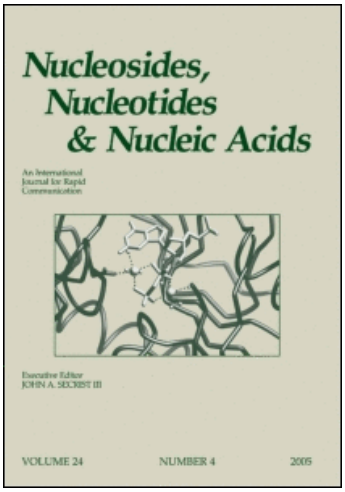

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Chemoselective Oxime and Thiazolidine Bond Formation: A Versatile and Efficient Route to the Preparation of 3′-Peptide-Oligonucleotide Conjugates

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ABSTRACT

Oligonucleotides carrying an aldehyde moiety at the 3′-end were synthesized by the oxidation of a 1,2-diol precursor. These were coupled to peptides bearing a cysteine residue for thiazolidine formation and an aminooxy group for oxime formation. The conjugation reaction proved very efficient and selective, thereby allowing the preparation of 3′-peptide-oligonucleotide conjugates in good yield. The conjugation was achieved in aqueous solution without using any protection strategy. Moreover, the present approach neither requires the use of peptide in excess nor changes the hybridization properties of the conjugates.

Key Words: DNA; Conjugation; Oxime; Peptide; Thiazolidine.

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INTRODUCTION

Due to the specific molecular recognition properties for the target nucleic acid, the oligonucleotides possess the best potential for the rational design of selective gene expression inhibitors. In fact, the oligonucleotides are being extensively used as molecular tool to study the role of various genes and have found useful application as therapeutic agents (antisense, antigene, siRNA strategies).[1–3] However, critical hurdles remains, such as poor cellular uptake efficiency, cell and tissue specific delivery and biostability that have impeded the effective use of oligonucleotide for therapeutic applications. The design of delivery strategies that can improve the cellular uptake and targeting is therefore, crucial for attaining the desired pharmalogical activity. Conjugation with appropriate reporter group has been envisioned to achieve this objective. A number of tethered ligand (i.e. cholesterol derivatives, folic acid, PEG, polyamines...) has been described for the purpose.[4]

In this context, peptide-oligonucleotide conjugates (POCs) have received considerable attention.[5] Peptides containing cationic groups (polylysine, polyarginine) and hydrophobic groups (aromatic amino acid-containing peptides) have been attached to oligonucleotides with the aim of changing the pharmacokinetic distribution. Peptide ligands of cellular receptors have also been conjugated to enhance cellular uptake and targeting. Lastly, peptide conjugation has been used to improve the nuclear localization or can increase the affinity for the DNA or RNA target.[5]

The synthesis of peptide-oligonucleotide conjugates however represents a synthetic challenge even though different methods have been described earlier.[5] Stepwise solid phase synthesis has been successfully applied in some cases but it requires the design of orthogonal protection strategies compatible with the peptide and oligonucleotide synthesis.[6,7] Preparation of POCs using the fragment coupling strategy has emerged as more suitable alternative. In this approach, the peptide and the oligonucleotide are prepared independently followed by subsequent coupling reaction of the two purified moieties, thus affording the desired conjugate. The coupling reaction is critical for the preparation of conjugates in good yield, and various methods (involving disulfide, maleimide, thioether and amide bond formation) have been proposed for this purpose.[8–12] Nevertheless, these methods suffers from certain limitations such as competing reagent hydrolysis and/or cross reactivity with other functionality present within the oligonucleotide or the reporter group. This greatly limits the scope of their application. To circumvent these limitations, we have investigated the use of the oxime and thiazolidine linkages. The efficiency of these methods has been demonstrated for the chemical ligation of peptides[13] and for the conjugation of peptides with carbohydrates[14] as well as for the chemical immobilization of DNA on glass support.[15,16] The use of these strategies for the conjugation of peptides at the 5′-extremity[17] or at the 2′-position inside the sequence of oligonucleotides[18] has been recently reported. The major advantage of such coupling reaction is that conjugation can take place between fully unprotected peptides and oligonucleotides with minimal manipulation and without using an additional preactivation step (as required, for example in case of amide bond formation).

