Interchangeable PEG-Supported Synthesis of Peptide-Oligonucleotide Chimeras

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A procedure has been developed for the preparation of peptide-PEG-oligonucleotide conjugates: the two sequences were synthesized at the extremities of the same commercial high-molecular-weight polyethylene glycol (PEG) starting both from the peptide as well as from the oligonucleotide component. The commercial PEG was modified at its terminal hydroxyl functions with two orthogonal protecting groups (DMT and Fmoc). The selective removal of those groups allowed the synthesis of the chimeras following two different, interchangeable strategies. Comparison of the final conjugates suggested that their production was equally effective.

Introduction

The use of oligonucleotides as gene-regulation agents has been greatly hampered by their poor cellular uptake as well as by their rapid degradation in vivo. One promising approach to facilitate cellular delivery is to conjugate the oligonucleotide to a peptide that possesses cell-penetration properties.[1] Unfortunately, the methods used to synthesize peptide and nucleic acids are not fully compatible with each other. In general, two synthetic approaches are available — total in-line synthesis or the conjugation of pre-assembled fragments. Many examples have been reported of in-line processes using an automatic synthesizer, where the peptide was usually assembled first on the same solid support and the oligonucleotide thereafter. So far, however, no general methods have been reported that are suitable for all the possible sequences. A new approach that utilizes an original solid support has been recently described as a possible general procedure,[2] but these methods are difficult to use in presence of trifunctional amino acids.[3] In a further attempt, an efficient total stepwise solid-phase synthesis of oligonucleotide-(3′-N)-peptide conjugates that makes use of either a controlled pore glass support or macroporous polystyrene beads extending a previous homoserine linker approach was described.[4]

Fragment condensation allows the separate synthesis, deprotection and purification both of the peptide and of the oligonucleotide and their joining together with a variety of linkages.[5,6] Some limitations are also present in this approach: for example, the different solubility of the two components of the conjugates can inhibit the coupling reactions. Additionally, the conditions are milder than during the direct synthesis, but fragment condensation can require multiple steps, causing a quite low overall yield value.

Even if the peptide conjugation improves the interaction of the oligonucleotide with the target cells, these molecules still suffer from a reduced lifetime within the body. The biostability of oligonucleotides can be enhanced by introducing ligands and appropriate tethers[7] or by chemical modification of the backbone, as in the case of the phosphorothioate oligomers.[8] The in vivo stability of the oligonucleotides can also be increased by their delivery through liposomes. Some cationic lipids are currently used since they not only enhance the rate of uptake into cells, but also modify the cellular distribution and increase the rate of hybridization to the target.[9] Liposomes have been modified to maximize oligonucleotide delivery; in particular, derivatization with hydrophobic polymers, such a polyethylene glycol (PEG), permits longer lifetimes and minimizes unwanted uptakes.[10] However, liposomes show some toxicity and serum instability, and their accumulation in the liver and spleen cannot be fully prevented.[11]

An alternative method can be represented by the use of macromolecular carriers: these polymeric material should be stable in the bloodstream and water soluble.[12] For example, a linear poly(ethyleneimine) has been used to deliver oligonucleotides into neurons,[13] and a hydroxypropyl methacrylate polymer has been conjugated through a disulfide linkage and efficiently taken up by cultured cells.[14] A detailed overview of the polymer carriers used for the delivery and stabilisation of the oligonucleotides has recently been published[15] where, among the various neutral polymers, the use of PEG was highlighted.[16]

