Liquid-Phase Synthesis and Characterization of a Conjugated Chimeric Oligonucleotide-PEG-Peptide

Sara Drioli,[a] Ilaria Adamo,[a] Maurizio Ballico,[a] François Morvan,[b] and Gian M. Bonora*[a]

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The preparation of a new chimeric conjugate in which a peptide and an oligonucleotide sequence are linked to the same, high-molecular weight poly(ethylene glycol) is reported. First, a new amino function was introduced on PEG selectively protected at the other OH extremity. Then, the peptide was synthesized at the amino-modified end, followed by removal of the OH-protecting group of PEG and synthesis of the oligonucleotide sequence. The final oligonucleotide deprotection was achieved without affecting the integrity of the peptide chain.

The use of oligonucleotides in the antisense and anti-gene strategy is being extensively investigated for its wide therapeutic potential,[1] although the adequate delivery of these molecules to individual cells is still a difficult task.[2]

To improve cellular delivery, the use of cationic lipids is a common procedure, despite several disadvantages due to their toxicity.[3] Derivatization of liposome surfaces with amphiphilic polymers, such as poly(ethylene glycol) (PEG), permits a longer lifetime and minimizes unwanted side-effects.[4,5]

The use of synthetic polymers as agents for the delivery of oligonucleotides has been widely explored during the last 20 years;[6] among the neutral polymers, PEG chains provide protection from degradation by nucleases and enhance the cellular penetration of linked biomolecules.[7] The use of high-molecular-weight PEG as a conjugating agent for oligonucleotides has recently been proposed since it has the potential to improve the activity and stability of these pharmacologically active biopolymers without producing any toxic effects.[8] Moreover, in the so called HELP (High Efficiency Liquid Phase) procedure, the preparation of synthetic nucleic acid sequences using high-molecular-mass PEG as a soluble support has been proposed to scale up their syntheses for industrial applications.[9]

Recently, the use of proteins or peptides with the ability to penetrate cell membranes has been extensively investigated to enhance the delivery of oligonucleotides.[10] In particular, several peptides have been successfully used for the intracellular release of macromolecules.[11] Also, large proteins are able to enhance the cellular uptake,[12] although the use of peptides can be more advantageous owing to their reduced size and simpler structure. The synthesis of peptide-oligonucleotide conjugates is a challenging task, especially if the method must be scaled up. Two different approaches are commonly adopted, namely total in-line synthesis and fragment conjugation.[13] In the in-line synthesis of the two sequences the key problem is finding the right combination of protecting group[14] and new support and synthetic strategies are currently being investigated.[15] On the other hand, the conjugation of the two components after their separate synthesis requires special linkages and several purification steps, while the solubility properties can hamper the coupling reaction, and the overall yield is usually low.[16,17] Recently, an alternative procedure has been proposed in which a protected peptide is coupled to the oligonucleotide bound to the solid support, allowing for its incorporation at defined sites of the oligonucleotide.[18]

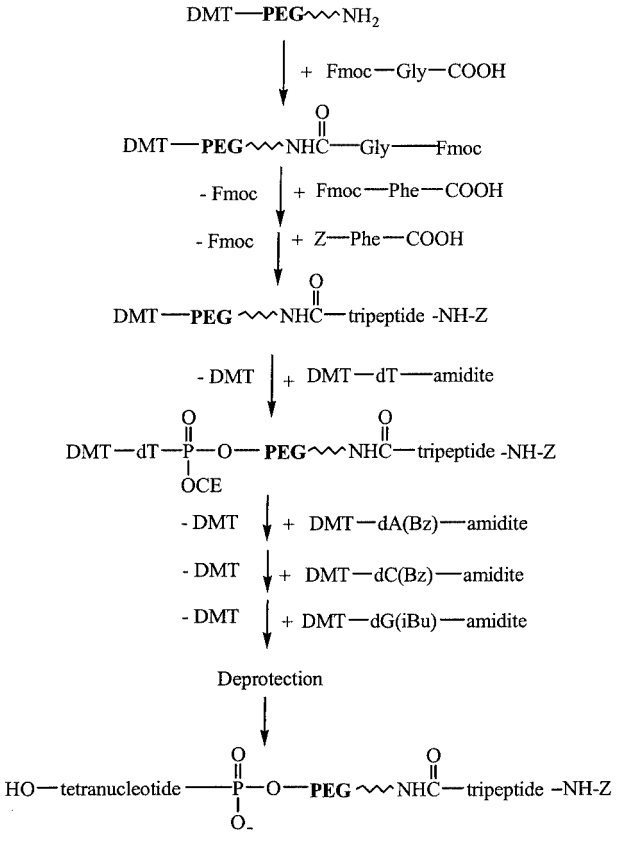
A few years ago a new procedure was devised that utilizes a polystyrene-PEG support in which a bifunctional linker orthogonally protected with a Fmoc/DMT system allows the sequential synthesis of a peptide-oligonucleotide conjugate,[19] but in that case PEG was part of a larger insoluble support. Because of our previous experience with peptide and oligonucleotide synthesis on PEG supports,[20,21] we decided to explore the feasibility of this synthetic task using a pure, commercial high-molecular-weight PEG, selectively modified at its extremities with the same orthogonal protecting groups, whose preparation we recently achieved.[22] All reactions were performed on the same PEG moiety, which is used both as soluble support and conjugating agent; thus, the final product was not released from PEG at the end of the synthesis. Hence, a liquid-phase synthesis, i.e. a synthesis supported on a soluble polymer, of