In this paper, we report on the extension of the oxime and thiazolidine bond formation for the preparation of 3′-peptide-oligonucleotide conjugates. For this purpose, an aldehyde precursor was anchored at the 3′-extremity of the oligonucleotide module,

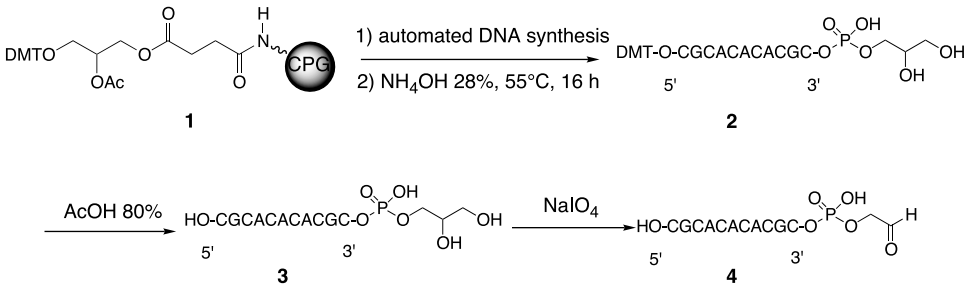
while the peptide module carried the corresponding complementary function: the aminooxy for oxime conjugation or the 1,2-aminothiol for thiazolidine bond formation. Two different peptides were used to emphasize the interest of this 3′-conjugation strategy: 1) a cyclopentapeptide containing an arginine-glycine-aspartic acid tripeptide motif (RGD), which is well known as a powerful and selective ligand of the $\alpha_v\beta_3$ integrin receptor and has been studied for tumor targeting[19] and; 2) a nuclear localization signal sequence Ala-Pro-$(Lys)_3$-Arg-Lys-Val-Glu-Asp-NH_2 (NLS) containing peptide which has been used for cell transfection and nucleus targeting.[20]

RESULTS AND DISCUSSION

Aldehyde Containing Oligonucleotide

For the introduction of the aldehyde moiety, a more convenient post-oxidation strategy was used because the classical acetal protection of aldehyde group could lead to depurination during the acidic deprotection step.[21] In a recent paper, we had reported on the use of a 1,2-amino alcohol containing solid support as a precursor of the aldehyde. The oxidative cleavage of the 1, 2 amino alcohol gives the corresponding aldedyde function at the 3′-end of the ODN.[22] However, further studies with this support have shown irreproducible results. It was observed that the efficiency of the support for the introduction of the 1, 2-amino alcohol moiety at the 3′-end decreases dramatically over a period of 3–4 months of storage even at low temperature. The solid support 3′-glyceryl CPG **1** bearing a 1,2-diol was therefore preferred for the introduction of the aldehyde moiety as the oxidation of the 1, 2-diol group to generate aldehyde is a well-known reaction.

The undecamer d(5′CGCACACACGCX3′) **3**, in which X represents the diol containing 3'-linker, was synthesized according to standard β-cyanoethylphosphoramidite chemistry using the commercial glyceryl solid support **1** at 1 μM scale (Scheme 1). The coupling efficiency as determined from trityl response was found higher than 98%. After deprotection of the nucleobases under the usual conditions (ammonia treatment for 16 h at 55°C), the intermediate oligonucleotide **2** bearing the 5′ trityl protecting group was purified by reverse-phase HPLC (the HPLC analysis of the crude undecamer **2** is depicted in Fig. 1A). Removal of the 5'-protection was then achieved by 80%



Scheme 1. Preparation of the aldehyde containing oligonucleotide **4**.

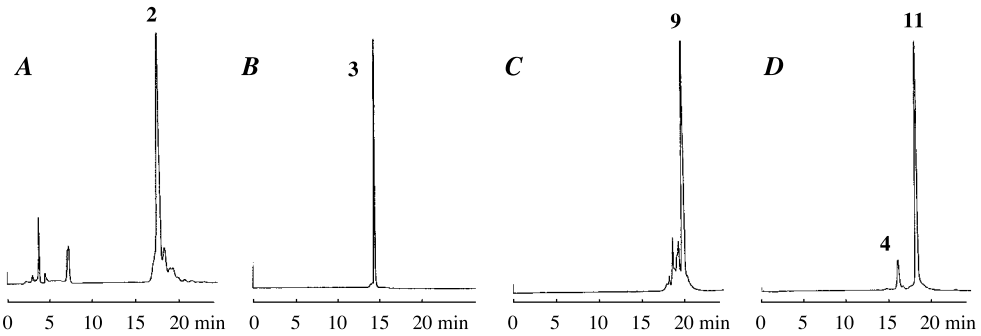


Figure 1. HPLC profiles (detection at 260 nm): ***A***) crude 5′-trityl protected undecamer **2**; ***B***) purified 3′-diol undecamer **3**; ***C***) crude reaction mixture of aldehydic undecamer **4** with RGD peptide **5**; ***D***) crude reaction mixture of aldehydic undecamer **4** with RGD peptide **7**. HPLC conditions: Analytical C_{18} reversed-phase HPLC using a gradient of 0–30% of acetonitrile over 20 min, at a flow rate of 1 mL min^{-1}.

