Highly Efficient Synthesis of Peptide – Oligonucleotide Conjugates: Chemoselective Oxime and Thiazolidine Formation**

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Abstract: A convergent strategy for the synthesis of peptide – oligonucleotide conjugates (POC) is presented. Chemoselective ligation of peptide to oligonucleotide was accomplished by oxime and thiazolidine formation. Oxime conjugation was performed by treating an oxyamine-containing peptide with an aldehyde-containing oligonucleotide or vice versa. Ligation by thiazolidine formation was achieved by coupling a peptide, acylated with a cysteine residue, to an oligonucleotide that was derivatised by an aldehyde function. For both approaches, the conjugates were obtained in good yield without the need for a protection strategy and under mild aqueous conditions. Moreover, the oxime ligation proved useful for directly conjugating duplex oligonucleotides. Combined with molecular biology tools, this methodology opens up new prospects for post-functionalisation of high-molecular-weight DNA structures.

Keywords: chemoselectivity · conjugation · oligonucleotides · peptides

Introduction

The use of oligonucleotides (ODN) for the inhibition of gene expression represents an attractive therapeutic approach. Oligonucleotides can target mRNA (antisense strategy), double stranded DNA (triple-helix strategy) or proteins (aptamer strategy). However, a number of hurdles have to be overcome. Cell-specific delivery, cellular uptake efficiency, stability against nucleases, intracellular distribution and target affinity constitute the main properties required for a therapeutic oligonucleotide approach.[1] A number of chemically modified oligonucleotides have been prepared for these purposes. These include modification of the backbone (i.e., phosphorothioate analogues, PNA…), modification of the sugar (i.e., 2′-*O*-alkylated sugar, α-nucleoside…) and attachment of a variety of reporter groups at the 3′ or 5′ extremities.[2]

In this context, peptide – oligonucleotide conjugates (POC) have received considerable attention as they can deliver a large spectrum of properties to oligonucleotides, such as enhancement of cellular uptake, improvement of nuclear localisation or an increase in affinity for the DNA or RNA target.[3]

Different methods for the preparation of peptide – oligonucleotide conjugates have been described.[3] Some have been prepared successfully by stepwise solid-phase synthesis. However, the standard chemistry required for peptide and DNA synthesis is not fully compatible; this greatly limits the scope of this approach.[3, 4] Therefore, the fragment-coupling approach has emerged as a more general, suitable strategy. It involves separate preparation of the peptide and of the oligonucleotide, and subsequent coupling of the two purified moieties. Various linkages, such as disulfide,[5] maleimide,[6] thioether[7] and amide[8] have been used for this purpose.

Although many methods of conjugation have been successfully exploited, they still present drawbacks that restrict their general applications. For instance, the major problem in using thiol- or amine-based chemistry for conjugation is the lack of regiospecific ligation upon reaction with peptides that contain multiple cysteine or lysine residues. To circumvent these limitations, the development of more selective and efficient reactions, as well as easier procedures and work up, is still of great interest. Ideally, the conjugation should take place between fully unprotected peptides and oligonucleotides, with minimal chemical manipulation and under physiological conditions, thus allowing single-step synthesis of various conjugates. Considering these criteria, we investigated the use of two new linkages: the oxime linkage and the thiazolidine linkage. Both have been used efficiently for the chemical ligation of peptides[9] and for the conjugation of peptides with carbohydrates.[10] The high efficiency and

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[**] Abbreviations: Aloc = allyloxycarbonyl, Boc = *t*-butyloxycarbonyl, DIEA = diisopropylethylamine, EDT = ethanedithiol, Fmoc = 9-fluorenylmethyloxycarbonyl, MBHA = *p*-methylbenzylhydrylamine, Pmc = 2,2,5,7,8-pentamethylchroman-6-sulfonyl, PyBOP = benzotriazol-1-yl-oxy-tris-pyrolidino-phosphoniumhexafluoro phosphate, TIS = triisopropylsilane, Trt = trityl.

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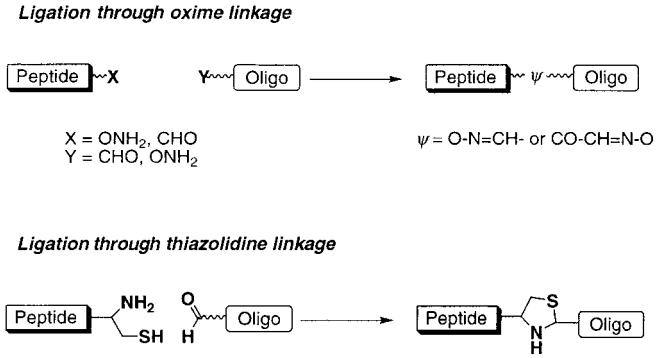
selectivity of the aminooxy–aldehyde coupling reaction has been demonstrated by using this reaction for the determination of very low amounts of carbonyl derivatives in water and ice,[11] as well for the quantification of abasic sites in DNA.[12] This methodology has been extended to the labelling of oligonucleotides and RNA,[13] and to the anchoring of oligonucleotides on polymer supports.[14]

In this paper, the preparation of peptide–oligonucleotide conjugates by oxime bond formation (i.e., reaction between an aldehyde and an oxyamine) and thiazolidine formation (i.e., reaction between an aldehyde and a 1,2-aminothiol) is described (Scheme 1). For this purpose, an aldehyde and an aminooxy function were incorporated into the oligonucleotide module at the 5′-extremity, while the peptide module carried the corresponding complementary function; that is, the aminooxy or the 1,2-aminothiol for reaction with the aldehyde oligonucleotide, or the α-*N*-glyoxylyl for reaction with the aminooxy-containing oligonucleotide (Scheme 1). The interest of this new approach is emphasised by using two biologically relevant peptides: a) a cyclopentapeptide containing an arginine-glycine-aspartic acid tripeptide motif (RGD), which is known to be a powerful and selective ligand of the $\alpha_v\beta_3$ integrin receptor.[15] Such peptides have been studied for tumour targeting[16] as well as for DNA delivery;[17] and b) a nuclear localisation signal sequence (NLS): the basic peptide APKKKRKV which is derived from the simian virus 40 antigen. The synthesis of oligonucleotides carrying this NLS sequence has been recently described, and the hybridisation properties of the conjugates with the complementary strand have been studied.[18] A cytomegalovirus luciferase-NLS gene containing this sequence has also been recently prepared for transfection into cells.[19]



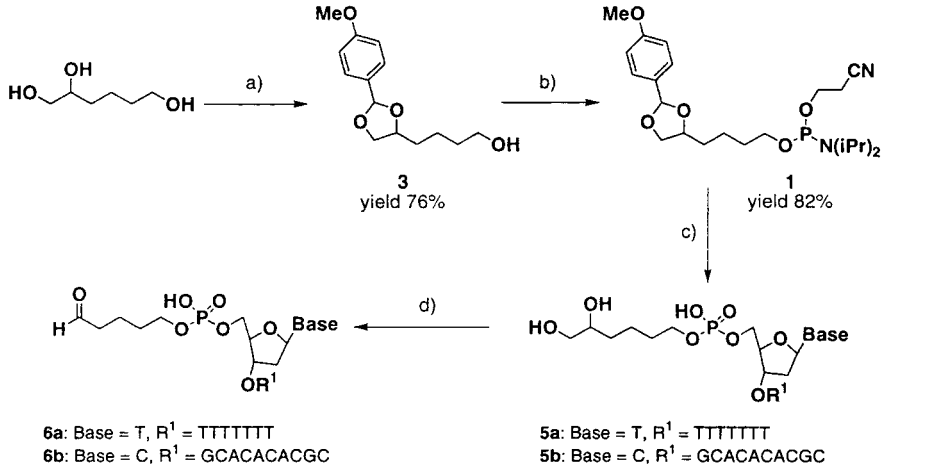
Scheme 1. Strategy for POCs synthesis by using oxime and thiazolidine linkages.