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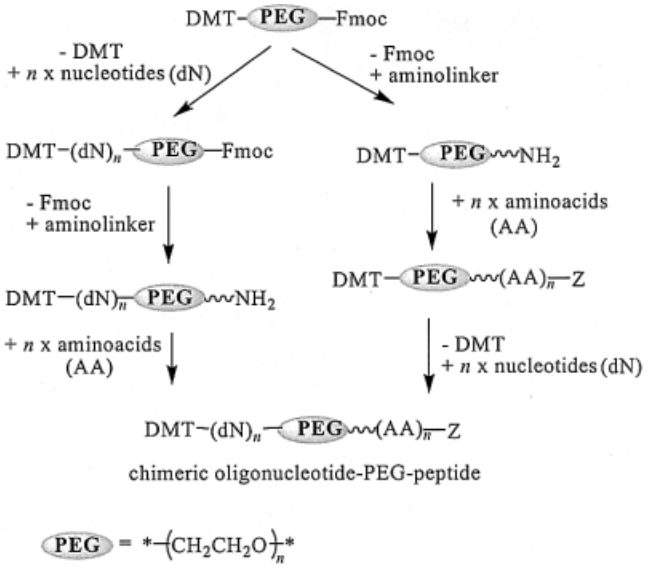
The introduction of a large PEG chain by classical solid-phase procedures suffers from the phase heterogeneity of the process that implies poor reactivity, unpredictable kinetic effects and a low final yield.[17] Alternatively, some examples of post-synthetic PEG-conjugation of these molecules have been reported, but it appears quite difficult to apply this approach when a large-scale synthesis of such products is required.[18,19] As a possible solution, the use of PEG in a liquid-phase procedure where it acts both as soluble synthetic support and as final conjugating agent has been successfully proposed.[20]

Hence, on the basis of our previous experience on the use of PEG,[21] we decided to investigate the liquid-phase, polymer-supported synthesis of chimeric PEG-conjugates carrying an oligonucleotide and a peptide sequence on the same polymer. In a previous paper, a protocol for the synthesis of such PEG-conjugates was reported taking advantage of the preparation of pure, selectively protected polydispersed PEGs.[22] In this paper we report the synthesis of the same peptide-PEG-oligonucleotide, but using a new protocol that allows its production starting from the oligonucleotide or from the peptide, respectively. The final aim is to ascertain the influence of the order of synthesis on the final product, if any, and to decide the best procedure for the preparation of these PEG-conjugated chimeras.

Results and Discussion

Recently,[23] we described the synthesis of DMT-d(GCAT)-PEG-Gly-L-Phe-D-Phe-Z, where the oligonucleotide was the simplest sequence containing all the four natural deoxyribonucleotides, while the peptide was the shortest sequence reported as being active toward receptors of the cell surface,[24] as demanded for the improvement of the oligonucleotide delivery.

Here we have investigated if the order of synthesis of two biopolymers on the same PEG had any effect on the efficiency of the process and on the quality of the final conjugate. Moreover, we exploited the use of 1,3-diaminopropane, a linear isomer of the molecule employed in the previous study. The starting support was protected with two orthogonal protecting groups — DMT and Fmoc — whose alternate deprotection allowed the synthesis of the same final chimera, but following the two alternative procedures reported in Scheme 1.



Scheme 1. General scheme showing the two alternative syntheses of a chimeric oligonucleotide-PEG-peptide on the same PEG-protected polymer

Modification of PEG

To perform the planned syntheses a sufficient amount of pure DMT-PEG-OH was initially prepared following the published procedure.[23] An Fmoc group was introduced at the free OH extremity of PEG to produce the orthogonally protected polymer DMT-PEG-Fmoc. This protected polymer was needed for the two planned syntheses, that is "peptide-first" and "oligonucleotide-first", since the two groups, fully compatible with the two synthetic procedures, can be removed without damaging the other biopolymeric sequence conjugated on PEG. The final removal of the protecting groups confirmed the efficacy of the procedure, as judged by NMR spectroscopy and ESI mass spectrometry. The mass increment with respect to starting PEG was calculated following the published procedure.[25] Some selected data are reported in Table 1.

