New Strategy for the Synthesis of 3′,5′-Bifunctionalized Oligonucleotide Conjugates through Sequential Formation of Chemoselective Oxime Bonds

Om Prakash Edupuganti, Yashveer Singh, Eric Defrancq,* and Pascal Dumy[a]

Abstract: A convenient strategy for the synthesis of bifunctionalized oligonucleotide conjugates bearing two different reporters at the 3′ and 5′ ends of the oligonucleotide is presented. The method involves the preparation of oligonucleotides bearing an aldehyde and/or aminooxy functionality at each end, followed by reaction to form oxime bonds with appropriately functionalized reporters. The conjugation reactions are carried out under mild aqueous conditions with good reaction yield.

Keywords: chemoselectivity · conjugation · DNA · oligonucleotides · oxime · peptides

Introduction

The use of synthetic oligonucleotides for the specific inhibition of gene expression represents an attractive therapeutic approach for the treatment of cancer and various other viral diseases.[1–3] These oligonucleotides may cause selective inhibition of gene expression either by targeting the mRNA by an antisense or siRNA mechanism,[4,5] double stranded DNA by triplex formation[6] or proteins by aptamer selection.[7] These strategies suffer, however, from the poor cell penetration and cellular targeting of these agents and sensitivity towards nuclease activity.[8] A very promising approach to overcome the problem of poor uptake is to attach these oligonucleotides to a variety of available cell penetrating and localizing peptides.[9] It has been reported that such peptide–oligonucleotide conjugates show enhanced cell specific targeting, cellular uptake efficiency and stability to degradation in comparison to unmodified oligonucleotides.[9–11] Besides, in many cases, improved binding strength with target sequence has been observed.[12] Similarly, conjugation with carbohydrates has also been explored to enhance cell targeting via lectin recognition.[13]

In this context, the chemical synthesis of peptide–oligonucleotide conjugates (POCs) has generated considerable interest. Different methods for the preparation of POCs have been described and can be categorized into two broad approaches. The first involves the total synthesis of peptide and oligonucleotide fragments on the same solid support. The synthesis of peptide is followed by that of oligonucleotide,[14] or vice versa,[15] and in many cases a branched linker may be attached to the support to permit the independent growth of the peptide and oligonucleotide fragments.[15] The method suffers from the poor compatibility of the peptide and oligonucleotide chemistries and has not found much favour in spite of some promising advances in this area.[16] The second approach (the fragment approach) involves the separate solid phase assembly of peptide and oligonucleotide, deprotection and purification (when necessary), and the coupling of the two fragments in solution phase. This is done by introducing mutually reactive groups into each fragment during or after the solid phase synthesis and involves the formation of linkages like disulfide,[17] thioether,[18] amide,[19] maleimide,[20] oxime,[21] thiazolidine,[21a,b] and hydrazone.[21b,22] This fragment approach is more frequently used on account of excellent coupling efficiencies and ease of purification. The method also offers an opportunity to ligate a number of other reporter molecules (carbohydrates, fluorophores, etc.) onto the oligonucleotide.

Complementary to this monoconjugation, it would be of great interest to anchor two different reporter groups to the oligonucleotides. For instance, attachment of two peptides with different properties may improve the biological activity of the oligonucleotide (for example, enhancement of cell penetration and targeting, increasing of the concentration into the nucleus or stabilization of the duplex) by using a specific peptide according to the property desired. Similarly, bisconjugates could combine a fluorescent moiety with a peptide residue, the fluorophore serving as a probe to detect the transport of the peptide–oligonucleotide conjugate. In this context, the preparation of phosphorothioate oligonu-

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cleotides substituted with a 5′-protected thiol function and a 3′-amino group has been described. The 3′-amino functionality was used to ligate a peptide (NLS) and the 5′-thiol to anchor a fluorescent reporter group.[23a] 3′,5′-Bisconjugates have also been prepared by on-column derivatization using photolabile solid phase synthesis supports.[23d] To our knowledge, little has been reported on the synthesis of oligonucleotides bearing two different peptides at the 3′ and 5′ ends. Therefore, the effort to develop suitable synthetic methods for the preparation of such bifunctionalized peptide–oligonucleotide conjugates should be of great interest.

Earlier work from our laboratory has focussed on the development of oxime and thiazolidine linkages for the efficient preparation of peptide–oligonucleotide conjugates. It has been shown that chemoselective oxime and thiazolidine linkages can be successfully employed to prepare peptide–oligonucleotide conjugates bearing peptides at either the 3′ or 5′ terminus of the oligonucleotide.[21,24] The methodology has been further explored by our group for the labelling of oligonucleotides and RNA[25] and for anchoring the oligonucleotide on glass support.[26] Earlier results from our group and others have shown that oxime and thiazolidine bonds do have certain advantages over other types of linkages. For instance, the oxime bonds give high efficiency of coupling, require the use of no activation or stabilization steps and do not suffer from non-regiospecific ligation as is the case with thio or amine based ligation.[21a,b] Since the oxime ligation is carried out at slightly acidic pH at which the free amino groups in peptides are protonated, it also helps to solubilize the peptide in water either alone or with a cosolvent.[21b]

In a recent work, we have described the preparation of 3′,5′-bisfunctionalized oligonucleotide conjugates bearing same groups at both the termini.[21d] The method involves simultaneous conjugation at the two extremities of the oligonucleotide functionalized with aldehyde groups. However, the procedure has only limited applicability as it does not permit the anchoring of different groups at the two extremities of the oligonucleotide. The preparation of such conjugates would require the use of different orthogonal protecting groups so that the generation of the aldehyde function and subsequent conjugation at the two termini of the oligonucleotide could be carried out in a sequential fashion. Herein, we present a new method for the preparation of 3′,5′-derivatized oligonucleotide conjugates bearing two different reporter groups, both with two different peptides and with a peptide and a fluorescent probe. The method is based on the sequential formation of chemoselective oxime bonds using two different strategies (Figure 1). The first involves the sequential generation of an aldehyde function on both ends of the oligonucleotide and subsequent conjugation reaction with the reporter group containing an aminooxy moiety (Figure 1a). The second requires the sequential generation of an aldehyde and an aminooxy function on the oligonucleotide followed by conjugation with appropriately derivatized reporters (Figure 1b).

