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A Straightforward Synthesis of 5′-Peptide Oligonucleotide Conjugates Using N^{α}-Fmoc-Protected Amino Acids

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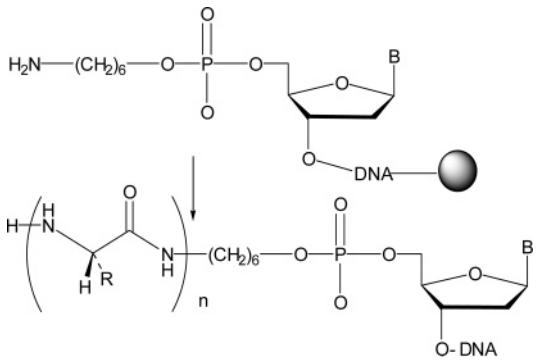
ABSTRACT



5′-Peptide oligonucleotide conjugates were prepared stepwise on a single support using N^{α}-Fmoc-protected amino acids and unprotected phosphate groups. The method uses commercially available reagents and is successful with most natural amino acids. The simplicity of the method may encourage researchers to prepare new oligonucleotide–peptide conjugates with novel properties.

The use of synthetic oligonucleotides to control gene expression has triggered the search for new oligonucleotide derivatives to improve their therapeutic potential.[1] Oligonucleotide−peptide conjugates are chimeric molecules consisting of oligonucleotides covalently linked to peptides. As a result, synthetic oligonucleotides acquire some of the biological and/or biophysical properties of peptides. Thus, linking peptides to oligonucleotides may have beneficial effects such as: (a) facilitating oligonucleotide transport through cell membranes,[2] (b) increased stability to exonucleases,[3] (c) improved binding to complementary sequences,[4] and (d) greater rate of hybridization.[5]

Two strategies can be followed to synthesize oligonucleotide−peptide conjugates.[4] In the postsynthetic conjugation approach, the two moieties are prepared independently and specific groups (such as thiols and maleimido) are specifically incorporated to link both molecules.[6] In the stepwise approach, oligonucleotide−peptide conjugates are prepared by stepwise addition of amino acids and nucleobases in solid

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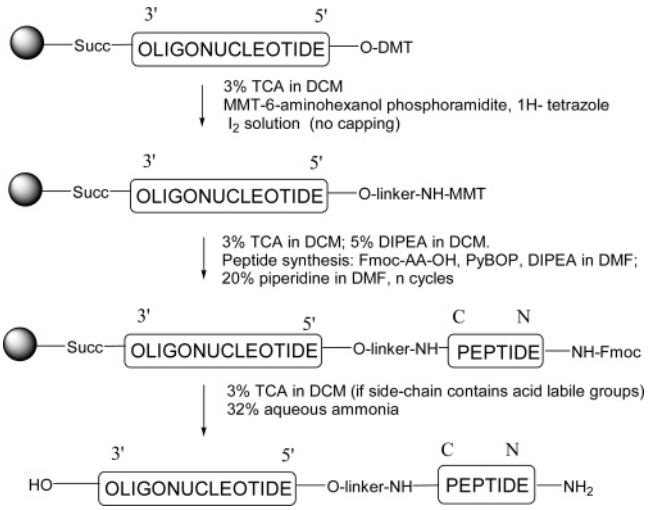
phase on the same solid support.[7] In this case, the problem is the incompatibility of the standard schemes of protection for peptides and oligonucleotides. For example, at the end of the solid-phase peptide synthesis a treatment with acid is usually required, which can provoke partial depurination of DNA. In the synthesis of oligonucleotide 3′-peptides, this problem could be averted by using N^{α}-*tert*-butoxycarbonyl (Boc)-protected amino acids with base-labile groups for the protection of side chains, a base-labile linker, and standard phosphoramidites[7a−e] or by using N^{α}-9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids.[7f−h] But these methods could not be applied for the incorporation of peptides at the 5′ end or in the middle of the oligonucleotide sequence, as required for some applications such as gene delivery.[8]