Results and Discussion

Oxime-bond formation was achieved by inserting the complementary reactive nucleophilic or electrophilic moieties into both modules for comparison. On the one hand, the aldehyde and the aminooxy group were introduced at the 5′-extremity of the oligonucleotides by using phosphoramidites **1** and **2**, respectively (Schemes 2 and 3, below). On the other hand, the N-terminal position or the lysine side chain of the peptides was functionalised by an aldehyde and an aminooxy moiety through $NaIO_4$ oxidative cleavage of a serine residue and by using the *N*-Boc-*O*-(carboxymethyl)hydroxylamine **15**, respectively (Schemes 4 and 5, below). For thiazolidine formation, the peptide was derivatised with a cysteine at the N-terminal position or at the lysine side chain, with the oligonucleotide bearing the aldehyde function at the 5′-end.

Functionnalisation at the 5′-extremity of the oligonucleotides:

Aldehyde-containing linker: Preparation of oligonucleotides containing an aldehyde linker at the 5′-extremity has already been described.[20] The authors used a phosphoramidite linker bearing a *bis*-benzoyl-protected diol. After DNA synthesis, the aldehyde was generated by oxidative cleavage of the diol with $NaIO_4$. However, five steps were necessary for the preparation of the corresponding phosphoramidite. We therefore considered a more straightforward synthetic route for the preparation of the phosphoramidite. As depicted in Scheme 2, phosphoramidite **1** was prepared in a two-step reaction from commercial 1,2,6-hexanetriol. The 1,2-diol moiety was first protected as a 2-methoxybenzylidene acetal by using 2-methoxybenzaldehyde dimethylacetal in the presence of a catalytic amount of pyridinium paratoluene sulfonate to afford the protected diol **3**.[21a] We noted that protection by a 2,4-dimethoxybenzylidene acetal was unsuitable for automated oligonucleotide synthesis due to its instability. Phosphitylation of **3** with 2-cyanoethyl diisopropylchlorophosphoramidite afforded the phosphoramidite **1** in 62% overall yield.



Scheme 2. Preparation of aldehyde-containing oligonucleotides **6a** and **6b**. a) 4-methoxybenzaldehyde dimethyl acetal, PPTS; b) 2-cyanoethyldiisopropylchlorophosphoramidite, DIEA; c) automated oligonucleotide synthesis, then 80% aqueous AcOH; d) $NaIO_4$.

Phosphoramidite **1** was used to prepare the oligonucleotides **6a** and **6b** as illustrative examples. Coupling of **1** at the last step of the automated oligonucleotide synthesis was achieved in excellent yield. After deprotection of the nucleobases under the usual conditions (ammonia treatment for 24 h at 55 °C), the oligonucleotides **4a** and **4b** were purified by reverse-phase HPLC. The 2-methoxybenzylidene acetal protection allowed easy purification of the oligonucleotides at this stage due to the group's hydrophobic properties (Figure 1A). The 2-methoxybenzylidene acetal was then cleaved under mild acidic conditions. Treatment of the 5′-end-protected oligonucleotides **4a** and **4b** with 80% aqueous acetic acid for 1 h, conditions that are commonly used for dimethoxytrityl (DMT) deprotection, afforded the corresponding oligonucleotides **5a** and **5b**. The methoxybenzylidene group was found to be remarkably labile under these conditions—a longer time is generally required for hydrolysis of methoxybenzylidene derivatives.[21b] No side reaction was observed. Compounds **5a** and **5b** were characterised by electrospray ionisation mass spectrometry (ES-MS, Table 1). Oxidative cleavage of the diol was then performed by using a 50-fold excess of $NaIO_4$ at room temperature for 30 min; this led to selective formation of the desired aldehydes **6a** and **6b**.

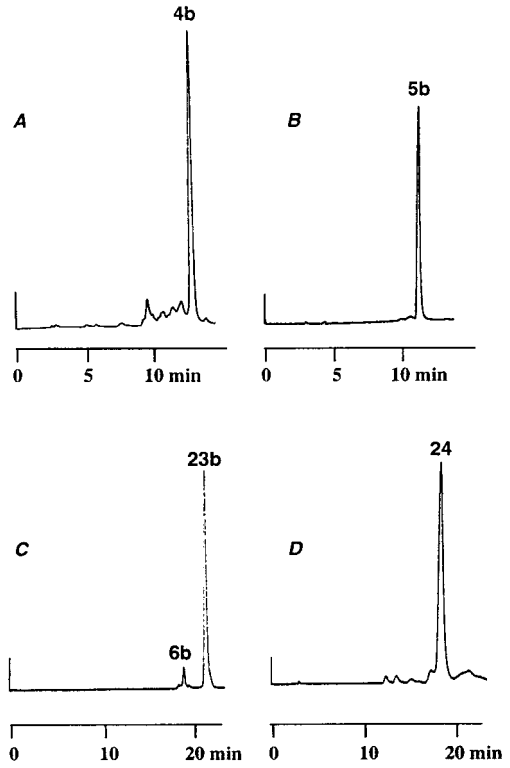


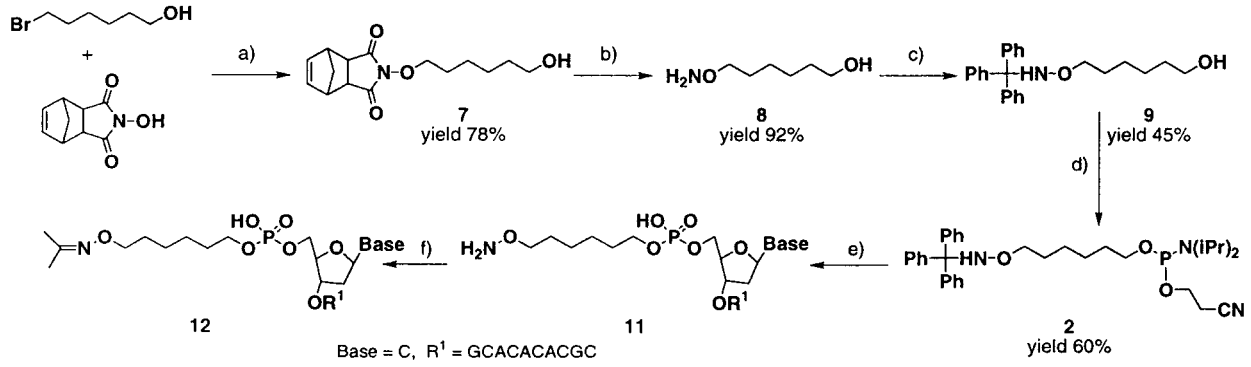
Figure 1. HPLC profiles (detection at 260 nm): A) crude protected undecamer diol **4b**; B) purified undecamer diol **5b**; C) crude reaction mixture of aldehyde-containing undecamer **6b** with RGD peptide **16;** D) crude reaction mixture of aldehyde-containing undecamer **6b** with NLS peptide **19**. For the HPLC conditions, see Experimental Section.