[a] Dept. of Chemical Science − University of Trieste
Trieste, Italy
[b] Lab. de Chimique Organique Biomoleculaire de Synthèse − Université de Montpellier II
Montpellier, France

the chimeric conjugate on the same PEG chain bearing a peptide on one side of the polyether moiety and an oligonucleotide sequence on the other, was achieved. The use of PEG as supporting polymer is extremely advantageous since it allows easy purification of intermediates from excess of reagents and soluble by-products. The sequential synthesis, purification and characterization of a sample PEG-conjugate is described here.

Results and Discussion

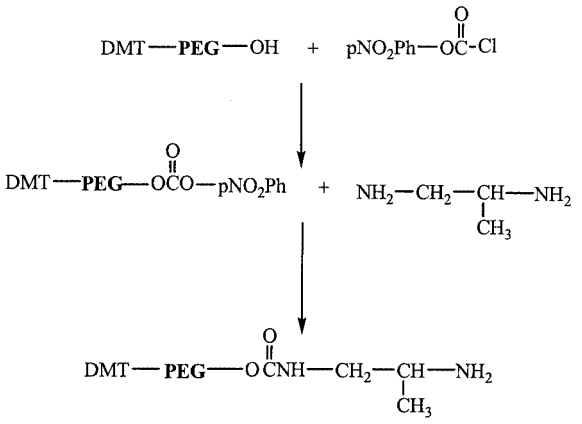
The preparation of the chimeric conjugate is described in Scheme 1. In the first attempt we synthesized the peptide sequence before the oligonucleotide to avoid any likely drawback due to the removal of the phosphate protecting groups after assembling the oligonucleotide chain. In fact, a modification of the overall solubility properties of the conjugate, before the assembling of the peptide, could be expected if some anionic phosphate groups were generated. For this reason we started from the monoprotected high-molecular-weight PEG derivative DMT-PEG-OH (M.W. = 6000 Da), obtained following the published procedure,[22] since the DMT protecting group is usually employed in oligonucleotide synthesis.