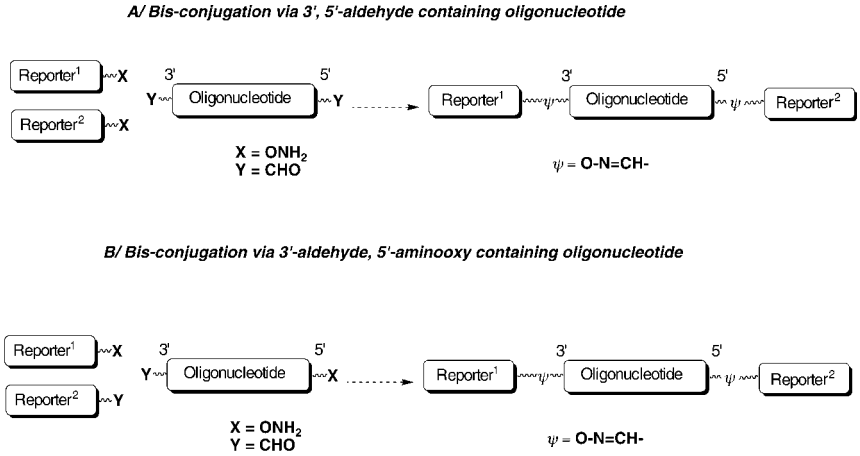
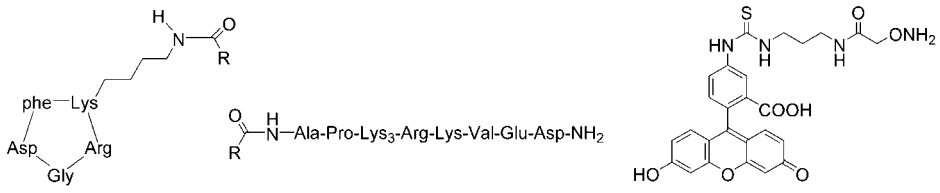


Figure 1. General strategy for the preparation of 3′,5′-bisfunctionalized oligonucleotides.

The relevance of the work has been emphasized by using two different peptides of biological significance (Figure 2). Firstly, a cyclopentapeptide consisting of an arginine-glycine-aspartic acid (RGD) motif known for selectivity towards the $\alpha_v\beta_3$ integrin receptor.[27] The RGD peptide has also been studied for tumour targeting[27b] and DNA delivery.[27c,d] Secondly, the NLS peptide, a nuclear localizing signal sequence with the basic peptide APKKKRKVED derived from the simian virus 40 antigen. The oligonucleotide conjugate with this sequence has been reported and affinity for the target sequence has been studied.[12b] A cytomegalovirus luciferase gene bearing this sequence has also been studied for transfection[28a] and the ability of this sequence for non-viral gene delivery has been thoroughly investigated recently.[28b] The fluorescein derivative **10** has been used as fluorescent reporter group.



P1: R = CH_2-ONH_2 **P2**: R = CH_2-ONH_2 **10**

P3: R = CHO **P4**: R = CHO

Figure 2. Structure of the different peptides **P1**–**P4** and fluorescein derivative **10**.

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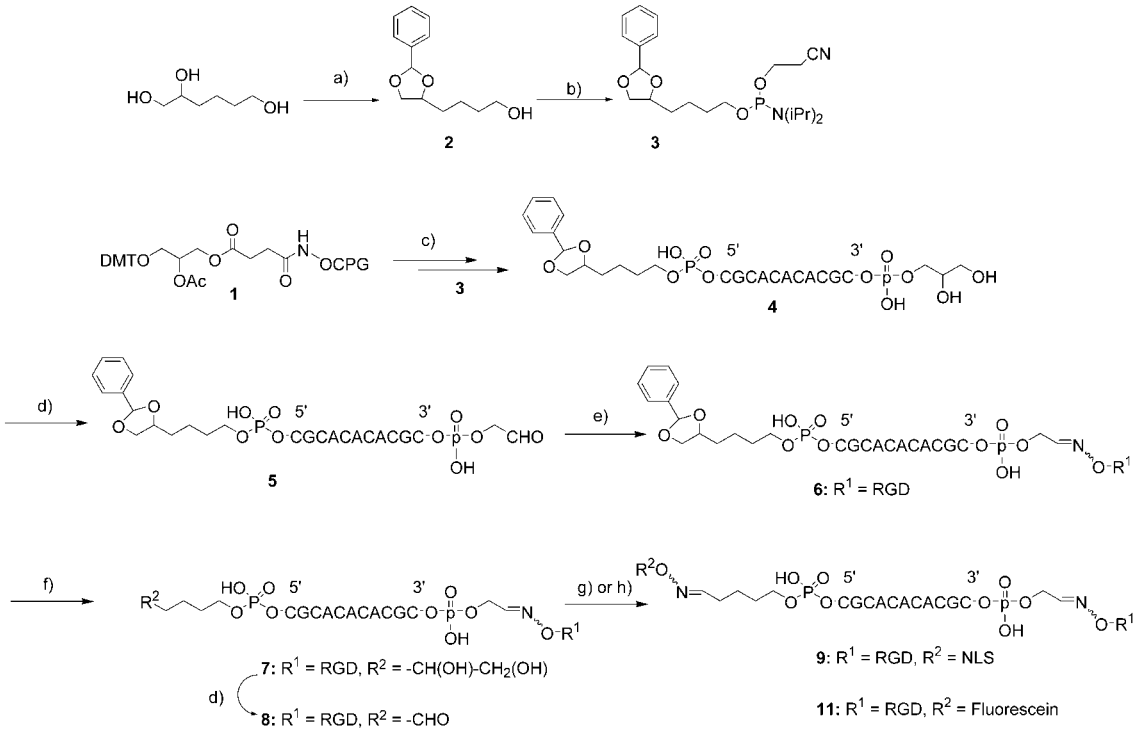
Results and Discussion