Table 1. Integration values of selected signals in the ^{1}H NMR spectra and mass increments (from ESI) of mono- and diprotected PEGs

Sample	^{1}H NMR chemical shifts (ppm)			Mass increments	
	protons	calcd.	found	calcd.	found
DMT-PEG-OH	DMT (aromatic)	4.0	3.7	302.0	303.0
	OH	1.0	1.1		
	PEG-CH_2-O-DMT	2.0	1.8		
Fmoc-PEG-OH	Fmoc (aromatic)	8.0	8.8	222.2	222.0
	Fmoc–CH–CH_2–O–CO + OH	4.0	4.5		
	CH_2–O–CO–O–Fmoc	2.0	1.9		
DMT-PEG-Fmoc	DMT (aromatic)	4.0	4.1	524.2	525.0
	PEG–CH_2–O–DMT	2.0	1.7		
	Fmoc–CH–CH_2–O–CO	3.0	2.8		
	CH_2–O–CO–O–Fmoc	2.0	1.9		

Synthesis of the Chimeric Peptide-PEG-oligonucleotide

Peptide-First

The chimeric PEG-conjugate was initially synthesized starting from the peptide sequence following the same procedure as reported in the previous synthesis.[23] As before, we introduced a diamino molecule capable of forming a stable urethane linkage with the OH group of PEG and a stable amide bond with the peptide. In the present procedure, a linear 1,3-diamino linker, isomeric with the 1,2-diaminopropane used in the previous study, was tested. The purpose of this modification was to eliminate any asymmetry and steric hindrance deriving from the substitution on the central carbon atom in the 1,2-linker. However, in this case its monoprotection was required since some dimerization (up to 10%) of the DMT-PEG-OH was observed in the presence of the excess of linker demanded for a quantitative yield. The monoprotection of the 1,3-diaminopropane was optimized to improve the literature procedure.[26] The direct reaction of the diamine with FmocCl was successfully performed by a careful optimization of the reaction conditions to avoid a massive production of the diFmoc-amine. Two consecutive acidic and basic extractions allowed the isolation of the monoprotected derivative in a pure form, as ascertained by ^{1}H NMR spectroscopy.

The reaction of PEG with the *N*-Fmoc-1,3-diaminopropane was obtained by activation of the free OH group of the polymer with *p*-NO_2-phenylchloroformate as previously reported.[29] The pH of the reaction must be kept basic to control the release of HCl from the reagent; in these conditions some β-elimination of the cyanoethyl (CE) protecting groups of the internucleosidic triester phosphates can be expected when the peptide is synthesized on a preformed PEG-oligonucleotide (see below). Hence, the tertiary amine to be used was carefully evaluated, varying its steric demand and nucleophilicity. The best result was obtained with trioctylamine (TOA): on a DMT-dT-PEG-OH sample no removal of the phosphate protections was detected. It is likely that the three long alkyl chains of the TOA hinder the nucleophilic attack on the CE moiety. After a further Fmoc deprotection, the modified DMT-PEG-1,3-diaminopropane was analyzed and the DMT to NH_2 ratio, as determined by UV absorption and TNBS analysis, was found to be 1:1 within the experimental error. The ESI mass spectra present a series of a single peak clusters in perfect agreement with the expected mass increment, while the ^{1}H NMR spectrum shows the expected set of signals (Figure 1).

The synthesis of the peptide as described in the previous paper produced the Z-protected DMT-PEG-tripeptide. Its analysis by mass spectrometry and NMR spectroscopy (Figure 2) confirmed the expected results.

The UV absorption of DMT, compared with the amount of terminal NH_2 of the peptide chain, gave an overall yield of 98%, corresponding to an average yield of at least 99% for each condensation step.

After removing the DMT protecting group, the oligonucleotide was synthesized using standard phosphoramidite , as before. As during the previous synthesis, the peptide modified the solubility properties of the supporting PEG. A reduced amount of the product was recovered during the purification processes of the intermediates based on the precipitation and filtration of the PEG-conjugates.

Oligonucleotide-First

The initial synthesis of the tetranucleotide was performed after acidic deprotection of DMT-PEG-Fmoc. The yield, evaluated by the UV absorption of the DMT group after each coupling, was near the quantitative value, as expected from similar liquid-phase processes. The ^{1}H NMR spectrum is in good agreement with the structure of the DMT-d(CGAT)-PEG-Fmoc, as proved by the integral values of some significant signals (Figure 3).