Scheme 1. Synthesis of the oligonucleotide-PEG-peptide

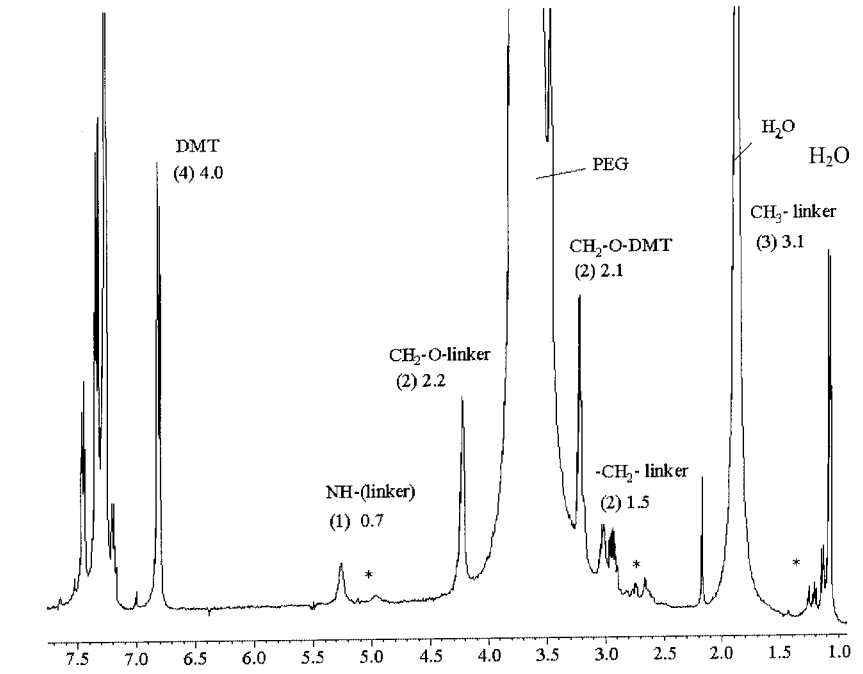
Introduction of the Amino Linker

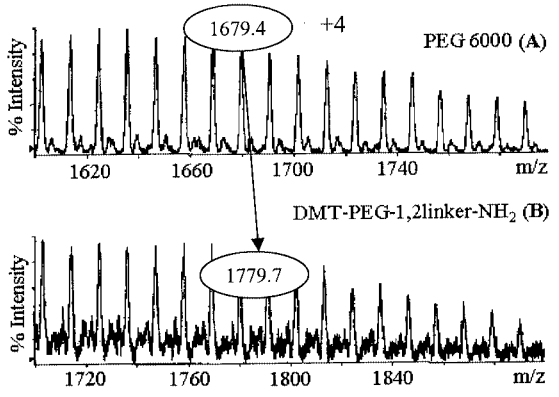
The synthesis of the peptide at the remaining, free OH terminal of the monoprotected PEG started with the introduction of a proper linker to provide the required stability to the bond between the PEG and the peptide. For this reason we introduced a diamino molecule capable of generating a stable urethane linkage with the activated OH group of PEG and a further stable amide bond with the first amino acid of the peptide. Moreover, to avoid any temporary protection of one of the two amino groups of the linker, 1,2-diaminopropane was employed, taking advantage of the different steric demand of the two units, as depicted in Scheme 2. Hence, a selective reaction between the less-hindered amino group and the activated polymeric terminal OH was expected.



Scheme 2. Selective introduction of the diamino linker

Activation of the hydroxyl group was achieved via formation of an active ester with pNO$_2$-phenylchloroformate.[23] A series of reactions was performed to determine the best conditions to achieve a single substitution of the diamino linker. Addition of solid activated DMT-PEG to a CH_2Cl_2 solution of the diamine was ineffective as it produced a modified PEG that, once precipitated, purified and analysed, showed an extensive dimerization; in fact, an NH_2 content lower than 50% with respect to the starting OH groups was observed. The same result was obtained with dropwise addition of the diamine solution to a concentrated solution of activated polymer. On the contrary, a far better result was obtained by adding a threefold excess of the neat amino compound in one portion to a 10% (w/v) solution of DMT-PEG-pNO$_2$ phenylcarbonate in CH_2Cl_2. In this case, the UV analysis, based on the TNBS test of the free NH_2 and on the absorbance of the DMT unit, gave a DMT/NH_2 ratio of 0.94. The ^{1}H NMR spectrum of the purified sample (Figure 1a) confirmed the identity of the product. It is possible to observe only traces of an undesired derivatization at the more hindered group (indicated with an asterisk). The formation of the urethane linker with the expected amino group was determined by NMR spectroscopy from the multiplicity of the urethane signal and from the downfield shifts of the CH_2 protons linked to the modified amino group, and confirmed by decoupling experiments (not shown). From the ESI-MS experiment a single, final product was observed and the theoretical m/z increments of the peaks of each cluster were in excellent agreement with the predicted value, as shown in Figure 1b.