Bisconjugation via 3′,5′-aldehyde functionality (Scheme 1): This strategy consists of a first conjugation reaction with the aldehyde at the 3′ end followed by the liberation of the second aldehyde moiety at the 5′ end and a subsequent second conjugation. The generation of aldehyde function at the 3′ end requires the modification of the solid support. Since depurination is observed during the acidic deprotection a post oxidation strategy is preferable for the introduction of the aldehyde moiety at the 3′ end. In the present work we have used the commercially available solid support **1** (3-[(4,4′-dimethoxytrityl) glyceryl-1-succinyl] long chain alkylamino controlled pore glass, CPG) to introduce the aldehyde function. The preparation of oligonucleotides bearing an aldehyde function at the 5′ end has been described earlier by using a modified phosphoramidite linker carrying a masked 1,2-diol group.[21a] The aldehyde was generated by mild oxidation with sodium periodate. However, the methoxybenzylidene acetal protection of the 5′-diol linker was found unsuitable for our strategy. In fact, cleavage of the 5′-diol protecting group occurred both during the purification step and the conjugation reaction. The benzylidene acetal protection was chosen, therefore, as it is reported to be more stable. The phosphoramidite linker **3** carrying a protected 1,2-diol was prepared in two steps starting from commercially available 1,2,6-hexanetriol. The 1,2-diol was first protected as benzylidene acetal **2** using benzaldehyde dimethyl acetal in the presence of a catalytic amount of PPTS. Phosphitylation with 2-cyanoethyl diisopropylchlorophosphoramidite afforded the desired modified phosphoramidite linker **3** carrying the protected diol. It should be noted that this linker would also aid in oligonucleotide purification because of its hydrophobic nature.

The 3′,5′-bifunctionalized undecamer d(5′XCGCACACACGCY3′), in which X represents the 5′-diol linker and Y the 3′-diol linker, was prepared by automated DNA synthesis, according to standard β-cyanoethylphosphoramidite chemistry by using the aforementioned support **1**. The phosphoramidite **3** was incorporated during the last step of the automated DNA synthesis. After cleavage from the solid support and deprotection of the nucleobases by usual ammonia treatment (28% ammonia, 16 h at 55°C), the oligonucleotide **4** carrying the free 3′-diol and the protected 5′-diol linker was purified by reverse phase HPLC. Oligonucleotide **4** was obtained as a single peak by reverse phase HPLC (Figure 3a). No hydrolysis of the 5′-benzylidene protected group was observed (which would give an oligonucleotide bearing a diol moiety at both ends, unsuitable for subsequent monoconjugation). Further oxidation with sodium periodate in water gave the desired 3′-aldehyde oligonucleotide **5** in 70% isolated yield. The 3′-conjugation reaction was then performed with the RGD peptide **P1** bearing an aminooxy function (Figure 2). Reaction was carried out in ammonium acetate buffer at slightly acidic pH (4.6) and was monitored by HPLC. The reaction proceeded to completion within 4 h to yield the 3′-RGD conjugate **6** as the major product (Figure 3b). No cleavage of the 5′-protecting group was observed in the slightly acidic conditions used for the coupling reaction. The 3′-RGD,5′-protected diol conjugate **6** was obtained after HPLC purification in 58% isolated yield. The conjugate **6** was then treated with 80%



Scheme 1. Preparation of conjugates **9** and **11**. a) benzaldehyde dimethylacetal, PPTS; b) 2-cyanoethyldiisopropylchloro phosphoramidite, DIEA; c) automated oligonucleotide synthesis; d) $NaIO_4$; e) RGD peptide **P1**; f) 80% aqueous AcOH; g) NLS peptide **P2**; h) fluorescein derivative **10**. PPTS = pyridinium paratoluenesulfonate; DIEA = diisopropylethylamine.