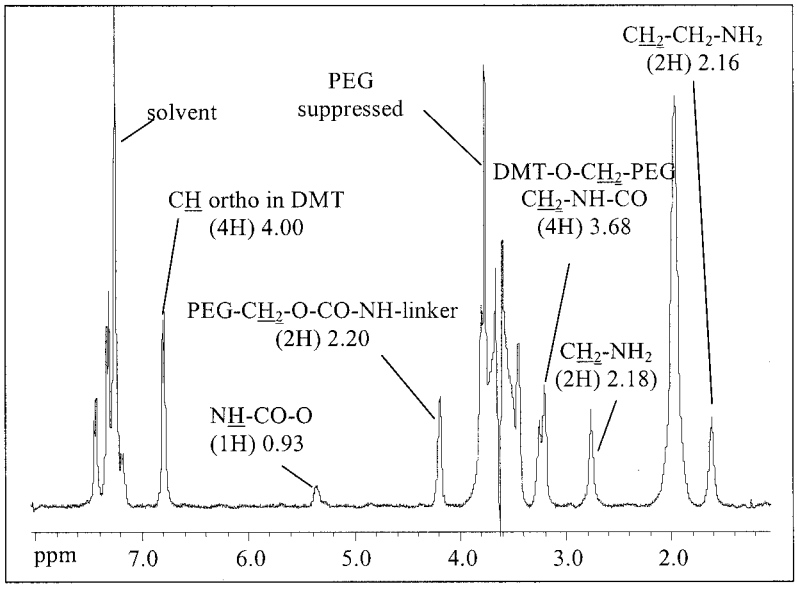


Figure 1. ^{1}H NMR spectrum of DMT-PEG-1,3-diaminopropane in $CDCl_3$

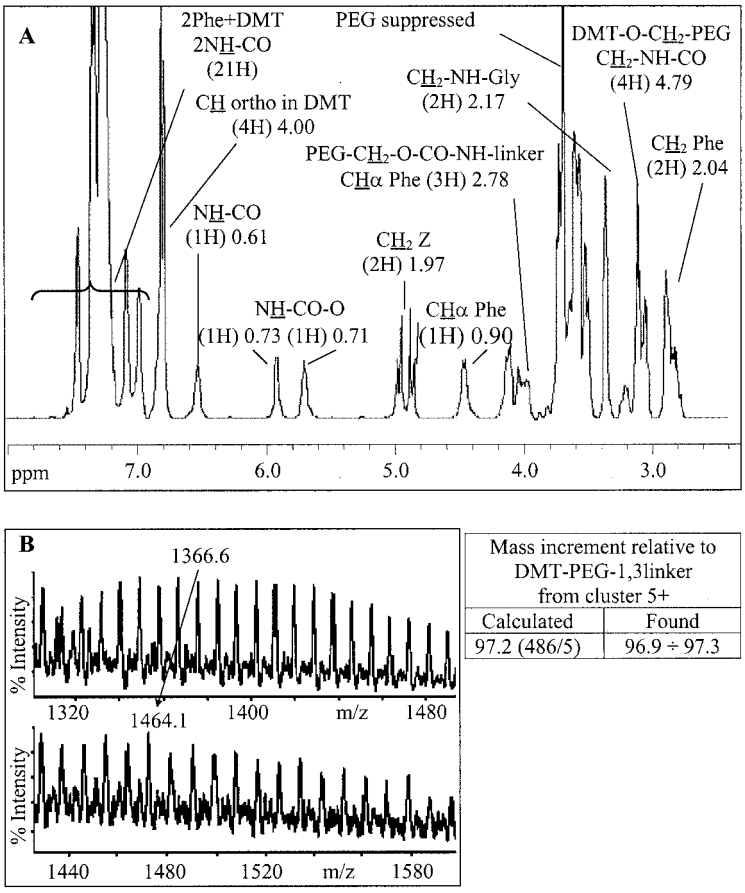


Figure 2. A) ^{1}H NMR spectrum of DMT-PEG-1,3-linker-Gly-L-Phe-D-Phe-Z in $CDCl_3$; B) part of the ESI spectrum