Table 1. ES-MS analysis.[a]

Compound	Calcd Mass	Found Mass
5a	2566.8	2568.2
5b	3466.3	3466.2
10	3708.7	3710.9
12	3506.6	3505.7
14	926.1	926.3
16	676.7	677.2
17	677.7	678.4
18	706.8	707.3
19	1271.5	1271.4
20	1285.5	1285.4
21	1254.4	1254.2
22	1301.6	1301.4
23a	3193.4	3192.5
23b	4093.0	4092.7
24	4687.8	4689.0
25	4107.2	4106.6
26	4702.0	4698.7
27a	3223.5	3223.6
27b	4123.1	4123.9
28	4717.9	4717.8

[a] Negative mode for oligonucleotides and conjugates, and positive mode for peptides. Eluent: H_2O/CH_3CN 50:50 (*v/v*); flow rate: 8 μL min^{-1}. The oligonucleotides and the conjugates were dissolved in $H_2O/CH_3CN/NEt_3$ 50:50:2 (*v/v/v*). The peptides were dissolved in H_2O/CH_3CN/TFA 50:50:1 (*v/v/v*).

Aminooxy-containing oligonucleotides: In a recent paper, we described a convenient synthetic route to aminooxy-containing oligonucleotides by incorporating a trityl-protected aminooxy group by using the phosphoramidite **2** as depicted in Scheme 3.[22] The oxyamine **8** was obtained from reaction of 6-bromohexanol with the dicarboxymide derivative, followed by cleavage of the protecting group with hydrazine. Selective *N* tritylation and subsequent phosphitylation with 2-cyanoethyl tetraisopropylphosphorodiamidite gave the phos-



Scheme 3. Preparation of oxyamine-containing oligonucleotide **11**. a) K_2CO_3, DMF; b) N_2H_4, EtOH; c) TrCl, pyridine; d) N,N′-diisopropylammonium tetrazolide, 2-cyanoethyldiisopropylphosphorodiamidite, e) automated oligonucleotide synthesis, then 80% aqueous AcOH; f) acetone, H_2O.

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phoramidite synthon **2**. It was noted that *N* protection with monomethoxytrityl chloride gave the corresponding aminooxy derivative, which proved highly unstable.

The undecamer d(XCGCACACACGC) **10**, in which X represents the 5′-aminooxylinker, was then prepared according to standard β-cyanoethylphosphoramidite chemistry by incorporating phosphoramidite **2** at the final step of the automated synthesis. After deprotection with concentrated ammonia for 24 h at 55 °C, the oligonucleotide **10** was purified by reverse-phase HPLC (Figure 2A shows the crude protected aminooxy undecamer **10**; B) purified aminooxy undecamer **11**; C) crude reaction mixture of aminooxy undecamer **11** with RGD peptide **17**; D) purified peptide **25**. For the HPLC conditions, see Experimental Section.

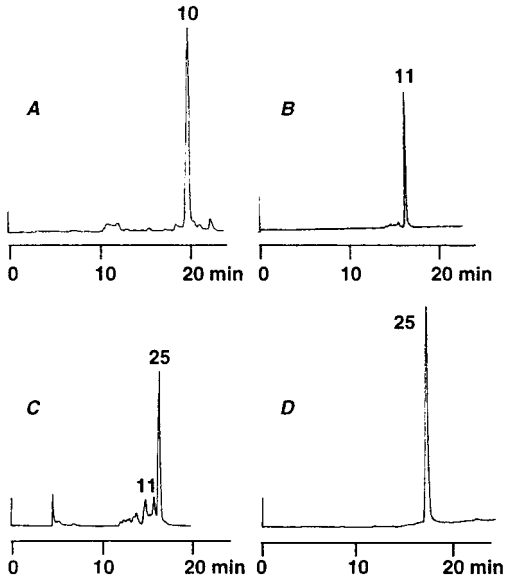
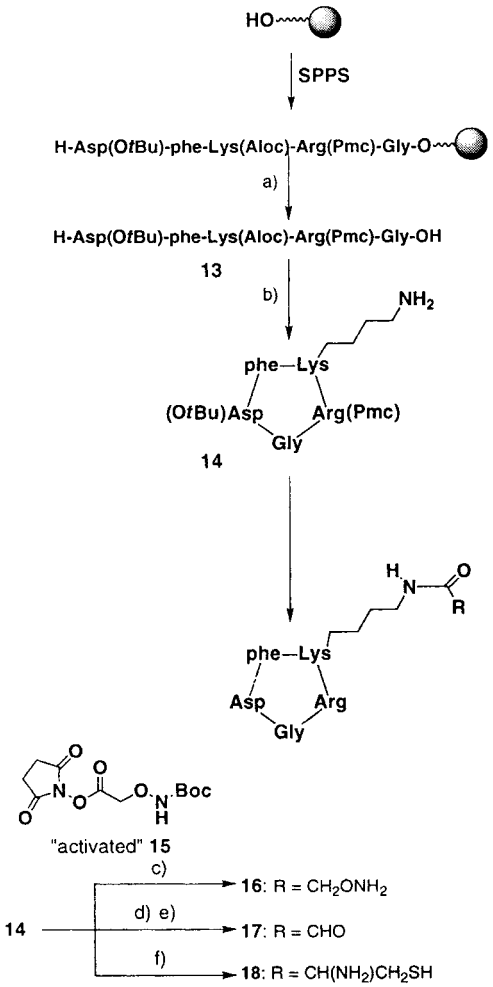


Figure 2. HPLC profiles (detection at 260 nm): A) crude protected aminooxy undecamer **10;** B) purified aminooxy undecamer **11;** C) crude reaction mixture of aminooxy undecamer **11** with RGD peptide **17;** D) purified peptide **25**. For the HPLC conditions, see Experimental Section.

ed aminooxy undecamer **10**) and characterised by ES-MS (Table 1). Removal of the trityl protection was then performed in 80% aqueous AcOH at room temperature for 8 h to afford the free aminooxy oligonucleotide **11** (Figure 2B). We noticed that compound **11** reacts readily with even traces of carbonyl compounds. Therefore, it was best characterised by the adduct **12** formed immediately after addition of acetone (ES-MS, Table 1).