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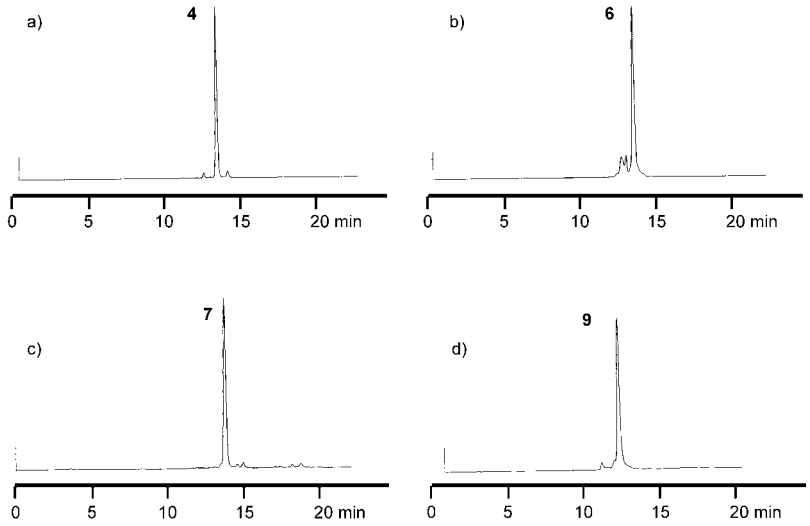

Figure 3. HPLC profiles (detection at 260 nm): a) purified undecamer 3′-diol,5′-protected diol **4**; b) crude reaction mixture of 3′-aldehyde containing undecamer **5** with RGD peptide **P1** (1st conjugation); c) crude reaction mixture of deprotection of **6**; d) crude reaction mixture of 5′-aldehyde containing undecamer **8** with NLS peptide **P2** (2nd conjugation). For the HPLC conditions, see Experimental Section.

aqueous acetic acid solution for 1 h to remove the 5′-protecting group and give the 3′-RGD,5′-diol conjugate **7**. It is important to note that in these slightly acidic conditions, which are commonly employed for 5′-detritylation, no hydrolysis of the oxime bond at the 3′-end was observed (Figure 3c shows the crude deprotection mixture which reveals the presence of a single major peak). Another oxidation step using sodium periodate in water gave the 3′-RGD,5′-CHO conjugate **8**. No degradation of the oxime bond was noted during this oxidative treatment. The second conjugation reaction was carried out with the NLS peptide **P2** carrying an aminooxy function (Figure 2) under similar conditions as for RGD incorporation at the 3′ end (i.e., ammonium acetate buffer at pH 4.6). The course of the reaction was followed by reverse phase HPLC and exclusive formation of the desired 3′-RGD,5′-NLS conjugate **9** was observed (Figure 3d). The same protocol was then applied with the aminooxy containing fluorescein derivative **10** leading to the 3′-RGD,5′-fluorescein conjugate **11**. The conjugates **9** and **11** were obtained after purification by HPLC in 55–60% isolated yields. All the oligonucleotide derivatives prepared herein were characterized by ES-MS analysis (see Supporting Information), which showed an excellent agreement between the experimentally determined molecular weights and the calculated values (Table 1).

Table 1. ES-MS analysis.[a]

Oligonucleotide	M_{calcd}	M_{found}
3′-diol,5′-protected diol **4**	3707.5	3708.8
3′-CHO,5′-protected diol **5**	3676.4	3676.3
3′-RGD,5′-protected diol **6**	4351.1	4351.1
3′-RGD,5′-diol **7**	4263.0	4262.7
3′-RGD,5′-CHO **8**	4230.9	4231.0
3′-RGD,5′-NLS **9**	5483.4	5483.6
3′-RGD,5′-fluorescein **11**	4749.5	4750.2
3′-diol,5′-protected aminoxy **13**	3862.2	3861.9
3′-CHO,5′-protected aminoxy **14**	3830.2	3829.1
3′-NLS,5′-protected aminoxy **15**	5082.6	5081.8
3′-NLS,5′-RGD **16**	5482.3	5481.0
3′-diol,5′-NLS **17a**	4854.2	4853.2
3′-diol,5′-RGD **17b**	4261.2	4260.0
3′-CHO,5′-NLS **18a**	4822.2	4822.3
3′-CHO,5′-RGD **18b**	4229.2	4229.1
3′-fluorescein,5′-NLS **19**	5341.3	5340.9

[a] The analysis was done in negative mode. The oligonucleotides and conjugates were dissolved in $CH_3CN/H_2O/NEt_3$ 50:50:2 (*v*/*v*/*v*). Eluent: CH_3CN/H_2O 50:50 (*v*/*v*).

Bisconjugation via 3′,5′-hetero-bifunctionalized oligonucleotide (Scheme 2): This bifunctionalization strategy offered two ways to prepare 3′,5′-bisconjugates. One consists of starting the conjugation with the aldehyde at the 3′ end followed by the deprotection of the aminooxy group at the 5′ end and subsequent coupling with an aldehyde-containing reporter. The second involves the "reverse way" where the deprotection and coupling at the 5′ end is carried out first, followed by the oxidation of the 3′-diol and subsequent coupling with an aminooxy-containing reporter. In this strategy it should be noted that the peptide used for the 5′-conjugation contains a glyoxylic aldehyde group proceeding from the oxidation of a 1,2-aminoalcohol residue of a serine. The formed oxime is thus a glyoxylic oxime which has been reported to be more stable than an aliphatic one.[29]

Introduction of the 3′ linker was achieved using the same strategy as above with the glyceryl support **1**. Anchoring the aminooxy moiety at the 5′ end using the phosphoramidite **12**, in which the aminooxy moiety is protected as a trityl, has been reported already.[21a] The trityl protection has the advantage that it can be removed under the same conditions as the classical DMT group used in DNA synthesis. Furthermore, the purifications by reverse phase HPLC of crude oligonucleotides were facilitated by the hydrophobic properties of the trityl group. The 3′,5′-bifunctionalized undecamer d($^{5'}$XCGCACACACGCY$^{3'}$), in which X represents the 5′-trityl protected aminooxy linker and Y the 3′-diol linker, was prepared by automated DNA synthesis according to standard β-cyanoethylphosphoramidite chemistry. After cleavage from the support and deprotection of bases using the standard protocol, the bifunctionalized 3′-diol,5′-trityl protected aminooxy oligonucleotide **13** was purified by reverse phase HPLC (Figure 4a).