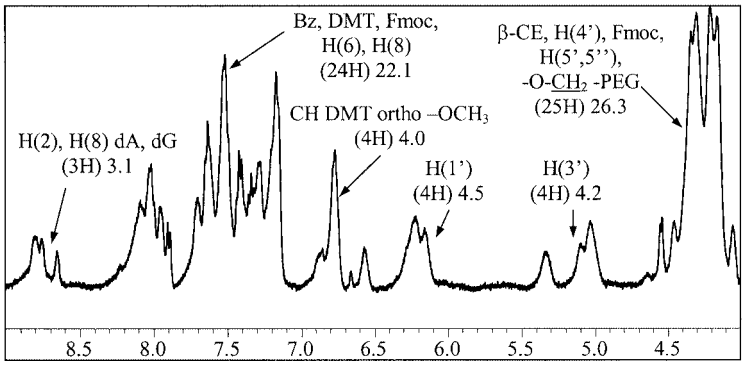


Figure 3. Partial ^{1}H NMR spectrum of DMT-d(CGAT)-PEG-Fmoc in $[D_6]$DMSO

The subsequent Fmoc deprotection required additional investigations due to the close similarity between the deblocking procedure and the removal of the CE protecting groups of the triester phosphates of the oligonucleotide. From a survey of the literature data, a procedure based on morpholine[27] was tested on DMT-dT-PEG-Fmoc, analyzing by ^{1}H NMR spectroscopy the ratio of the CE to the other nucleosidic signals. This procedure was compared with the standard piperidine/DMF method, which results in removal of 25% of the CE groups. Morpholine allowed the complete removal of the Fmoc group without damaging the protection of the triester phosphate. The ESI mass analysis validated those data. These conditions were therefore applied to the deprotection of DMT-d(CGAT)-PEG-Fmoc. The *N*-Fmoc-1,3 diaminopropane was then introduced. The use of TOA during the activation of the free, terminal OH group confirmed the efficacy of this procedure. However, during the successive condensation reaction, and in contrast to previously reported results,[28] at least 60% of the phosphate protecting groups were removed, as determined from the ^{1}H NMR spectrum. This is likely due to the nucleophilicity of the free amino group of the linker that was used in excess to obtain a quantitative modification of the terminal PEG hydroxyls. A possible

solution requires the introduction of the Fmoc-linker in advance, instead of starting from DMT-PEG-Fmoc, as indicated in Scheme 1; in that case, however, the protected polymer will lose its more general feature. During the peptide synthesis, the successive Fmoc deprotection of the linker with morpholine/DMF solution did not damage any CE protection; the overall and average yields were the same as those observed during the other procedure. However, the presence of a small number of ionisable diester bonds along the oligonucleotide chain produced during the reaction with *N*-Fmoc-1,3-diaminopropane modified the advantageous solubility properties of PEG. A reduced recovery of the final product was observed due to a less-effective precipitation of the chimeric PEG-conjugate during the purification of the intermediates.

Analysis and Comparison of Products

The chimeric PEG-conjugates were compared to verify if any significant difference between the two procedures could be observed. HPLC studies of the two crude products gave almost superimposable chromatograms, as shown in Figure 4.

The ^{1}H NMR analysis supported the substantial identity between the two products, as can be observed in Figure 5.

The amount of product obtained from the two procedures was similar, with a small increment for the procedure starting with the oligonucleotide synthesis, regardless of two additional steps required in the overall protocol. However, the difference on final recovery was not so high to clearly discriminate among the two procedures.

Conclusion

This study has confirmed that it is possible to synthesize by the versatile liquid-phase process a chimeric oligonucleotide-PEG-peptide using the high molecular weight polymer both as a synthetic support and as a conjugating agent. Also, it appears possible to design a properly modified poly(ethylene glycol) on which the order of synthesis of the two biopolymers can be planned independently from the support. In particular, using two orthogonal protecting groups at the extremities of the same PEG chain, it is possible to initiate the synthesis starting from the oligonucleotide as well from the peptide side, without any substantial difference.