Peptides synthesis:

RGD peptide: (Scheme 4) The protected cyclic peptide **14** represents the key intermediate into which the reactive moiety (i.e., ONH_2, CHO or SH) can be anchored through the amino side-chain group of the lysine residue. The synthesis of compound **14** has been reported.[23] It was prepared with some modifications by cyclisation of the linear peptide H-Asp(O*t*Bu)-phe-Lys(Aloc)-Arg(Pmc)-Gly-OH **13**.[23c] The linear side-chain-protected peptide was assembled by solid-phase peptide synthesis starting with a glycine residue at the C terminus to prevent racemisation during the subsequent head-to-tail cyclisation. The latter was carried out at high dilution (0.5 mM) in DMF to prevent dimerisation, with PyBOP as

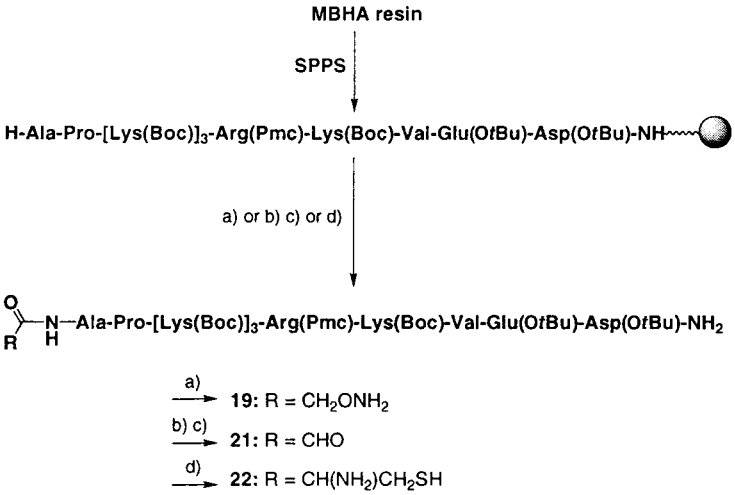


Scheme 4. Preparation of functionalised RGD peptides **16**, **17** and **18**. a) 1% TFA, CH_2Cl_2; b) PyBOP, DIEA, high dilution, then tetrakis(triphenylphosphine)Pd(0); c) **15**, then 50% TFA; d) Boc-Ser(O*t*Bu)-OH, PyBOP, then 50% TFA/TIS; e) $NaIO_4$; f) Fmoc-Cys(Trt)-OH, PyBOP, then piperidine/DMF, then 90% TFA/EDT/TIS.

coupling reagent. The Aloc moiety was then cleaved by using tetrakis(triphenylphosphine)palladium(0) in the presence of phenylsilane as scavenger.[24] Purification by reverse-phase HPLC yielded the key intermediate **14.** Introduction of the aminooxy group was achieved by coupling the activated ester of *N*-Boc-*O*-(carboxymethyl)-hydroxylamine, **15**, onto the lysine side chain. First, the aminooxy moiety was protected by a Boc group from commercial carboxymethoxylamine hemihydrochloride to give the acid **15**.[25] Before the coupling reaction, the acid was reacted with *N*-hydroxysuccinimide in the presence of dicyclohexyl carbodiimide to give the corresponding activated ester. Reaction of the activated ester of **15** with the peptide **14** was carried out in DMF. The protecting groups were then removed in 50% trifluoroacetic acid (TFA) in the presence of triisopropylsilane. The resulting aminooxy peptide **16** was purified by HPLC. The serine residue was introduced by using commercially available Boc-Ser(O*t*Bu)-OH and PyBOP, and then the protecting groups were removed in 50% TFA. The 1,2-amino alcohol was then oxidised by using $NaIO_4$; this led to the selective formation of the aldehyde-containing peptide **17**. The cysteine residue was

introduced by using Fmoc-Cys(Trt)-OH and PyBOP. Removal of the Fmoc protecting group by using piperidine in DMF and subsequent cleavage of *t*Bu and Pmc protecting groups in 90% TFA with ethanedithiol as scavenger afforded the corresponding peptide **18**. The three peptides **16**, **17** and **18** were characterised by ES-MS (Table 1).

NLS peptide (Scheme 5): The NLS-containing peptides were assembled manually on a MBHA resin to provide the C-terminal-amidated peptide by using a Fmoc/*t*Bu strategy.



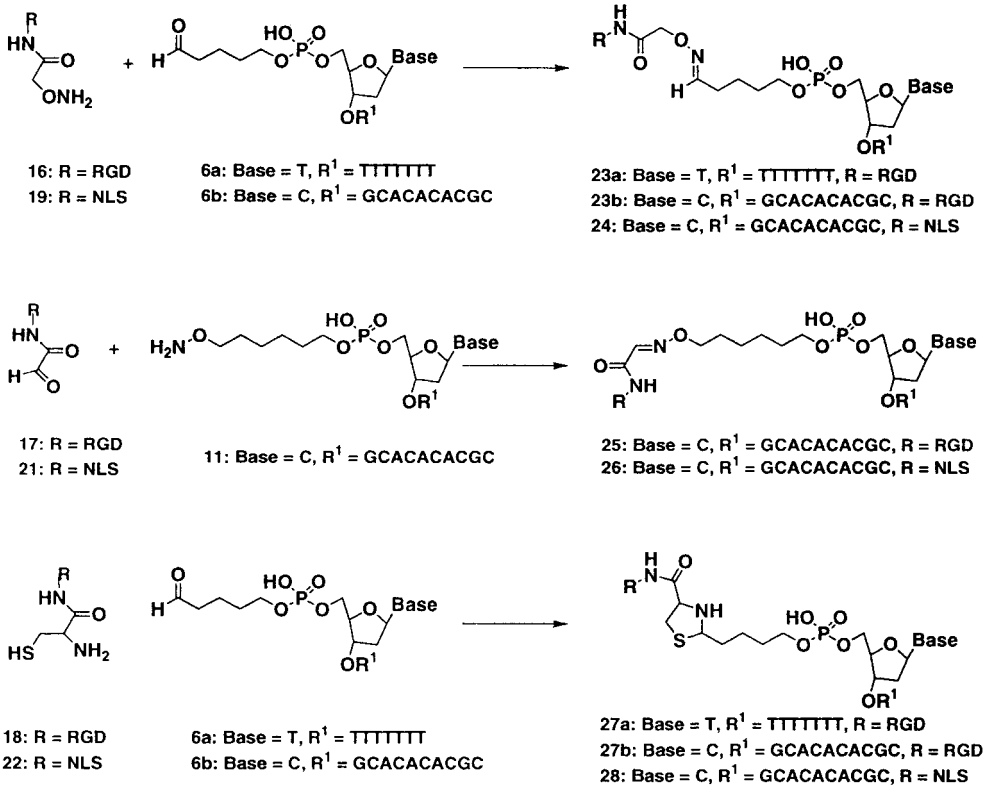
Scheme 5. Preparation of functionalised NLS peptides **19**, **21** and **22**. a) **15**, then 90% TFA/TIS; b) Boc-Ser(O*t*Bu)-OH, PyBOP, then 90% TFA/TIS; c) $NaIO_4$; d) Fmoc-Cys(Trt)-OH, PyBOP, then piperidine/DMF, then 90% TFA/EDT/TIS.