Strategy starting with 3′ conjugation: The oxidative cleavage of the diol at the 3′ end of the oligonucleotide was carried out under the same conditions as above, by using

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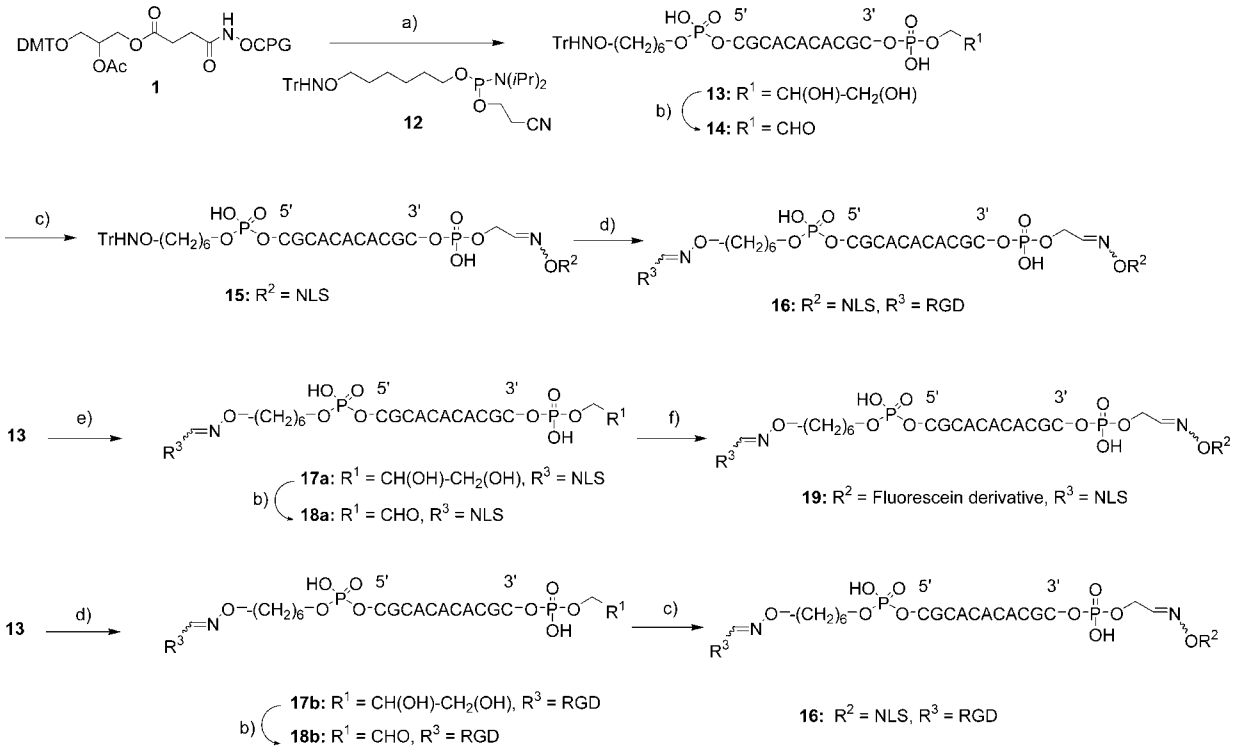

Scheme 2. Preparation of conjugates **16** and **19**. a) automated oligonucleotide synthesis; b) $NaIO_4$; c) NLS peptide **P2**; d) 80% aqueous AcOH, RGD peptide **P3**; e) 80% aqueous AcOH, NLS peptide **P4**; f) fluorescein derivative **10**.

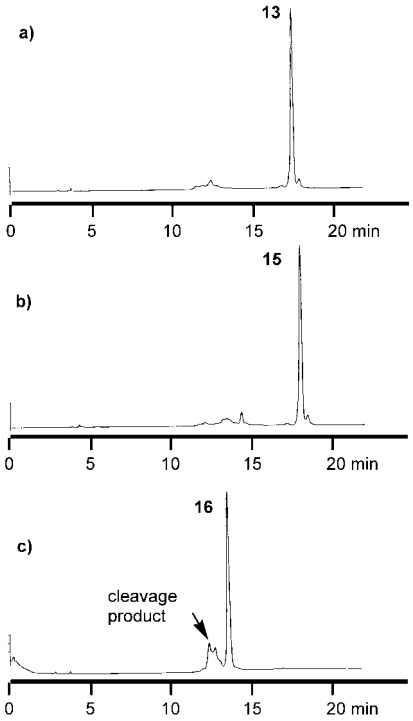

Figure 4. HPLC profiles (detection at 260 nm): a) purified undecamer 3′-diol,5′-protected aminooxy **13**; b) crude reaction mixture of 3′-aldehyde containing undecamer **14** with NLS peptide **P2** (1st conjugation); c) crude reaction mixture of conjugate **15** with RGD peptide **P3** (2nd conjugation). For the HPLC conditions, see Experimental Section.

