyl)-hydroxylamine followed by deprotection in acidic conditions to obtain **2**.

The aminooxy linker of **2** was used for the reaction with 5′-aldehyde containing oligonucleotide **8** to prepare the oligonucleotide glycocluster **1**. The antisense oligonucleotide used herein is complementary to the initiation codon region of the firefly luciferase mRNA.[20] The 5′-aldehyde oligonucleotide, 5′-d(XTGGCGTC TTCCATTT)-3′ **8** (X is the aldehyde-containing linker), was prepared by a previously reported procedure.[16a]

The conjugation reaction of **8** with the peptide lactose cluster **2** was carried out in a 0.1 M ammonium acetate buffer at room temperature. The reaction was monitored by RP HPLC, which showed the exclusive formation of the product **1** in about 8 h (Supporting Information). The product **1** was obtained in good yield after HPLC purification. Using a similar procedure, the oligonucleotide **8** was reacted with aminooxylated lactose **6** to obtain the lactose oligonucleotide conjugate **9**. The conjugates prepared herein were characterized by ESI MS analysis, and an excellent agreement between the calculated and observed molecular weights was observed (Supporting Information).

The stability of the duplex formed by the oligonucleotide−glycocluster **1** with the complementary sequence was investigated by thermal denaturation experiments. The T_m value for the duplex formed by the conjugate **1** was 59.0 °C, and this was identical to the T_m value (59.0 °C) for the duplex formed by the unmodified oligonucleotide. The T_m decreased sharply (ΔT_m = 13.8 °C) as a single mismatch base was introduced into the complementary sequence (A at G9). The same decrease in the T_m value (ΔT_m = 14.0) was obtained when the unmodified oligonucleotide was hybridized with the complementary mismatch sequence (Supporting Information). The CD spectra for the duplex formed by the conjugate **1** or unmodified oligonucleotide with the complementary sequence showed a pattern characteristic identical to that of a B-type duplex (Supporting Information). The individual CD spectrum showed negative and positive excitation of similar magnitude appearing, respectively, at 245 and 275 nm. The results clearly indicate that the binding affinity and the recognition specificity of the glycocluster **1** are not compromised after conjugation with the glycocluster, unlike the earlier results.[9,11] Besides, the duplex conformation remains unperturbed, contrary to the previous report.[9]

The binding interaction of the oligonucleotide glycocluster **1** to specific lectins obtained from *Arachis hypogaea* (peanut)[21] was investigated after noncovalent immobilization of **1** on the surface of microtiter plates. The peroxidase activity was detected by the addition of a standard HRP substrate. The efficient binding of the conjugate **1** to the specific lectins is clearly evident from the results shown in Figure 2 (bar A). In addition, the binding of the conjugate **1** to the specific lectin shows concentration dependence (inset, Figure 2). Furthermore, the conjugate **1** does not bind to the nonspecific lectins (bar B). This proves that the binding produced by the conjugate **1** is resulting from specific

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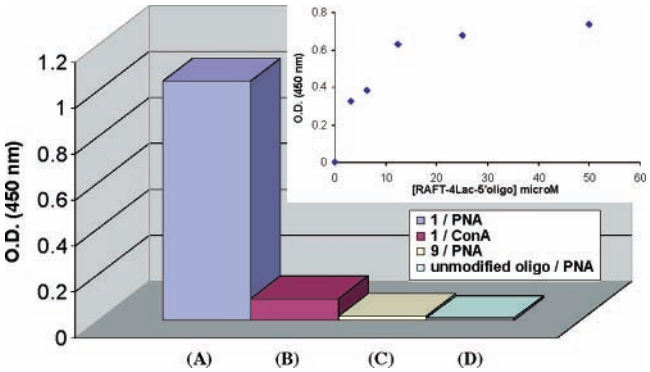

Figure 2. Lectin binding studies. (A) Glycopeptide−oligonucleotide conjugate **1** with specific HRP-labeled lectin (PNA). (B) Glycopeptide−oligonucleotide conjugate **1** with nonspecific HRP-labeled lectin (ConA). (C) Oligonucleotide conjugate with monovalent lactose **9** with specific HRP-labeled lectin (PNA). (D) Unmodified oligonucleotide with specific HRP-labeled lectin (PNA). PNA = lectin from *Arachis hypogaea* (peanut); ConA = concanavalin A lectin from *Canavalia ensiformis* (Jack bean). [C] = 100 μM, lectin 5 μg/mL.

interactions. Moreover, the binding of the conjugate **9** (presenting a monovalent lactose moiety) is negligible (bar C) and is quite the same when compared to the nonmodified oligonucleotide (bar D). This suggests that the monovalent derivative **9** is not recognized by the lectins. This points toward the fact that the four lactose residues are interacting with the lectins cooperatively as a tetravalent lactose cluster.

In conclusion, a convenient strategy for preparing oligonucleotide conjugates with glycoclusters is reported. The strategy involves the attachment of multiple lactose units to a regioselectiveley addressable peptide template RAFT and attachment of this peptide glycocluster to the 5′-terminus of oligonucleotides. The advantage of using the template is that it permits the conformationally controlled presentation of the lactose cluster. The attachment of the peptide−lactose cluster to the oligonucleotide does not influence the binding affinity, recognition specificity, or conformation of the oligonucleotide. The oligonucleotide glycocluster shows efficient binding to the lectins due to the cooperative binding effect of the four lactose residues. The results reported herein could be potentially useful in the studies related to the design of a receptor-mediated delivery strategy for the oligonucleotide.

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Supporting Information Available: Experimental procedure, RP HPLC profiles and ESIMS spectra for **1**, **2**, **8**, and **9**, CD spectra, and T_m data. This material is available free of charge via the Internet at http://pubs.acs.org.

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