The reactive functions were introduced directly onto the support at the N-terminal position by using the activated ester of **15**, Boc-Ser(O*t*Bu)-OH or Fmoc-Cys(Trt)-OH, which led, after deprotection and cleavage from the resin, to peptides **19**, **20** and **22**, respectively. The aldehyde-containing peptide **21** was obtained from oxidation of the intermediate amino alcohol **20** with $NaIO_4$. Peptides **19**–**22** were checked for their purity by HPLC and were characterised by ES-MS (Table 1).

Conjugation reactions (Scheme 6):

Oxime linkage: We first studied the conjugation of the aldehyde-containing oligonucleotides **6a** and **6b** with the RGD-containing peptide **16**. A slight excess (1.5 equiv) of the aminooxy peptide **16** was reacted with the 8-mer **6a** and the 11-mer **6b** in aqueous solution at pH 5. Reactions were carried out in slightly acidic conditions as the optimal pH is around 4–5 for oxime-bond formation.[26] 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 oxime **23b**, as depicted in Figure 1C. The same selectivity was observed for conjugation with **6a**. Subsequent purification by HPLC afforded the conjugates **23a** and **23b** in almost 50% isolated yield. The same protocol was then applied to the NLS peptide **19.** Reaction of the 11-mer **6b** with the aminooxy peptide **19** in aqueous solution selectively afforded the conjugate **24** (Figure 1D). The conjugates **23a**, **23b** and **24** were characterised by ES-MS. In all cases, the experimentally determined molecular weights were in excellent agreement with the calculated values (Table 1).

The "reverse strategy" was conducted by reacting the oxyamine-containing oligonucleotide **11** and the aldehyde-



Scheme 6. Conjugation reactions: synthesis of POCs **23**–**28**.

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containing peptides **17** and **21**. The reactions were performed under the aforementioned conditions and led selectively to the expected conjugates **25** (Figure 2C) and **26**, respectively. The conjugates **25** and **26** were purified by RP-HPLC and characterised by ES-MS (Table 1). In both cases, the yields were close to 50% after purification by HPLC.

The chemical stability of the oxime linkage was then studied by incubating the conjugates **23b** and **25** in a phosphate buffer at pH 4 and pH 7. Surprisingly, no apparent difference of stability was observed for both conjugates, although it was anticipated that the glyoxylic oxime **25** would be more stable than the classical oxime **23b**. No significant hydrolysis or degradation products (from depurination) were observed even after 72 h of incubation at 37 °C.

Oxime-bond formation by using these two strategies permits rapid and clean preparation of peptide – oligonucleotide conjugates. However, it should be emphasised that the approach involving coupling of an aminooxy oligonucleotide to an aldehyde peptide appeared less convenient to work up. In fact, we noticed that free aminooxy oligonucleotides are very often prone to react with traces of carbonyl compounds present in HPLC solvents or in the atmosphere. Therefore, the first approach is recommended for the synthesis of oxime conjugates.

Thiazolidine linkage: Formation of the thiazolidine linkage was first performed with the RGD peptide **18** and the 8-mer **6a**. The reaction was conducted at room temperature in sodium acetate buffer at pH 5.4 with a fourfold excess of peptide **18**.[27] The reaction was very rapid ($t_{1/2} < 15$ min) and led to selective formation of the conjugate **27a** (Figure 3A).

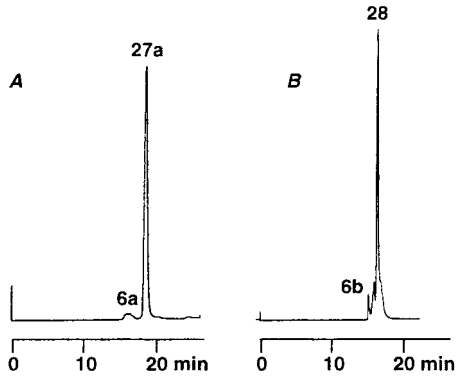

Figure 3. HPLC profiles (detection at 260 nm): A) crude reaction mixture of aldehyde-containing oligonucleotide **6b** with RGD peptide **18;** B) crude reaction mixture of aldehyde-containing undecamer **6b** with NLS peptide **22**. For the HPLC conditions, see Experimental Section.

The same procedure was then applied to the 11-mer **6b** with the RGD peptide **18** and the NLS peptide **22**. Exclusive formation of conjugates **27b** and **28** was observed, respectively (Figure 3B shows the HPLC profile of the crude reaction mixture in the case of conjugation with NLS peptide **22**). The three conjugates **27a**, **27b** and **28** were characterised by ES-MS (Table 1).

The chemical stability of the thiazolidine linkage was also studied by incubating the conjugate **27a** under the same conditions as used for **23b** and **25**. In contrast to the oxime bond, it was found that the thiazolidine bond is less stable at pH 4. In fact, the conjugate **27a** is up to 50% hydrolysed back to the starting material after 72 h of incubation at pH 4 at 37 °C. However, **27a** proved to be stable at higher pH value (e.g., pH 7) as emphasised by the lack of hydrolysis or by product formation under this condition.

Hybridisation properties of the POC: The hybridisation properties of the conjugates were studied by melting temperature (T_m) measurements to evaluate the influence of the two peptides RGD and NLS as well as of the two linkages on the stability of the duplex oligonucleotide. The oxime conjugates **23b** and **24** and the thiazolidine conjugates **27b** and **28**, containing, respectively, the RGD and the NLS peptide, were hybridised with their complementary strand d(GCGTGTG-TGCG). The melting temperatures of the resulting duplexes were determined (Table 2). The oligonucleotide **5b** containing the diol linker at the 5′-end was studied for comparison. All the peptide – oligonucleotide conjugates showed a slightly higher melting temperature. The slight increase of stability could be attributed to the positive charges of the lysine and arginine side chains present within each peptide. Such stabilisation of conjugates carrying basic amino acids has been previously reported.[6a, 18] It is also notable that the nature of the linkage does not appear to perturb the stability of the double strand, as no substantial difference was observed between **23b** and **27b** or between **24** and **28**.