Recently, a method for the solid-phase synthesis of oligonucleotide−5′-peptide conjugates has been described.[9] It is based on the use of a protecting group [2-(biphenyl-4-yl)propan-2-yloxycarbonyl, Bpoc] for the α-amino group of the amino acids that can be removed under mild acid conditions suitable for DNA. Similarly, the monomethoxytrityl (MMT) group has been used for the synthesis of DNA/PNA chimeras.[10]

In this paper, we describe the use of common Fmoc-amino acids for the stepwise synthesis of oligonucleotide-5′-peptide conjugates. Although the Fmoc-removal conditions are not orthogonal to the base-labile cyanoethyl protection of the DNA phosphotriester moieties, we found that the presence of unprotected phosphate groups did not hinder the assembly of small peptide sequences on oligonucleotide supports.

^{N}PPLPPGP^{C} is a proline-rich peptide that has recently been prepared in our laboratory for NMR structural studies using standard Fmoc chemistry. We chose this as a model to test the methodology because it does not contain trifunctional amino acids. The self-complementary octanucleotide sequence ($^{5'}$CCAATTGG$^{3'}$) was prepared on controlled pore glass (CPG), polystyrene (LV200), and polystyrene-polyethylenglycol (PS-PEG) supports using standard 2-cyanoethyl

Scheme 1. Solid-Phase Synthesis of 5′-Peptide Oligonucleotide Conjugates



phosphoramidite protocols. After the assembly of the oligonucleotide sequence, an amino group was added to the 5′-end using the N-6-MMT-aminohexyl phosphoramidite (Scheme 1). Aliquots of the 5′-amino support carrying the 5′amino oligonucleotide were placed in a syringe, and the tripeptide PGP was assembled using standard Fmoc-chemistry (Scheme 1). It was also assembled using N^{α}-trityl-protected amino acids.[11] Unfortunately, during the latter assembly reactivity of the carboxyl functions was poor due to steric hindrance of the trityl group, and severe truncation by acetylation was observed. Acetylation (from capping reagents) together with isobutyrylation and benzoylation of the peptide sequence was described by Zamarella et al.[9] as one of the more severe side reactions occurring during the assembly of peptide at the 5′-end of the oligonucleotides. Surprisingly, the peptide sequence assembled using N^{α}-Fmoc-protected amino acids showed only mild truncation by acetylation at the 5′-aminooligonucleotide site even after repetitive capping with acetic anhydride after each amino acid addition (see Figure S1, Supporting Information). We believe that the piperidine treatment applied during the removal of the Fmoc group eliminates the "reactive" acetyl groups in the support and the assembly of the peptide sequences is thus more efficient. We also found that polystyrene (LV200) and CPG supports gave good results, the former being slightly better. Unfortunately, the oligonucleotide−peptide conjugate could not be obtained on the PS-PEG support.

Next, we prepared the heptapeptide sequence as well as related tri- and tetrapeptide sequences with trifunctional amino acids on CPG supports carrying 5′-amino oligonucleotides (see Table 1). Whenever possible, protecting groups labile to mild acidic conditions (such as 1% trifluoroacetic acid) were used to protect the side chains of the N^{α}-Fmoc-protected amino acids.

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Table 1. Characterization of Oligonucleotide 5′-Peptide Sequences