Experimental Section

Abbreviations: CE = cyanoethyl; DCE = dichloroethane; DMAP = 4-dimethylaminopyridine; DMT = 4,4′-dimethoxytrityl; EDC = *N*-(3-dimethylaminopropyl)-*N*-ethyl carbodiimide;

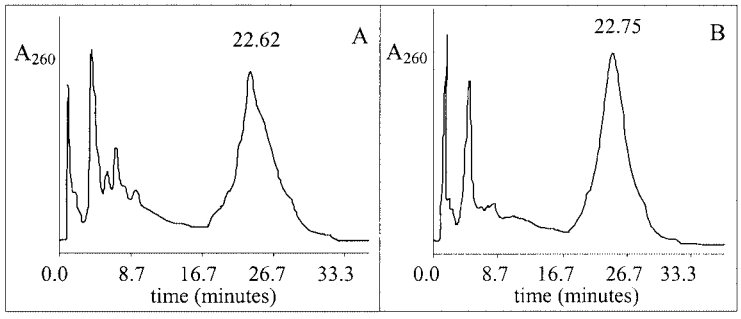


Figure 4. Ion-exchange HPLC of the two chimeric PEG-conjugates: A) peptide first, B) oligonucleotide first

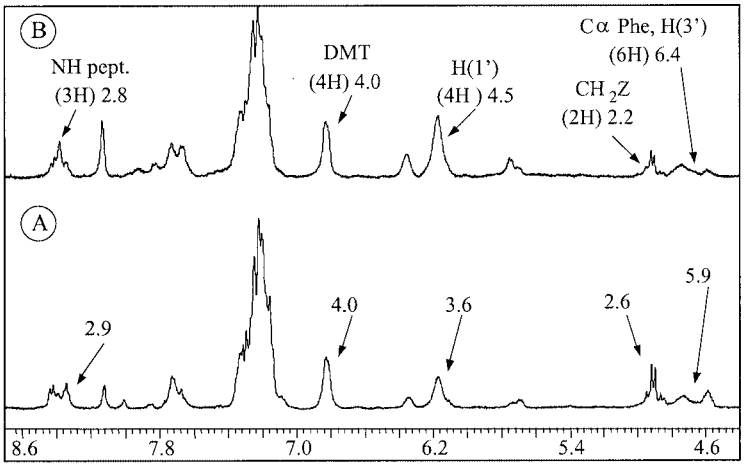


Figure 5. Part of the ^{1}H NMR spectra of the two chimeric PEG-conjugates in [D_6]DMSO: A) peptide first, B) oligonucleotide first

Fmoc = 9-fluorenylmethoxycarbonyl; HOBT = 1-hydroxybenzotriazole; MTBE = methyl *tert*-butyl ether; TBHP = *tert*-butyl hydroperoxide; TNBS = trinitrobenzensulfonic acid; Z = benzyloxycarbonyl; TCA = trichloroacetic acid.

General Remarks: NMR spectra were recorded on a JEOL EX-400 (400 MHz) spectrometer using TMS as internal standard. ESI mass spectra were obtained on a PE-API 1 spectrometer by infusion of a solution of the polymeric sample in a 1:1 solution of MeOH/5% CH_3COONa (ionising potential: 5600 V; infusion: 0.1 mL/h; curtain gas: N_2, 1.2 mL/min; nebulizing gas: air, 0.6 L/min). PEG, common reagents and anhydrous solvents were purchased from Fluka, Buchs (Switzerland). Phosphoramidites and 1*H*-tetrazole were obtained from Pharmacia, Uppsala (Sweden). Protected amino acids were obtained from Chem-Impex Intl., Wood Dale (USA).

HPLC was performed on a Hewlett Packard Series 1100, equipped with a UV Lambda-Max 481 LC (Waters), using a Resource Q (Pharmacia) column (1 mL). Eluent A: NaCl 0.05 M (pH 12.0); eluent B: NaCl 0.5 M (pH 12.0); gradient: 25% B for 15 min, from 25 to 60% B for 5 min, 60% B for 5 min.

DMT was evaluated from its 498 nm absorption in a 3:2 solution of $HClO_4$/EtOH. Fmoc was evaluated from its 264 nm absorption in H_2O or in MeOH solution.