Table 2. Melting temperatures of duplexes formed by hybridisation of the indicated oligonucleotides with the complementary strand d(GCGTGTG-TGCG).[a]

Parent oligonucleotide **5b**	$T_m = 66.0\,^{\circ}C \pm 1\,^{\circ}C$
POC **23b** (oxime linkage)	$T_m = 68.0\,^{\circ}C \pm 1\,^{\circ}C$
POC **27b** (thiazolidine linkage)	$T_m = 69.0\,^{\circ}C \pm 1\,^{\circ}C$
POC **24** (oxime linkage)	$T_m = 66.5\,^{\circ}C \pm 1\,^{\circ}C$
POC **28** (thiazolidine linkage)	$T_m = 66.5\,^{\circ}C \pm 1\,^{\circ}C$

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

Conjugation with duplex oligonucleotides: The undecamer **6b** was hybridised with the complementary strand, and the duplex was reacted with the RGD peptide **16** under the same conditions as for the single strand. The reaction was complete within 2 h and showed the same efficiency as previously observed when the single strand was used. The absence of variation of the melting temperature during the course of reaction clearly indicates that the coupling occurs without disrupting the duplex structure.

Conclusion

Conjugation of oligonucleotides with peptides has been readily performed through oxime or thiazolidine bond formation. These one-step ligations are compatible with the use of unprotected peptides and oligonucleotides. Thanks to

the high chemoselectivity of these reactions, the rapid and convenient production of POCs without the need for extensive chemical manipulation is thus allowed. The aldehyde- and aminooxy-containing oligonucleotides are easily accessible from phosphoramidites **1** and **2**. Moreover, oxime and thiazolidine ligations have already been carried out under denaturing or high-salt conditions; this may extend the scope of the present approach to less-soluble peptides or conjugates as well as to higher molecular weight systems.[28] Together the high efficiency and versatility of this strategy over conventional conjugation methods is of great interest for devising new molecular systems based on ODN. Furthermore, the conjugation with oxime-bond formation could also be performed on duplexes' secondary structure. This latter result opens up attractive prospects for the post-functionalisation of high-molecular-weight structures, such as DNA, combined with the molecular biology tools availability.

Experimental Section

Materials and methods: All commercially available chemical reagents were used without purification. 2-Cyanoethyl diisopropylchlorophosphoramidite and 2-cyanoethyl tetraisopropylphosphorodiamidite were purchased from Aldrich. 4-Methoxybenzaldehyde dimethyl acetal and the *N*-Boc-O-(carboxymethyl)hydroxylamine, **15**, were prepared as described.[21, 25] Compound **15** is also commercially available from Fluka as (Boc-aminooxy)acetic acid. Thin-layer chromatography (TLC) was performed on silica gel 60 F_{254} plates (Merck), and preparative column chromatography was performed on silica gel 60 (Merck, 200–63 μm). HPLC purification, as well as HPLC analysis of oligonucleotides and conjugates, was performed on a Waters system equipped with two M 510 pumps, an M 490E detector and an M 680 system controller. HPLC purification and analysis of peptides were done on a Waters system consisting of a Delta 600 pump, a 2487 dual λ detector and a 600 E-system controller. The oligonucleotides and the conjugates were purified on a μ-bondapak C-18 column (Macherey–Nagel Nucleosil: 10 × 250 mm, 7 μm) with two systems of solvent. System I: 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. System II: solvent A, 20 mM sodium phosphate/MeOH 95:5 (v:v); solvent B, MeOH; flow rate, 4 mL min^{-1}; a linear gradient from 0 to 35% B in 20 min was applied. The peptides were purified on a Delta Pak™ C-18 column (Waters: 25 × 200 mm, 15 μm) by using solvent system III: solvent A, H_2O/TFA 99.9:0.1 (v:v); solvent B, CH_3CN/H_2O/TFA 90:10:0.1 (v:v:v); flow rate 22 mL min^{-1}; a linear gradient from 5 to 100% B in 30 min was applied. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AC 200 and Avance spectrometers at 200 and 300 MHz, respectively. Mass spectra were measured on a Delsi–Nermag R10-10 for EI and DCI, and on a VG Platform (Micromass) for ESI. Analysis was performed in the negative mode for the oligonucleotides and the conjugates and in the positive mode for the peptides. The eluent was 50% aqueous acetonitrile and the flow rate was 8 μL min^{-1}. The oligonucleotides and the conjugates were dissolved in 50% aqueous acetonitrile, and 1% of NEt_3 was added. The peptides were dissolved in 50% aqueous acetonitrile, and 1% of TFA was added.

4-[2-(4-Methoxy-phenyl)-[1,3]-dioxolan-4-yl]-butan-1-ol (3): 1,2,6-hexanetriol (3.10 g, 23.0 mmol) and a catalytic amount of pyridinium *p*-toluene sulfonate (0.1 g) were added to a solution of 4-methoxybenzaldehyde dimethylacetal (2.10 g, 12.0 mmol) in DMF (20 mL). The solution was stirred at 50 °C under argon for 20 h, then the solvent was evaporated under vacuum. The oily residue was dissolved in CH_2Cl_2, and the organic layer was washed successively with aqueous $NaHSO_3$, aqueous $NaHCO_3$ and brine, and evaporated to give **3** as a white oil. Yield: 2.22 g, 76%; ^{1}H NMR ($CDCl_3$): δ = 7.37 (dd, 2H; Ar-H), 6.87 (d, 2H; Ar-H), 5.84 and 5.73 (2s, 1H; O-CH-O), 4.24–4.03 (m, 2H; CH_2O), 3.78 (s, 3H; OCH_3), 3.66–3.60 (m, 3H; CH-O and CH_2O), 1.68–1.42 (m, 7H; 3 CH_2 and OH); ^{13}C NMR ($CDCl_3$): δ = 160.8 and 160.7 (q), 130.9 and 130.3 (q), 128.4 and 128.2 (2 CH), 114.1 (2 CH), 104.3 and 103.41 (CH), 77.5 and 76.7 (CH), 71.1 and 70.4 (CH_2), 63.0 (CH_2), 55.7 (CH_3), 33.6 (CH_2), 32.9 (CH_2), 22.4 (CH_2); MS (DCI/NH_3): m/z = 253 $[M+H]^+$.

Phosphoramidite (1): DIEA (375 μL, 2.10 mmol) and 2-cyanoethyl diisopropylchlorophosphoramidite (315 μL, 1.40 mmol) were added under argon to a solution of compound **3** (270 mg, 1.10 mmol) in anhydrous CH_2Cl_2 (3 mL). The solution was stirred for 2 h at room temperature, and CH_2Cl_2 (20 mL) was then added. The organic layer was washed with 10% aqueous $NaHCO_3$ solution, then with brine and dried (Na_2SO_4). The solvent was evaporated under vacuum, and the crude mixture was purified by silica gel column chromatography (CH_2Cl_2/cyclohexane/Et_3N 74:24:2) to give compound **1** as a white oil. Yield: 0.40 g, 82%; ^{1}H NMR ($CDCl_3$): δ = 7.37 (dd, 2H; Ar-H), 6.87 (d, 2H; Ar-H), 5.83 and 5.72 (2s, 1H; O-CH-O), 4.24–4.05 (m, 2H; CH_2O), 3.78 (s, 3H; OCH_3), 3.65–3.55 (m, 5H; CH-O and 2 CH_2O), 2.61 (m, 2H; CH_2CN), 1.68–1.52 (m, 8H; 3 CH_2 and 2 CH), 1.18–1.13 (m, 12H; 4 CH_3); ^{31}P NMR ($CDCl_3$): δ = 145.5; MS (FAB, NBA matrix): m/z = 453 $[M+H]^+$.