no.	oligonucleotide−peptide sequence	side-chain protecting group[a]	HPLC (min)[b]	mass (MALDI) found/calcd	T_m (°C)[c]
1	^{N}PDP^{C}-5′CAATTG3′	D(PhiPr)	9.0	2278.3/2278.4	
2	^{N}PGP^{C}-5′CCAATTGG3′		10.4	2838.8/2839.7	37.1
3	^{N}PPLPPGP^{C}-5′CCAATTGG3′		11.8	3243.3/3244.2	36.5
4	^{N}PKP^{C}-5′CCAATTGG3′	K(Tfa)	10.7	2908.2/2910.8	38.6
5	^{N}PKEP^{C}-5′CCAATTGG3′	K(MMT) E(PhiPr)	9.9	3036.5/3038.9	40.0
6	^{N}PQ(Trt)P^{C}-5′CCAATTGG3′	Q(Trt)	21.7	3150.3/2910.8[d]	39.6
7	^{N}PH(Trt)P^{C}-5′CCAATTGG3′	H(Trt)	21.6	3160.5/2919.8[d]	40.2
8	^{N}PMTP^{C}-5′CCAATTGG3′	T(Trt)	12.2	3013.1/3014.9	37.7
9	^{N}PYQP^{C}-5′CCAATTGG3′	Y(ClTrt)	11.8	3073.3/3074.0	44.1
10	^{N}PHP^{C}-5′CCAATTGG3′	H(Boc)	10.6	2918.3/2919.8	39.0
11	^{N}PHP^{C}-5′CCAATTGG3′	H(Fmoc)	10.7	2917.8/2919.8	40.0
12	^{N}PWP^{C}-5′CCAATTGG3′		13.9	2967.5/2968.9	43.9
13	^{N}PHCP^{C}-5′CCAATTGG3′	H(Tos) C(MMT)	11.6	3020.6/3022.8	40.1
14	^{N}AKKKKLDP^{C}-5′CCAATTGG3′	K(Tfa) D(PhiPr)	10.2	3495.2/3495	38.5
15	5′CCAATTGG3′		8.8		36.5

[a] Abbreviations: PhiPr, 2-phenylisopropyl ester; Trt, trityl; Tfa, trifluoroacetyl; MMT, monomethoxytrityl; Boc, *tert*-butyloxycarbonyl; Tos, tosyl; Fmoc, 9-fluorenyloxycarbonyl; ClTrt, 2-chlorotrityl. [b] Retention time (min) in HPLC (see the Supporting Information). [c] 1 M NaCl, 0.1 M sodium phosphate, pH 7.0. [d] Mass calculated for the trityl-protected derivative.

Amino acids with a carboxylic acid side chain (Asp, Glu) were protected as 2-phenylisopropyl (PhiPr) esters. First, the hexanucleotide ^{N}PD(PhiPr)P^{C}-5′CAATTG3′ was assembled. The support was then treated with 3% trichloroacetic acid (TCA) in CH_2Cl_2 for 10 min to remove the PhiPr group and then with ammonia. The desired oligonucleotide−peptide conjugate was present as the major component of the crude. Unfortunately, ammonia removed the PhiPr group if TCA treatment was omitted. It was important ensure that the PhiPr group was completely removed by the TCA treatment since hydrolysis by ammonia may lead to the corresponding amide instead of carboxylic acid. The amide peptide is difficult to detect. Using a model peptide (PheGluPro-amide) prepared on polystyrene, we demonstrate that the PhiPr group is completely removed by 3% TCA giving glutamic acid (see the Supporting Information). Therefore, after ammonia deprotection the desired oligonucleotide−peptide conjugates carrying glutamic acid are obtained.

Unprotected Fmoc-Asn and Fmoc-Gln can be used for the synthesis of peptides, although these amino acids are frequently protected with the trityl (Trt) group. We attempted to use both unprotected and Trt-protected glutamine (entries 6 and 9, Table 1), but we observed that the trityl group of glutamine was not removed by 3% TCA (entry 6, Table 1). Stronger acidic conditions will be needed to remove the Trt group which are not appropriate for DNA. In contrast, the desired tetrapeptide (entry 9, Table 1) was obtained using unprotected Fmoc-glutamine.

Two side-chain derivatives of lysine were tested successfully. The derivative of lysine carrying the trifluoroacetyl (Tfa) group is already used for the synthesis of oligonucleotide−3′-peptide conjugates because it is removed in ammonia.[7] Sequence ^{N}PKP^{C}-5′CCAATTGG3′ (entry 4, Table 1) was obtained in good yields using Fmoc-Lys(Tfa)-OH. Furthermore, the derivative of lysine carrying the acid labile MMT group was appropriate for the preparation of the oligonucleotide carrying the tetrapeptide ^{N}PKEP^{C}-5′CCAATTGG3′ (entry 9, Table 1). This result was expected because the MMT group also protects the 6-aminohexyl moiety. The nuclear localization signal (NLS) peptide AKKKKLDP was also prepared using Fmoc-Lys(Tfa)-OH and Fmoc-Asp-(PhiPr)-OH (entry 14, Table 1).