acetic acid treatment for 1 h. Oxidative cleavage of the diol moiety was carried out by using a 20-fold excess of $NaIO_4$ in water at room temperature for 1 h leading to the selective formation of the aldehyde containing oligonucleotide **4** in almost 90% isolated yield. The aldehyde containing oligonucleotide **4** was characterized by ESMS analysis (Table 1).

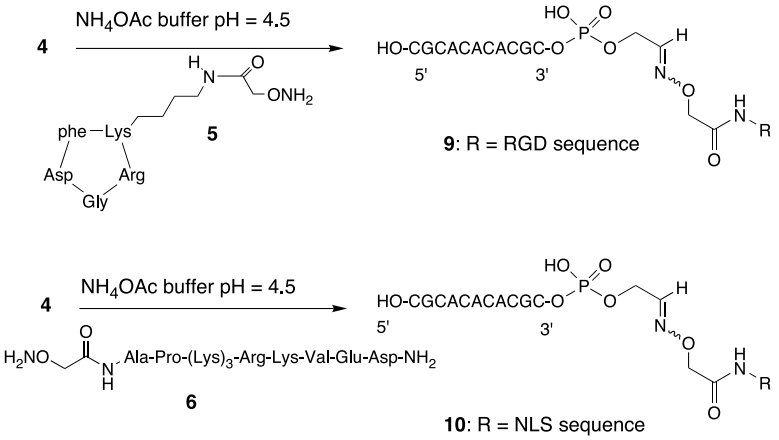
Conjugation Reaction

Oxime Linkage

The conjugation experiment was first performed using the RGD-containing peptide **5** (Scheme 2). A slight excess (2 equiv) of the aminooxy peptide **5** was reacted with the aldehyde containing undecamer **4** in ammonium acetate buffer solution (pH = 4.5). The course of the reaction was followed by reverse-phase HPLC. After 5 h, the HPLC profile of conjugation reaction mixture showed a single major peak along with minor

Table 1. ESMS analysis of the oligonucleotides **3**, **4** and **9–12** and melting temperatures (*T*m) of duplexes formed by hybridization of the indicated oligonucleotides with the complementary strand d(GCGTGTGTGCG).

	$(M-H)^-$		
Oligonucleotide or conjugate	Calcd	Found	Tm ± 1°C
Undecamer 3′-diol **3**	3424.3	3424.8	61
Undecamer 3′-aldehyde **4**	3392.2	3392.3	–
RGD-oxime conjugate **9**	4050.9	4051.7	60
NLS-oxime conjugate **10**	4644.7	4645.7	60
RGD-thiazolidine conjugate **11**	4081.0	4081.1	60
NLS-thiazolidine conjugate **12**	4674.8	4675.1	59



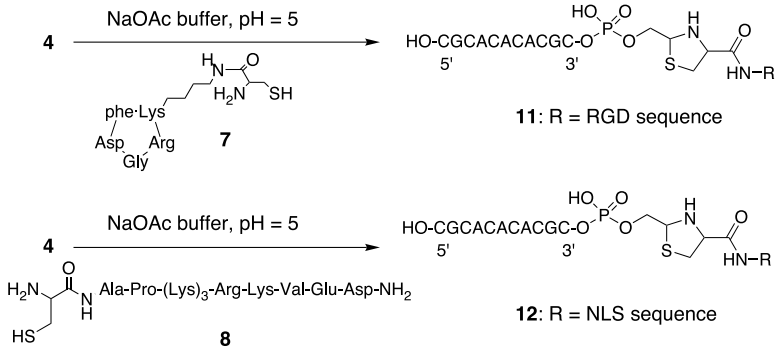
Scheme 2. Preparation of the oxime conjugates **9** and **10**.

impurities (Fig. 1C). Subsequent purification by reverse-phase HPLC afforded the conjugate **9** in almost 50% isolated yield.