4-(6-Hydroxyhexyloxy)-4-aza-tricyclo [5.2.1.0^{2,6}]dec-8-ene-3,5-dione (7): A mixture of *endo*-*N*-hydroxy-5-norbornene-2,3-dicarboximide (5.02 g, 28.0 mmol) and K_2CO_3 (7.70 g, 56.0 mmol) in DMF (250 mL) was stirred at 50 °C under argon for 1 h. 6-Bromohexanol (5.07 g, 28.0 mmol) was added, and the mixture was then stirred for 4 h at 50 °C. After filtration, the solvent was evaporated under vacuum. EtOAc was added to the residue obtained, and the organic layer was washed with 0.1N NaOH, then with brine. The organic layer was dried (Na_2SO_4) and evaporated to give compound **7** as a pale yellow oil. Yield: 6.00 g, 78%; ^{1}H NMR ($CDCl_3$): δ = 6.10 (m, 2H; CH=CH), 3.90 (t, 2H; CH_2O), 3.60 (t, 2H; CH_2O), 3.40 (m, 2H; 2 CH-C=O), 3.15 (m, 2H; 2 CH), 1.80–1.30 (m, 10H; 5 CH_2); ^{13}C NMR ($CDCl_3$): δ = 172.1 (q), 134.2 (CH), 76.8 (CH_2), 61.8 (CH_2), 51.0 (CH_2), 44.4 (CH), 42.3 (CH), 32.1 (CH_2), 27.6 (CH_2), 25.0 and 24.9 (CH_2); MS (EI): m/z = 279 $[M]^+$.

6-Aminooxyhexan-1-ol (8): Compound **7** (6.00 g, 22.0 mmol) was dissolved in EtOH (75 mL), and hydrazine (1.40 g, 44.0 mmol) was added. The solution was refluxed for 2 h, then filtered and evaporated under vacuum. Purification by silica gel column chromatography (EtOAc/MeOH 95:5) of the crude mixture afforded **8** as a white oil. Yield: 2.60 g, 92%; ^{1}H NMR ($CDCl_3$): δ = 3.59–3.52 (m, 4H; 2 CH_2O), 1.54–1.43 (m, 4H; 2 CH_2), 1.31–1.25 (m, 4H; CH_2CH_2); ^{13}C NMR ($CDCl_3$): δ = 76.4 (CH_2), 62.9 (CH_2), 33.0 (CH_2), 28.7 (CH_2), 26.2 (CH_2), 26.0 (CH_2); MS (DCI): m/z = 134 $[M+H]^+$.

6-(*N*-Tritylaminooxy)-hexan-1-ol (9): Trityl chloride (6.60 g, 24.0 mmol) was added to a solution of compound **8** (2.60 g, 20.0 mmol) in dry pyridine (50 mL) cooled to 0 °C. The solution was stirred at room temperature under argon for 4 h. MeOH (3 mL) was then added dropwise, and the solvent was evaporated under vacuum. The residue obtained was dissolved in EtOAc, and the organic layer was washed with H_2O, then with brine. The organic layer was then dried (Na_2SO_4) and evaporated. The crude mixture was purified by silica gel column chromatography (EtOAc/cyclohexane/NEt_3 40:60:1) to give compound **9** as a white powder. Yield: 3.40 g, 45%; ^{1}H NMR ($CDCl_3$): δ = 7.34–7.25 (m, 15H; Ar-H trityl), 6.23 (br s, 1H; NH), 3.66 (t, 2H; CH_2O), 3.57 (m, 2H; CH_2O), 1.47–1.42 (m, 4H; 2 CH_2), 1.21–1.16 (m, 4H; 2 CH_2); ^{13}C NMR ($CDCl_3$): δ = 144.5 (q), 129.1 (CH), 127.6 (CH), 126.8 (CH), 77.0 (q), 73.9 (CH_2), 63.0 (CH_2), 32.7 (CH_2), 28.4 (CH_2), 25.9 (CH_2), 25.5 (CH_2).

Phosphoramidite (2): *N*,*N*′-Diisopropylammonium tetrazolide (0.24 g, 1.7 mmol) and 2-cyanoethyl tetraisopropylphosphorodiamidite (1.00 g, 3.9 mmol) were added to a solution of compound **9** (1.00 g, 2.4 mmol) in CH_2Cl_2 (20 mL). The solution was stirred under argon at room temperature for 16 h, then diluted with CH_2Cl_2 (100 mL). The organic layer was washed twice with H_2O, then with brine and dried (Na_2SO_4). The solvent was evaporated under vacuum and the residue obtained was purified by silica gel column chromatography (EtOAc/cyclohexane/NEt_3 30:70:1) to afford phosphoramidite **2** as a white oil. Yield: 1.60 g, 60%; ^{1}H NMR ($CDCl_3$): δ = 7.33–7.26 (m, 15H; Ar-H trityl), 6.26 (s, 1H; NH), 3.82 (m, 2H; CH_2O), 3.68–3.58 (m, 4H; CH_2O), 2.62 (t, 2H; CH_2CN), 1.60–1.44 (m, 5H; 2 CH_2 and CH), 1.21–1.17 (m, 17H; 4 CH_3, 2 CH_2 and CH); ^{31}P NMR ($CDCl_3$): δ = 145.4; MS (FAB, NBA matrix): m/z = 575 $[M]^+$.

Oligonucleotides synthesis: Automated DNA synthesis was carried out on an Expedite DNA synthesiser (Perkin–Elmer) by using standard β-cyanoethyl nucleoside phosphoramidites chemistry on a 1 μM scale. After cleavage from the solid support and deprotection by treatment with

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concentrated ammonia (30%) for 24 h at 55 °C, the oligonucleotides **4a, 4b** and **10** were purified by HPLC (system I).

Diol-containing oligonucleotides (5a) and (5b): The 5′-protected oligonucleotides **4a** and **4b** were treated with an 80% AcOH aqueous solution for 1 h. The residue obtained after lyophilisation was dissolved in water, and the aqueous layer was extensively washed with Et_2O to remove the methoxybenzylidene by-product. Subsequent lyophilisation afforded the oligonucleotides **5a** and **5b**, which were characterised by ES-MS (Table 1).

Aldehyde-containing oligonucleotides (6a) and (6b): $NaIO_4$ (50 equiv, 3.5 mg) was added to a solution of oligonucleotide **5a** (0.320 μmol) in water (500 μL), and the solution was stirred at room temperature for 15 min. The resulting oligonucleotide **6a** was then purified by HPLC (system II); yield: 0.280 μmol, 86%. The undecamer **6b** was prepared in the same manner; yield: 78%.

Aminooxy oligonucleotide (11): The 5′-trityl-containing oligonucleotide **10** was treated with an 80% AcOH aqueous solution for 8 h. The solvent was then evaporated by lyophilisation to give compound **11**, which was used without further purification for the next conjugation reaction. An aliquot of the oligonucleotide **11** was incubated with acetone and the corresponding oxime ether **12** was characterised by ES-MS (Table 1).