A	B
Calc.	Calc.
-	(404-2)/4 = 100.5
Found	Found
-	100.5 ± 0.2

Mass increment from PEG 6000

Figure 1. A) ^{1}H NMR spectrum of the DMT-PEG-1,2-linker-NH_2 in $CDCl_3$; B) ESI-MS of the same sample

Synthesis of the Peptide

As a sample peptide we decided to synthesize the simplest sequence reported in the literature as being active toward receptors of the cell surface, which was the first type of target used to enhance oligonucleotide delivery. Hence we planned to synthesize the tripeptide Z-D-Phe-L-Phe-Gly on the DMT-PEG-1,2-linker, a specific inhibitor of cell fusion, previously proposed in a different peptide-oligonucleotide conjugate.[24] The synthesis was performed using the Fmoc derivatives of the first two amino acids, while the last one, a D-Phe, was introduced as a Z derivative, as demanded by the original active sequence. The coupling was almost quantitative when using a threefold excess of the amino acid in the presence of EDC/HOBT as condensing agents. An overall yield of 98%, corresponding to an average yield of 99%, was measured from the absorbance of the unchanged NH_2 groups. The liquid-phase process was exactly the same as previously described.[25] All the synthetic procedures were perfectly compatible with the soluble polymer-supported process and 95% of the expected amount of product was recovered upon completion of the synthesis.

The identity of the product was ascertained through the inspection of the ^{1}H NMR and ESI/MS spectra. A nearly 1:1 ratio between the signals of the two terminal groups DMT and Z was observed in the NMR spectrum (Figure 2) within the experimental error, while the mass increment measured for the DMT-PEG-peptide, compared to the DMT-PEG-linker, matches the theoretical value with an average inaccuracy of 0.2%. The NMR spectrum was measured on a DMSO solution due to the broadening of the

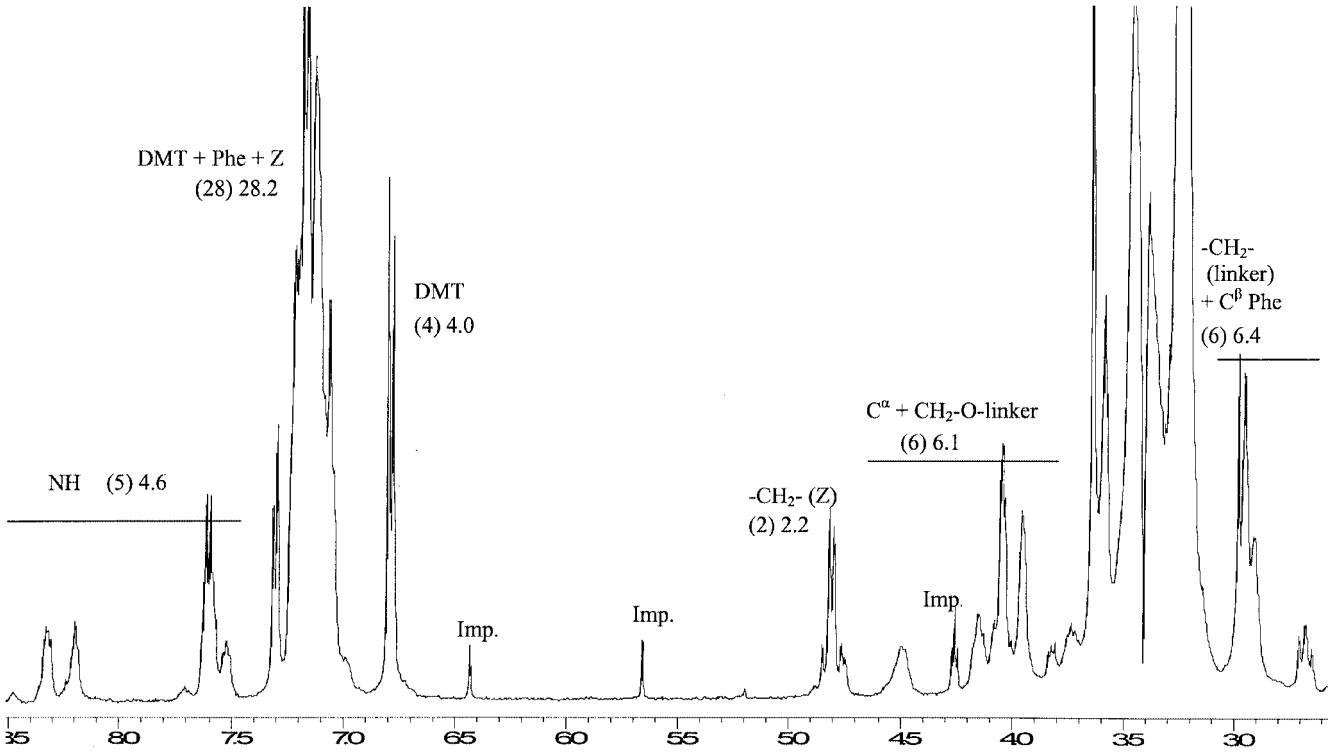


Figure 2. ^{1}H NMR spectrum of the DMT-PEG-tripeptide in [D_6]DMSO

lines observed in $CDCl_3$ and to avoid superimposition of some of the signals. The amino acid analysis of the sample gave the correct ratio expected for the sequence (1.97 Phe to 1.00 Gly).