The same protocol was applied for the NLS peptide **6**. Reaction of the aldehyde containing undecamer **4** with the aminooxy peptide **6** in ammonium acetate buffer selectively afforded the conjugate **10**. The conjugates **9** and **10** were characterized by ES-MS analysis, which showed that experimentally determined molecular weights are in excellent agreement with the calculated values (Table 1).

Thiazolidine Linkage

For the conjugation reaction involving the formation of the thiazolidine linkage, the RGD-containing peptide **7** was used (Scheme 3). The reaction was carried out at room temperature in sodium acetate buffer with a 3-fold excess of the peptide **7**. It has been reported that the reaction between an 1, 2-aminothiol and an aldehyde proceeds rapidly at pH 4–5.[23] Consequently, the pH was adjusted at pH = 5. Also, acidic



Scheme 3. Preparation of the thiazolidine conjugates **11** and **12**.

conditions should avoid the disulphide formation in the cysteine containing peptide **7**. HPLC analysis revealed the exclusive formation of the conjugate **11** (Fig. 1D shows the HPLC profile of the crude reaction mixture after 5 h). Using similar protocol, oligonucleotide **4** was reacted with NLS peptide **8** to afford the conjugate **12**. The two peptide-oligonucleotide conjugates **11** and **12** were characterized by ES-MS analysis (Table 1).

Hydrolytical Stability of Oxime and Thiazolidine Conjugates

The chemical stability of the oxime and the thiazolidine linkages were studied by incubating the conjugates **9** and **11** (as a representative example) in aqueous buffers of pH 4 and 7. At neutral pH, no significant hydrolysis or degradation products could be observed with **9** even after 72 h of incubation at 37°C. This was further confirmed by ESMS analysis of the final incubated sample that revealed the presence of the starting material containing oxime bond. In contrast the thiazolidine conjugate **11** was completely hydrolyzed to the starting material after 24 h of incubation under similar conditions. At pH 4, the oxime conjugate showed a significant hydrolysis (around 30%) after 72 h of incubation at 37°C whereas the thiazolidine conjugate **11** was again completely hydrolyzed to the starting material after 10 h of incubation under similar conditions. The hydrolysate were analysed by ESMS analysis of the final incubated sample that showed the presence of the aldehyde containing oligonucleotide **4**. Contrary to the previously reported observations,[18] our results indicate that the oxime bond is much more stable than thiazolidine at pH = 4 and 7.

Hybridization Properties of the POC

The hybridization properties of the different peptide-oligonucleotide conjugates **9**–**12** were studied by melting temperature (T*m*) measurements to evaluate the influence of the reporters as well as the type of linkages at the terminal 3′-phosphate. The oxime conjugates **9**–**10** and the thiazolidine conjugates **11**–**12** were hybridized with their complementary strand d(5′GCGTGTGTGCG3′) and the melting temperatures of the resulting duplexes were determined (Table 1). The oligonucleotide **3** containing the 3′-diol linker was studied for comparison. All the peptide-oligonucleotide conjugates showed a melting temperature close to that observed for the oligonucleotide **3**. The nature of the linkage between peptide and oligonucleotide fragments thus do not perturb the duplex stability, as no substantial difference was observed between **9** and **11** or between **10** and **12**. Interstingly, the cationic peptide NLS was not found to affect the stability of the duplex.

CONCLUSION

3′-Conjugation of oligonucleotide with peptide has been readily performed through oxime or thiazolidine bond formation. These one-step ligations are fully compatible with the use of unprotected oligonucleotide and peptide. The high chemoselectivity of these reactions allow rapid and convenient preparation of 3′-peptide-oligonucleotide conjugates without resorting to extensive chemical manipulation. The oligonucleotides

bearing the aldehyde at 3′-end are easily accessible by using the commercial glyceryl solid support through the oxidation of the intermediate 3′-diol. 3′-Conjugation should also be advantageous due to the higher stability to nucleases. Together with the already reported 5′- and 2′-functionalization strategies,[17,18] this 3′-conjugation represents a versatile and highly efficient tool for post-DNA modification and could be of great interest to devise new molecular systems based on modified oligonucleotide.