Peptide synthesis: Solid-phase peptide synthesis was performed by using Fmoc/*t*Bu chemistry on a 348 Ω peptide synthesiser (Advanced ChemTech) with SASRIN resin (loading: 0.5 mmol g^{-1}, scale: 150 mg), in the case of RGD peptides, or manually with MBHA resin, in the case of NLS peptides (loading: 0.4 mmol g^{-1}, scale: 150 mg). The coupling reactions were performed for 30 min by using a twofold excess of amino acid protected by *N*-Fmoc and PyBOP and a fourfold excess of DIEA in DMF (1.5 mL). The *N*-Fmoc protecting groups were removed by treatment with a piperidine/DMF solution (1:4 v/v, 10 mL g^{-1} resin) for 10 min, three times.

RGD peptides (16), (17) and (18): The key intermediate cyclic peptide **14** was obtained from the linear H-Asp(O*t*Bu)-phe-Lys(Aloc)-Arg(Pmc)-Gly-OH, **13**. The linear peptide **13** was cleaved from the resin by reaction with trifluoroacetic acid (1%) in CH_2Cl_2 for 20 min. The volatile solvent was removed, and the resulting crude product was triturated, then washed with Et_2O three times and used without further purification. Cyclisation was performed at 0.5 mM concentration in DMF with PyBOP (1.2 equiv). The pH was adjusted to 8 – 9 by addition of DIEA, and the solution was stirred at room temperature for 1 h. The Aloc moiety was then removed by using tetrakis(triphenylphosphine)palladium(0) (0.2 equiv) and phenylsilane (25 equiv). Purification by HPLC with system III gave the cyclic peptide **14** (77%). Introduction of the aminooxy moiety was achieved by reaction of **14** with the *N*-succinimido-activated ester of the protected *O*-(carboxymethyl)hydroxylamine **15** (2 equiv) in DMF for 1 h in the presence of DIEA. The protecting groups were then cleaved by treatment with 50% TFA aqueous solution for 1 h; this led to the aminooxy-containing peptide **16** (68%). Introduction of the serine moiety was done by using Boc-Ser(O*t*Bu)-OH (2 equiv) and PyBOP (2 equiv) with DIEA (3 – 4 equiv) in DMF at 10^{-2} M. Cleavage of the protecting groups was then achieved by treatment with TFA/H_2O/TIS (95:2.5:2.5) solution for 2 h. Subsequent oxidation was then performed by using $NaIO_4$ (1.5 equiv) in water for 1 h. Purification by HPLC afforded the aldehyde peptide **17** (30% overall yield), which was characterised by ES-MS (Table 1).

The cysteine residue was introduced by using Fmoc-Cys(Trt)-OH (2 equiv), PyBOP (2 equiv) and DIEA (3 – 4 equiv) in DMF at 10^{-2} M. The Fmoc group was then cleaved by treatment with a piperidine/DMF solution (1:4, v/v) for 10 min. The other protecting groups were then cleaved in TFA/EDT/H_2O/TIS (90:5:2.5:2.5) for 2 h; this led to peptide **18**, which was purified by HPLC (60%). The structure of **18** was confirmed by ES-MS (Table 1).

NLS peptides (19), (21) and (22): In case of NLS peptides, the side-chain protecting groups were as follows: Boc for Lys, Pmc for Arg, *t*Bu for Asp and Glu. Derivatisations were carried out directly on the solid support from the N terminus position.

The aminooxy moiety was introduced by reaction with the activated ester of **15** (2 equiv) in DMF for 40 min. Cleavage from the resin and deprotection of the side-chain were then achieved by using TFA/H_2O/TIS (90:8:2) for 2 h; this gave the aminooxy peptide **19**. Introduction of the serine moiety was performed by using Boc-Ser(O*t*Bu)-OH (2 equiv) in DMF and PyBOP (2 equiv). Cleavage from the resin and deprotection of the side-chain group were done as above. Subsequent oxidation of the intermediate aminoalcohol **20** with $NaIO_4$ (30 equiv) in water for 3 h afforded the aldehyde peptide **21**, which was purified by HPLC.

The cysteine residue was introduced by using Fmoc-Cys(Trt)-OH (2 equiv) and PyBOP (2 equiv). The Fmoc group was then removed by treatment with a piperidine/DMF solution (1:4, v/v) for 10 min. Cleavage from the resin and deprotection of the side-chain groups were then performed in TFA/EDT/H_2O/TIS (90:5:2.5:2.5) for 2 h; after purification by HPLC, this gave the peptide **22**.

Conjugation through oxime-ether linkage:

*From aldehyde oligonucleotides **6a** and **6b***: A solution of RGD peptide **16** (1.5 equiv) in water (100 μL) was added to a solution of oligonucleotide **6a** (21 OD) in water (500 μL, pH adjusted to 5 with AcOH). The mixture was stirred at room temperature, and the progress of the reaction was followed by HPLC. The starting material disappeared in 1 h. Purification by HPLC with system II afforded the conjugate **23a** in 68% yield (15 OD). The conjugate **23b** was obtained in the same manner from oligonucleotide **6b** in 66% yield. Conjugation with NLS peptide **19** was achieved by using the same protocol with the undecamer **6b** and led to the conjugate **24** in 50% yield.

*From aminooxy oligonucleotide **11***: A solution of the RGD peptide **17** (0.34 μmol) in water (200 μL) was added to a solution of oligonucleotide **11** (20 OD, 0.17 μmol) in AcOH (500 μL). The mixture was stirred at room temperature for 3 h. The crude mixture was then purified by HPLC by using system I to give the conjugate **25** in 51% yield. Conjugation with the NLS peptide **21** was achieved in the same manner and led to conjugate **26** in 50% yield.

Conjugation by thiazolidine linkage:

*Conjugation with RGD peptide **18***: A solution of RGD peptide **18** (4 equiv) in sodium acetate buffer (130 μL) was added to a solution of oligonucleotide **6a** (25 OD) in sodium acetate buffer (100 μL, pH 5.4). The mixture was stirred at room temperature for 30 min until the starting material disappeared. Purification by HPLC with system II afforded the conjugate **27a** in 56% yield (14 OD). By using a similar protocol, reaction of the oligonucleotide **6b** gave the conjugate **27b** in 50% yield.

*Conjugation with NLS peptide **22***: Conjugation with NLS peptide **22** was achieved by using the same procedure as with the undecamer **6b** and led to the conjugate **28** in 64% yield.

Melting studies: The melting curves (absorbance versus temperature) were measured at 260 nm on a Lambda 5 UV/visible spectrophotometer equipped with a Perkin – Elmer C570 – 070 temperature controller and by 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) that contained 1 mM EDTA and 100 mM NaCl. All measurements were done at a concentration of 12 μM. Before each melting experiment, samples were heated at 80 °C for 5 min, then cooled slowly.

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