Synthesis of the Oligonucleotide

The oligonucleotide sequence synthesized was the simplest and shortest sequence containing all the four natural deoxyribonucleotides in order to investigate the influence of the final deprotection of the heterocyclic bases on the overall synthetic process. After removal of the DMT protecting group, the product was assembled by standard phosphoramidite chemistry, following the HELP procedure.[26] The overall and average yields, based on the absorbance of the DMT group, were 94% and 98%, respectively, in good agreement with the values normally observed in this process; the recovery of the product, however, was not complete as no more than 60% of the expected product was obtained. This could be due to the modification of the solubility properties of the supporting PEG caused by the peptide or by the simultaneous presence of the peptide and the growing oligonucleotide. In any case, these data will be compared with further syntheses of similar chimeric PEG conjugates that are currently under investigation.

The final product was studied by NMR spectroscopy and mass spectrometry. As seen in Figure 3, only some of the signals can be identified, but a careful investigation of the integrated values allowed us to confirm the successful synthesis of the tetranucleotide. Instrumental limitations, however, did not allow for mass increment evaluation of the final product by ESI/MS.

Final Deprotection

A crucial point of the procedure was the final deprotection of the oligonucleotide, since this usually requires harsh conditions which are not fully compatible with presence of peptide bonds. As previously reported,[19] a substantial amount of product was lost using the standard conditions, i.e. a concentrated aqueous NH_3 solution, overnight, at 55 °C. Hence, we decided to apply a procedure that was successfully proposed for similar peptide-oligonucleotide conjugates.[27] The right conditions were determined from a simple PEG-supported protected nucleotide, namely benzoylated adenine, bearing at the other extremity a Z-Phe residue bonded through the 1,2-amino linker. From a series of experiments, we optimized the deblocking conditions at 55° C and at room temperature. Thanks to the NMR transparency of PEG, and to its solubility, the removal of the benzoyl group and the stability of the linkage between the polymer and the amino acid were easily monitored. After 1 hour at room temperature or 30 minutes at 55 °C, the deprotection of adenine was complete, while the linked amino acid was entirely unaffected. Therefore, the chimeric oligonucleotide-PEG-peptide was subjected to the final deprotection at room temperature in order to operate under the mildest possible conditions. The result is clearly shown in Figure 4, where the two NMR spectra of the fully protected (A) and nucleobase- and phosphate-deprotected conjugates