$NaIO_4$, leading to 3′-aldehyde-containing oligonucleotide **14**. The 3′-conjugation reaction was then performed with the NLS peptide **P2** in ammonium acetate buffer (pH 4.6). Under these conditions the 5′-trityl protection was found to be stable as confirmed by HPLC analysis (Figure 4b depicts the crude conjugation mixture which reveals a single major peak). The 3′-NLS,5′-trityl protected aminooxy conjugate **15** was obtained after purification by reverse phase HPLC in 50% yield. The 5′ conjugation was then achieved using a one-pot trityl cleavage and coupling reaction. This procedure has the advantage that it eliminates problems associated with the highly reactive aminooxy moiety. Reaction was carried out in 80% aqueous acetic acid in the presence of the RGD peptide **P3** containing the aldehyde function (Figure 2). The liberation of the aminooxy moiety at the 5′ end resulted in the immediate conjugation with the aldehydic peptide **P3** through the formation of the oxime bond. The unprotected oligonucleotide intermediate could never be observed. The course of the reaction was controlled by careful monitoring on analytical HPLC and revealed some cleavage of the oxime bond at the 3′ end (Figure 4c depicts the HPLC profile of the crude reaction mixture which shows the presence of less than 10% of the product resulting from cleavage of the 3′-oxime bond). Purification by reverse phase HPLC afforded the 3′-NLS,5′-RGD conjugate **16** in 50% isolated yield.

Strategy starting with 5′ conjugation: In this case, the first conjugation was carried out at the 5′ end of the oligonucleotide leading to the formation of a glyoxylic oxime. As this

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type of oxime has been described as more stable under acidic conditions we presumed that cleavage during the second conjugation should be less than that observed in the case of the strategy starting with 3′ conjugation. The first conjugation was performed using the same procedure as above (i.e., one-pot detritylation/coupling). Reaction of the 3′-diol,5′-trityl protected aminooxy oligonucleotide **13** was carried out in 80% aqueous acetic acid solution in the presence of the NLS peptide **P4** containing the aldehyde moiety at the N-terminus (Figure 2). Analysis by HPLC showed the selective formation of the 3′-diol,5′-NLS conjugate **17a** which was purified by reverse phase HPLC (Figure 5a). The

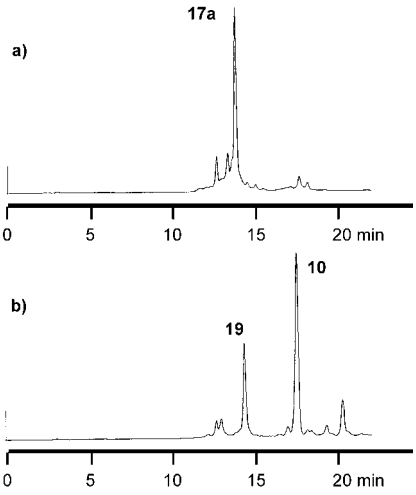

Figure 5. HPLC profiles (detection at 260 nm): a) crude reaction mixture of undecamer 3′-diol,5′-protected aminooxy **13** with the NLS peptide **P4**; b) crude reaction mixture of conjugate **18a** with fluorescein derivative **10**.

3′-diol was then oxidized under the mild $NaIO_4$ conditions described above leading to the 3′-CHO,5′-NLS conjugate **18a**. A second conjugation was then performed with the fluorescein derivative **10** in ammonium acetate buffer at pH 4.6. The course of reaction was monitored by HPLC and reaction completion was achieved after 4 h (Figure 5b). No traces of compound resulting from the cleavage of the 5′-oxime could be detected. Purification by HPLC afforded the desired 3′-fluorescein,5′-NLS conjugate **19**. The 3′-NLS,5′-RGD conjugate **16** was also prepared using the same strategy starting from the 3′-diol,5′-trityl protected aminooxy oligonucleotide **13**. All the oligonucleotides and conjugates **13**–**19** were characterized by ES-MS analysis which showed experimental molecular weights in excellent agreement with the calculated values (Table 1).

Hybridization properties of the POCs (Table 2): Hybridization properties of the conjugates were studied by melting temperature (T_m) measurements to evaluate the influence of the anchoring of two reporter groups on the stability of the duplex oligonucleotide. The conjugates **9** and **16** containing the RGD and the NLS peptide at the 3′ and 5′ ends respectively, or vice versa, were hybridized with their complementary strand d(GCGTGTGTGCG). The conjugates **11** and **19** containing the fluorescent label at the 5′ and 3′ ends, respectively, were also studied. The melting temperatures of the resulting duplexes were determined and the "natural" oligonucleotide d(CGCACACACGC) without any reporter was also studied for comparison. The melting temperature data are collected in Table 2. It is evident that duplexes containing the modified oligonucleotides are slightly more stable than the duplex containing the natural oligonucleotide. This could be attributed to the positive charges of the lysine and arginine side chains present within the peptides. The results reported herein are consistent with the earlier reported observations and further emphasize the fact that bisconjugation does not induce any instability in the duplex.

Table 2. Melting temperatures of the duplexes formed by hybridization of the indicated oligonucleotides with the complementary strand d(GCGTGTGTGCG).[a]

ON	T_m [°C]
unmodified undecamer	59.0±1
3′-RGD,5′-NLS **9**	62.0±1
3′-NLS,5′-RGD **16**	62.0±1
3′-RGD,5′-fluorescein **11**	61.0±1
3′-fluorescein,5′-NLS **19**	63.0±1

[a] Measurements were performed in sodium phosphate buffer (10 mM) containing EDTA (1 mM) and NaCl (100 mM) at pH 7.

Conclusion

In conclusion, we present a very convenient and facile strategy to prepare bifunctionalized peptide–oligonucleotide conjugates containing two different peptides at the 3′ and 5′ ends. The strategy is also applicable to the preparation of peptide–oligonucleotide conjugates carrying a fluorescent reporter group. The procedure involves sequential formation of oxime bonds in a one step ligation that is compatible with the use of unprotected peptide and oligonucleotide. The method thus allows the rapid and efficient preparation of POCs where the cell-penetrating and cell-targeting peptides can be anchored on the same oligonucleotide. Moreover, the bifunctionalization of oligonucleotides does not destabilize the duplexes. The results open up new prospects for the preparation and availability of a large variety of POCs for molecular biology studies.