The TNBS analysis was made on 2 mg samples of PEG-conjugates, accurately weighted and added to a 10 mL flask with 9 mL of borate buffer 0.1 M (pH 9.3) and with 0.25 mL of a 0.03 M solution of TNBS in the same buffer. The volume was adjusted to 10 mL, and, after 30 min, the absorbance at 421 nm (ε = 12850) gave the amount of free NH_2 groups.

DMT-PEG-Fmoc: The product, starting from a commercial OH-PEG-OH (mol. wt. = 6 kDa), was obtained following the published procedure.[22] The purification process, based on the separation of the intermediate DMT-PEG-succinate, was modified using the same QAE-Sephadex A50 column, but equilibrated with a 0.625 mM solution of 1,3-diaminopropane (pH 9.15). A stepwise gradient with a 1.25 mM solution was used to elute the product from the column. Once collected, the solution containing the product was taken to dryness on a rotary evaporator, and the residue recrystallized from CH_3CN/MTBE after removal of any undissolved material by filtration. The UV analysis of the DMT absorption at 498 nm allowed the evaluation of the modification of a single OH extremity up to 98%. The expected structure of the derivative was confirmed by ^1H NMR spectroscopy and ESI mass spectrometry. After removal of the succinate moiety in a 30% NH_4OH solution, the Fmoc group was introduced at the remaining OH function by reaction with 10 equivalents of FmocCl (solid) in anhydrous pyridine. The product was purified by recrystallisation from DCE/MTBE and analyzed as before.

***N*-Fmoc-1,3-diaminopropane:** Freshly distilled 1,3-diaminopropane (1.0 mL) was added to 50 mL of CH_2Cl_2. In an ice-bath, whilst stirring, 0.5 equivalents of FmocCl in 15 mL of CH_2Cl_2 was added at once and the mixture was left to react for 30 minutes. The *N*,*N*′-diFmoc derivative was filtered from the reaction mixture, and the solution extracted three times with 20 mL of a 10% (v/v) solution of HCl. The acidic solution was brought to pH 8.5 with a 0.2 N NaOH solution, extracted three times with CH_2Cl_2, and the solvents evaporated to dryness. The residue was suspended in anhydrous diethyl ether, filtered, washed with diethyl ether and desiccated. Yield 0.43 g (22%).

Synthesis of DMT-d(GCAT)-PEG-Gly-L-Phe-D-Phe-Z. Peptide-First Procedure

DMT-PEG-pNO_2 Phenylcarbonate: Pure DMT-PEG-OH (1.0 g) was activated by dissolving it in the minimum amount of CH_2Cl_2 and adding three equivalents of *p*-NO_2-phenylchloformate and two equivalents of TOA, adjusting the pH to 9 by further addition of TOA. The mixture was left to react under argon atmosphere, at room temperature, for 4 hours. The product was precipitated by slow addition, in an ice-bath and whilst stirring, of MTBE. It was then filtered, washed with diethyl ether and dried over KOH pellets under vacuum. Yield 0.89 g (99%).

DMT-PEG-1,3aminolinker-Fmoc: DMT-PEG-pNO_2 phenylcarbonate (1.0 g) was dissolved in 5 mL of CH_2Cl_2 and added, whilst stirring, to three equivalents of *N*-Fmoc-1,3-diaminopropane. The mixture was left to react under argon for 2 hours at room temperature. The product was precipitated by slow addition, in an ice-bath and whilst stirring, of MTBE. It was then filtered, washed with diethyl ether and dried over KOH pellets under vacuum. Yield 0.98 g (92%).

DMT-PEG-Gly-L-Phe-D-Phe-Z: DMT-PEG-1,3aminolinker-Fmoc (0.5 g) was dissolved in 20 mL of a 10% morpholine solution in DMF and left to react at room temperature for 20 minutes. The deprotected product was precipitated in an ice-bath with MTBE, filtered and washed with diethyl ether. A TNBS analysis confirmed the degree of deprotection. In a separate vessel, three equivalents of Fmoc-Gly-OH was dissolved in 3 mL of anhydrous CH_2Cl_2 and, whilst stirring in an ice-bath, three equivalents each of HOBT, EDC, and TEA were added in this order. This solution was added to the deprotected PEG derivative dissolved in 5 mL of CH_2Cl_2. The reaction was left stirring at room temperature overnight. The product was precipitated as described previously, filtered and washed with diethyl ether. The same procedure was employed for the introduction of Fmoc-L-Phe-OH and of Z-D-Phe-OH. Yield of DMT-PEG-tripeptide: 0.45 g (97%).