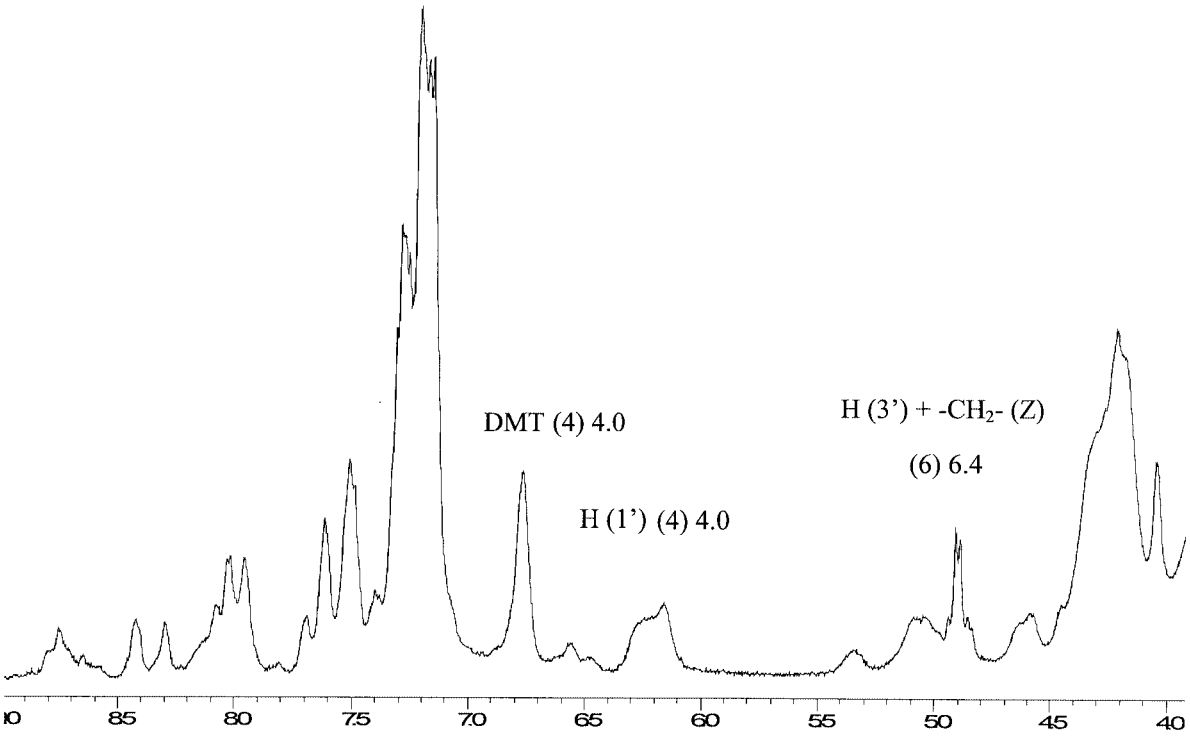


Figure 3. Partial ^{1}H NMR spectrum of the DMT-tetranucleotide-PEG-tripeptide in [D_6]DMSO

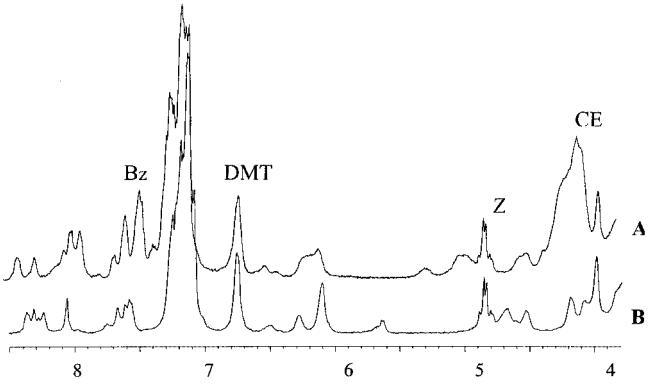


Figure 4. ^{1}H NMR spectra of the final chimeric conjugate with the oligonucleotide component fully protected (A) and deprotected (B)

(B) are properly aligned, confirming the efficacy of our procedure.

A further characterization of fully protected and deprotected products was obtained from a MALDI analysis. As indicated in Figure 5, the two samples showed good agreement between the calculated and the measured masses, within the limitation of the PEG polydispersity. Furthermore, the difference found between compounds A and B (about 500 daltons) was in agreement with the mass change corresponding to the removal of the benzoyl, isobutyryl and cyanoethyl groups (491 daltons).

Final analysis of the product was performed by IE HPLC of the crude conjugate. As shown in Figure 6 (curve A), a main peak due to the expected product was observed. The late running peak was assigned to the final product due to its chromatographic properties. Removal of the DMT group shifts the main peak to a shorter elution time (curve B) superimposable on the lower, early running peak observed in the previous analysis. This peak may be due to partial removal of DMT unit in the crude, terminally protected compound, and not to an impurity, since all the shorter and deleted sequences were capped before.

Removal of the DMT terminal protecting group was eventually carried out following the standard procedure, namely glacial acetic acid and water (4:1), without any problem.

The Z group was not removed since it is needed for the activity of the tripeptide as cellular fusion inhibitor.[24] However, as an example, the elimination of this group was achieved by hydrogenation on Pd/C (10% by weight) in MeOH.

In conclusion, this study has demonstrated that it is possible to plan a sequential liquid-phase synthesis of a peptide-oligonucleotide conjugate on the same high-molecular-weight PEG. Of course, this procedure, based on the use of a pure, selectively protected polymer allows the preparation of PEG-peptides and PEG-oligonucleotides carrying any further active molecule useful to enhance their pharmacological properties. Further investigations will be required to extend this approach to the synthesis of oligonucleotide-peptide-containing trifunctional amino acids, looking for the best solutions for the protection of the side chains, as previously discussed for a solid-phase-based approach.[14]