EXPERIMENTAL SECTION

All commercially available chemical reagents were used without purification. The 3′-glyceryl-CPG solid support **1** was purchased from Eurogentec. The RGD peptide **5** and **7** and the NLS peptide **6** and **8** were prepared by SPPS as previously described.[17] HPLC purifications as well as HPLC analysis of oligonucleotides and conjugates were performed on a Waters system equipped with two M510 pumps, a M490E detector and a M680-system controller. The oligonucleotides and the conjugates were purified on a μ-Bondapak C_{18} column (Macherey-Nagel Nucleosil: 10 × 250 mm, 7 μm). The following system of solvent was used: solvent A, 20 mM ammonium acetate/CH_3CN, 95:5 (v:v); solvent B (CH_3CN); flow rate, 4 mL/min; a linear gradient from 0 to 30% B in 20 min was applied. ElectroSpray Mass Spectra were recorded on an Esquire spectrometer (Bruker). The analysis was performed in the negative mode. The eluent was 50% aqueous acetonitrile and the flow rate was 8 μL/min. The oligonucleotides and the conjugates were dissolved in 50% aqueous acetonitrile and 1% of NEt_3 was added.

Oligonucleotides synthesis. Automated DNA synthesis was carried out on an Expedite DNA synthesizer (Perkin-Elmer) using standard β−cyanoethyl nucleoside phosphoramidites chemistry using the 3′-glyceryl-CPG solid support **1** on a 1 μM scale (14 mg, loading = 71 μmol). After cleavage from the solid support and deprotection by treatment with concentrated ammonia (28%) for 16 h at 55°C, the oligonucleotide **2** was purified by reverse-phase HPLC.

Diol containing oligonucleotide (3). The 5′-protected oligonucleotide **2** was treated with 80% AcOH aqueous solution for 1 h. The residue obtained after lyophilization was dissolved in water and the aqueous layer was extensively washed with Et_2O to remove the trityl by-product. Subsequent lyophilization afforded the oligonucleotide **3**.

Aldehyde containing oligonucleotide (4). To a solution of oligonucleotide **3** (420 μg, 0,12 μmol) in water (500 μL), $NaIO_4$ (514 μg, 2.4 μmol) was added and the solution was stirred at room temperature for 1 h. The oligonucleotide **4** was then purified by reverse-phase HPLC (402 μg, 0.11 μmol, 92%).

Oxime conjugates (9) and (10). To a solution of oligonucleotide **4** (402 μg, 0.11 μmol) in 2 mM ammonium acetate buffer (200 μL, pH = 4.5), a solution of RGD peptide **5** (160 μg, 0.22 μmol) in 2 mM ammonium acetate buffer (10 μL) was added. The mixture was stirred at room temperature and the reaction was monitored by HPLC. Disappearance of the starting material was achieved in 5 h. Purification by HPLC

afforded the conjugate **9** in 50% yield (204 μg). Conjugation with the NLS peptide **6** was achieved using the same protocol with the undecamer **4** leading to the formation of conjugate **10** in 46% isolated yield.

Thiazolidine conjugates (11) and (12). To a solution of oligonucleotide **4** (390 μg, 0.115 μmol) in 0.2 M sodium acetate buffer (200 μL, pH = 5), a solution of RGD peptide **7** (215 μg, 0.230 μmol) in 0.2 M sodium acetate buffer (100 μL) was added. The mixture was stirred at room temperature for 7 h until the complete disappearance of the starting material. Purification by HPLC afforded the conjugate **11** in 50% isolated yield (240 μg, 0.060 μmol). Conjugation with NLS peptide **8** was achieved using the same procedure with the undecamer **4** leading to the formation of conjugate **12** in almost 50% isolated yield.

Analysis of Hydrolytical Stability of Oxime and Thiazolidine Conjugates

The conjugates **9** and **11** (2 A_{260} units) were dissolved in 0.2 M sodium acetate buffer (100 μL, pH = 4) or 0.2 M sodium phosphate buffer (100 μL, pH = 7) and incubated at 37°C. The crude mixtures were analyzed by reverse-phase HPLC and then desalted on C_{18} for ESMS analysis.

Melting Experiments

The melting curves (absorbance versus temperature) were measured at 260 nm on a Varian Cary 400 Scan UV/visible spectrophotometer using a rate of 1°C/min (2–80°C). Melting experiments were carried out by mixing equimolar amounts of the two undecamer strands dissolved in 10 mM sodium phosphate buffer (pH = 7) containing 1 mM EDTA and 100 mM NaCl (concentration of DNA = 4 μM). Before each melting experiment, samples were heated at 80°C for 5 min, then cooled slowly.

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