DMT-d(GCAT)-PEG-Gly-L-Phe-D-Phe-Z: The DMT-PEG-1,3aminolinker-tripeptide-Z was dissolved in 10 mL of DCE and 10 mL of a 6% (w/v) solution of TCA in DCE was slowly added after cooling in an ice-bath The reaction was left at room temperature whilst stirring for 15 min. The product was precipitated with MTBE, filtered and washed with diethyl ether. The dried OH-PEG-aminolinker-tripeptide-Z was dissolved in 1 mL of anhydrous CH_3CN under argon atmosphere in a three-necked vessel and, through the self-sealing septa, a 3.5-fold excess of DMT-dT-phosphoramidite (0.2 M in CH_3CN) and 14-fold excess of 1*H*-tetrazole (0.45 M in CH_3CN) were injected. After 15 min of reaction under argon the product was precipitated with MTBE in an ice-bath, filtered, washed with diethyl ether and recrystallized from EtOH. Once verified that the reaction yield approached completion (if far from a quantitative value, it was allowed to react again under the same conditions), the product was capped by dissolving it in 5 mL of CH_3CN and adding, in this order, 0.2 mL of 2,6-lutidine, 0.2 mL of *N*-methyl imidazole and 0.2 mL of acetic anhydride. After 5 min the product was precipitated with MTBE, filtered and washed with diethyl ether. The phosphite triester obtained was then oxidized to the phosphate by dissolving it in 5 mL of CH_3CN and adding, in an ice-bath, whilst stirring, 0.3 mL of TBHP. After 15 min the product was precipitated with MTBE, filtered and washed with diethyl ether. The other DMT-deoxynucleosides were added following the same procedure. The final product was recrystallized from EtOH and dried under vacuum over KOH. Yield of DMT-oligonucleotide-PEG-peptide 0.25 g (82%)

Synthesis of DMT-d(GCAT)-PEG-Gly-L-Phe-D-Phe-Z. Oligonucleotide-First Procedure

DMT-d(G*C*AT)-PEG-Gly-L-Phe-D-Phe-Z: DMT-PEG-Fmoc (0.5 g) was dissolved in 10 mL of anhydrous DCE and, under vigorous stirring, in an ice-bath, 10 mL of 6% (w/v) TCA in DCE solution was added. After 15 minutes the deprotected PEG derivative was precipitated as usual, recrystallized from DCE/MTBE, washed with diethyl ether and analyzed by UV absorption to confirm the complete removal of the DMT group. The dried product was dissolved in 1 mL of anhydrous CH_3CN in a three-necked vessel and left to react with the four DMT-deoxyribophosphoramidites as in the synthesis described above. DMT-d(CGAT)-PEG-Fmoc was deprotected with a 10% morpholine solution in DMF following the previous conditions. Then, the resulting DMT-d(CGAT)-PEG-OH was activated with *p*-NO_2-phenylcarbonate and *N*-Fmoc-1,3-diaminopropane was introduced as in the previous synthesis (see above). After removal of the Fmoc protection, DMT-d(CGAT)-PEG-1,3-diaminopropane-NH_2 was reacted with the three protected amino acids following the reaction conditions described in the other synthesis of the chimeric PEG-conjugate (see above). The final product was recrystallized from EtOH and dried under vacuum over KOH. Yield of DMT-oligonucleotide-PEG-peptide 0.28 g (91%).

The deprotections of the triester phosphates, of the heterocyclic bases and of the terminal 5′-OH of the oligonucleotide were performed as in the published synthesis.[23] The final, deprotected products were lyophilized and kept under vacuum.

Acknowledgments

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