Experimental Section

Materials and methods: All solvents and reagents used were of the highest purity available. 1,2,6-Hexanetriol, pyridinium *para*toluenesulfonate and sodium periodate were obtained from Aldrich and used without further purification. The solid support **1** (3-[(4,4′-dimethoxytrityl) glyceryl-1-succinyl] long chain alkylamino CPG) was purchased from Eurogentec. The peptides **P1**–**P4** were prepared by solid phase peptide synthesis according to a reported method.[21a] The aminooxy fluorescein derivative **10**[25b] and the phosphoramidite **12**[21a] were prepared as previously described. The oligonucleotides and conjugates were purified on a μ-Bondapak C-18 column (Macherey-Nagel Nucleosil: 10×250 mm, 7 μm) using the

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following solvent system: solvent A, 20 mM ammonium acetate/CH_3CN 95:5 (*v*/*v*); solvent B (CH_3CN); flow rate, 4 mL min^{-1}; a linear gradient from 0 to 30% B in 20 min was applied. The purity of the product was assessed on analytical column using the same gradient at a flow rate of 1 mL min^{-1}. Mass Spectra were measured on a Delsi-Nermag R10-10 for EI and DCI and on an Esquire 3000 (Bruker) for ESI. The analysis was performed in the negative mode for the oligonucleotides and conjugates using 50% aqueous acetonitrile as eluent. The oligonucleotides and conjugates were dissolved in 50% aqueous acetonitrile and 2% NEt_3 was added. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AC 200 spectrometer.

4-[2-Phenyl-(1,3)-dioxolan-4-yl]-butanol-1-ol (2): 1,2,6-Hexanetriol (3.68 g, 27.52 mmol) and a catalytic amount of PPTS (0.3 g) were added to a solution of benzaldehyde dimethyl acetal (2.095 g, 13.76 mmol) in dry DMF (30 mL). The reaction mixture was stirred for 24 h at 55 °C under anhydrous conditions. The DMF was then removed under vacuum and the oily residue obtained was dissolved in EtOAc. The organic layer was washed successively with saturated aqueous $NaHSO_3$, 10% aqueous $NaHCO_3$ and brine, and dried over anhydrous Na_2SO_4. The pure product **2** was obtained by purification (2.1 g, 72%) on silica column using CH_2Cl_2. ^{1}H NMR ($CDCl_3$): δ = 7.52–7.44 (m, 2H; Ar-H), 7.42–7.34 (m, 3H; Ar-H), 5.92 and 5.80 (2 s, 1H; O-CH-O), 4.28–4.08 (m, 2H; CH_2O), 3.95–3.71 (m, 3H; CH-O and CH_2O), 1.84–1.42 (m, 6H; $3CH_2$); MS (DCI/NH_3): *m*/*z*: 222.8 $[M+H]^+$.

Phosphoramidite (3): DIEA (302 mg, 2.33 mmol) and 2-cyanoethyl diisopropylchlorophosphoramidite (359 mg, 1.5 mmol) were added under argon to a solution of compound **2** (260 mg, 1.16 mmol) in anhydrous CH_2Cl_2 (3 mL). The reaction mixture was stirred at room temperature for 4 h and CH_2Cl_2 (20 mL) was then added. The organic layer was washed successively with 10% aqueous $NaHCO_3$, then with brine and dried over anhydrous Na_2SO_4. The crude mixture was purified by silica gel column chromatography (AcOEt/cyclohexane/Et_3N 90:10:2) to give compound **3** as a white oil (0.320 g, 65%). ^{1}H NMR ($CDCl_3$): δ = 7.48–7.44 (m, 2H; Ar-H), 7.37–7.35 (m, 3H; Ar-H), 5.91 and 5.79 (2 s, 1H; O-CH-O), 4.27–4.07 (m, 2H; CH_2O), 3.87–3.75 (m, 2H; CH_2O), 3.70–3.44 (m, 5H; CH_2O, 3CH), 2.62 (t, 2H; CH_2CN), 1.70–1.45 (m, 6H; $3CH_2$), 1.18–1.15 (m, 12H; $4CH_3$); ^{31}P NMR ($CDCl_3$): δ = 145.5; MS (DCI/NH_3): *m*/*z*: 422.8 $[M+H]^+$.

Oligonucleotide synthesis: Automated DNA synthesis was carried out on an Expedite DNA synthesizer (Perkin–Elmer) using standard β-cyanoethyl nucleoside phosphoramidite chemistry on a 1 μM scale with final DMT on, using the modified solid support **1**. The modified phosphoramidites **3** and **12** (0.1 g mL^{-1} in dry acetonitrile) were coupled to the support bound oligonucleotide during the last step of the automated DNA synthesis using an extended coupling time (15 min). The oligonucleotides were cleaved from the solid support by treatment with a 28% ammonia solution for 2 h and finally deprotected by keeping the ammonia solution at 55 °C for 16 h. The 5′-protected oligonucleotides carrying free 3′-diol **4** and **13** were purified by reverse phase HPLC and characterized by ESMS analysis (Table 1).

Bis-conjugation via 3′,5′-aldehyde functionality

3′-Aldehyde containing oligonucleotide (5): $NaIO_4$ (20 equiv, 0.923 mg) was added to a solution of oligonucleotide **4** (0.8 mg, 0.215 μmol) in water (1 mL), and the solution was stirred at room temperature for 1 h. The resulting oligonucleotide **5** was immediately purified by HPLC (0.554 mg, 70%).