Next, four derivatives of histidine were tested. The Trt derivative described previously for the preparation of oligonucleotide-3′peptide conjugates[12] was too stable because, like Trt in Gln, it was not removed by 3% TCA (entry 7, Table 1). In contrast, the Boc-protected derivative was removed by 3% TCA (entry 10, Table 1). The base-labile Fmoc and the tosyl derivatives were also suitable for the preparation of conjugates carrying histidine (entries 11, 13 Table 1).

Amino acids carrying alcohol groups were protected with Trt (Thr) and chlorotrityl (ClTrt) (Tyr) groups. Both were removed by 3% TCA (entries 8 and 9, Table 1).

Fmoc-Trp and Fmoc-Met were successfully used without protection (entries 8 and 12, Table 1). It is important to notice that Met needs protection as sulfone when the peptide is assembled before the oligonucleotide because it is unstable during the phosphite-to-phosphate oxidation.[13] In our case, the peptide is assembled after the oligonucleotide, so there is no need to protect Met because there is no oxidation step. Unprotected Fmoc-arginine was also tested using carbodiimide activation with an excess of 1-hydroxybenzotriazole to keep the guanidine group of arginine in the protonated form.[7d] However, peptide elongation did not occur under these conditions. Carbodiimide activation is inefficient within CPG supports.[7g] Although we did not test it, arginine may be obtained by introduction of the guanidine group in ornithine.[14] Cysteine was protected by the acid-labile MMT

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group (entry 13, Table 1). This conjugate was highly sensitive to ammonia, and it was obtained in low yields (see below).

During the synthesis of the PGP−octanucleotide conjugate, we used the standard ammonia deprotection conditions (55 °C, overnight). These conditions were also used for the remaining sequences because the amide linkage between peptide and oligonucleotide is stable to ammonia. But when trifunctional amino acids such as histidine or cysteine were present in the peptide sequence the oligonucleotide part underwent severe hydrolysis, as decribed elsewhere.[7] This problem was averted by reducing the time of the ammonia treatment (1 h, at 55 °C) or lowering the temperature (overnight, at room temperature).

Finally, some authors have recommended the use of the sarcosyl linkage between oligonucleotide and solid support to avoid premature removal of the oligonucleotide during the piperidine treatment to eliminate the Fmoc groups. To assess the extent of this side reaction, we treated oligonucleotide supports with the piperidine solution, followed by analysis of the filtrates. We conclude that this side reaction is negligible in the synthesis of small peptides on oligonucleotide supports (see the Supporting Information).

Melting temperatures of self-complementary oligonucleotide−peptide conjugates are shown in Table 1. Melting curves were obtained under high salt conditions in order to be able to measure melting temperatures. In agreement with previous reports,[15] the presence of the peptide induced a small duplex-stabilizing effect, especially when tryptophan and tyrosine residues are present. Coulombic charge screening by high salt eliminates the beneficial effect of cationic side chains. For this reason, peptides carrying Lys and His do not stabilize the duplex structure under the conditions used in this work. On the contrary, high salt may push aromatic amino acids (Trp, Tyr) into the minor groove, thus stabilizing the duplex.

In summary, we have developed an efficient and general method for the synthesis of short oligonucleotide-5′-peptide conjugates using commercially available products such as standard DNA synthesis reagents, amino modifiers, and Fmoc-protected amino acids. The method allows the preparation of the oligonucleotide−peptide conjugates in any laboratory without the need to prepare special reagents. We believe that this method may have a strong impact on a large number of laboratories by opening the possibility of finding more and more applications for molecules of this type.

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Supporting Information Available: Detailed experimental protocols and representative HPLC chromatograms. This material is available free of charge via the Internet at http://pubs.acs.org.

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