3′-RGD,5′-protected diol conjugate (6): A solution of RGD peptide **P1** (4 equiv) in water was added to a solution of oligonucleotide **5** (0.5 mg, 0.136 μmol) in 0.1 M ammonium acetate buffer (0.5 mL, Ph 4.6). The reaction mixture was stirred at room temperature and monitored by HPLC. Completion of the reaction was achieved in 4 h. Purification by HPLC afforded the 3′-conjugate **6** (0.35 mg, 60%).

3′-RGD,5′-diol conjugate (7): Oligonucleotide **6** (0.32 mg, 0.07 μmol) was treated with an 80% AcOH aqueous solution (0.5 mL) for 1 h. The acetic acid was then lyophilized affording the oligonucleotide **7** (0.19 mg, 60%), which was used in the next step without further purification.

3′-RGD,5′-aldehyde conjugate (8): Oxidative cleavage of the 5′-diol was performed as above for **5**. Starting from oligonucleotide **7** (0.18 mg, 0.042 μmol), the oligonucleotide **8** was obtained after purification by HPLC (0.12 mg, 70%).

3′-RGD,5′-NLS conjugate (9): An aqueous solution of NLS-peptide **P2** (4 equiv) was added to a solution of oligonucleotide **8** (0.11 mg, 0.026 μmol) in 0.1 M ammonium acetate buffer (0.1 mL; pH 4.6). The reaction mixture was stirred for 3 h at room temperature leading to the formation of conjugate **9** which was purified by HPLC (0.08 mg, 55%).

3′-RGD,5′-fluorescein conjugate (11): Conjugation with fluorescein derivative **10** was achieved using the same protocol as for **9** and led to the conjugate **11** in 61% yield.

Bisconjugation via hetero-bifunctionalized oligonucleotide starting with 3′-conjugation

3′-Aldehyde,5′-trityl aminooxy containing oligonucleotide (14): The oxidation of the 3′-diol was achieved using the same protocol as above for **5**. Starting from the 3′-diol oligonucleotide **13** (0.84 mg, 0.217 μmol), the conjugate **14** was obtained (0.74 mg, 90%).

3′-NLS,5′-trityl aminooxy containing conjugate (15): A solution of the NLS peptide **P2** in water (2 equiv, 0.5 mg) was added, to a solution of the oligonucleotide **14** (0.74 mg, 0.193 μmol) in 0.1 M ammonium acetate buffer (0.7 mL, pH 4.6). The reaction mixture was stirred for 4 h at room temperature affording the 3′-NLS conjugate **15** in 50% yield (0.47 mg) after purification by HPLC.

3′-NLS,5′-RGD conjugate (16): The 3′-NLS conjugate **15** (0.45 mg, 0.088 μmol) was dissolved in an 80% aqueous AcOH solution (0.4 mL) and the RGD peptide **P3** in water (3 equiv, 0.175 mg) was added. The reaction mixture was stirred overnight at room temperature and the acetic acid was then lyophilized. The crude mixture was then purified by HPLC to give the 3′-NLS,5′-RGD conjugate **16** (0.24 mg, 50%).

Bisconjugation via hetero-bifunctionalized oligonucleotide starting with 5′-conjugation

3′-Diol,5′-NLS conjugate (17a): Oligonucleotide **13** (0.95 mg, 0.246 μmol) was dissolved in an 80% aqueous AcOH solution (0.8 mL) and the NLS peptide **P4** (3 equiv, 0.93 mg) in aqueous solution was added. The reaction mixture was stirred for 8 h at room temperature leading to the formation of the 5′-NLS conjugate **17a**, which was purified by HPLC (yield: 60%, 0.7 mg). The 3′-diol,5′-RGD conjugate **17b** was obtained in the same manner using RGD peptide **P3** (yield: 57%).

3′-Aldehyde,5′-NLS conjugate (18a): Oxidation of the 3′-diol moiety was achieved as described for **5** by using $NaIO_4$. The conjugate **18a** was obtained in 81% yield after purification by HPLC. The 3′-aldehyde,5′-RGD conjugate **18b** was prepared using the same protocol with the undecamer **17b** (yield: 74%).

3′-Fluorescein,5′-NLS conjugate (19): A solution of the fluorescein derivative **10** (2 equiv, 0.06 mg) in DMF was added to a solution of the 3′-aldehyde,5′-NLS conjugate **18a** (0.25 mg, 0.052 μmol) in 0.1 M ammonium acetate buffer (0.22 mL, pH 4.6). The mixture was stirred for 4 h at room temperature and the crude product was purified by HPLC to afford the conjugate **19** (0.11 mg, 40%).

3′-NLS,5′-RGD conjugate (16): An aqueous solution of NLS peptide **P2** (2 equiv, 0.12 mg) was added to a solution of oligonucleotide **18b** (0.2 mg, 0.047 μmol) in 0.1 M ammonium acetate buffer (0.3 mL, pH 4.6). The reaction mixture was stirred for 4 h at room temperature and the conjugate **16** was then purified by HPLC (0.180 mg, 70%).

Melting studies: The melting curves (absorbance versus temperature) were measured at 260 nm on a Lambda 5 UV/Vis spectrophotometer equipped with a Perkin–Elmer C570–070 temperature controller using a rate of 1 °C min^{-1} (from 2 to 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. All measurements were done at a concentration of 12 mM. Before each melting experiment, samples were heated at 80 °C for 5 min then cooled slowly. Experiments were carried out in duplicate.

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