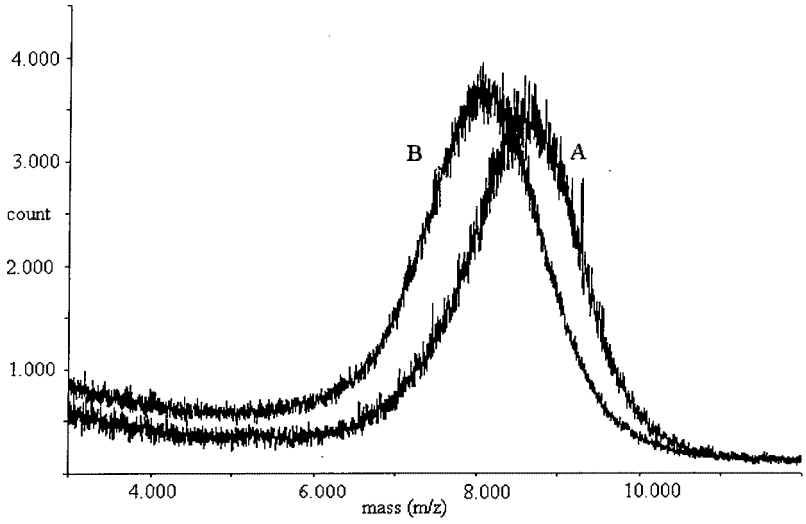


Figure 5. MALDI spectrum of the tetranucleotide-PEG-tripeptide protected (A) and deprotected (B)

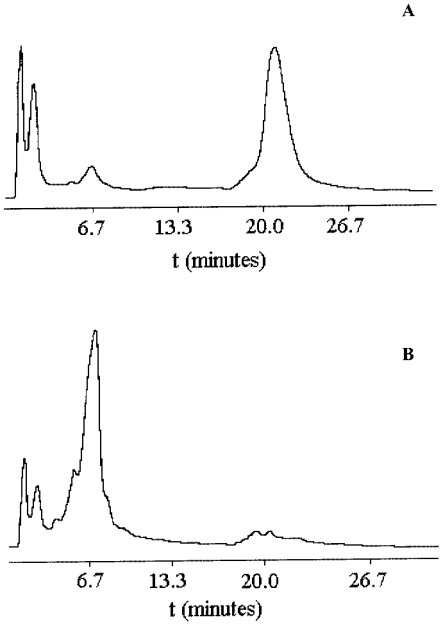


Figure 6. IE HPLC of the tetranucleotide-PEG-peptide DMT-on (A) and DMT-off (B)

Moreover, the reverse order of the synthesis of the two components on the polymeric support will be explored.

Experimental Section

Abbreviations: ACN = acetonitrile; Bz = benzoyl; CE = cyanoethyl; DCE = dichloroethane; DMAP = 4-dimethylaminopyridine; DMT = 4,4′-dimethoxytrityl; EDA = ethylendiamine; EDC = *N*-(3-dimethylaminopropyl)-*N*-ethyl carbodiimide; Fmoc = 9-fluorenyl-metoxycarbonyl; HOBT = *N*-hydroxybenzotriazole; *i*Bu = isobutyl, MTBE = methyl *tert*-butyl ether; NMI = *N*-methylimidazole; TBHP = *tert*-butyl hydroperoxide; TNBS = trinitrobenzenesulfonic acid; Z = benzyloxycarbonyl.

Materials and Methods: NMR spectra were recorded on a JEOL 400 MHz spectrometer using TMS as internal standard. ESI-MS spectra were obtained on a PE-API 1 spectrometer by infusion of a solution of the polymeric sample into 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). MALDI-TOF mass spectra were recorded on a Voyager DE mass spectrometer (Perseptive Biosystems, Framingham, MA, USA) equipped with an N_2 laser (337 nm). Maldi conditions are as follow: accelerating voltage: 24000V; guide wire: 0.05% of accelerating voltage; grid voltage: 94% of accelerating voltage; delay extraction time: 550 ns and 100 scans averaged in negative mode. Samples were mixed with 2,5-dihydroxybenzoic acid (DHB) as a matrix. Amino acid analyses were performed on a Carlo Erba amino acid analyser 3A30, equipped with a cationic AminoSEP Beckman 0.4 × 20 cm column, in a sodium citrate buffer. PEG, common reagents and anhydrous solvents were purchased from Fluka, Buchs (Switzerland). Phosphoramidites and 1H-tetrazole were obtained from Pharmacia, Uppsala (Sweden). Protected amino acids were obtained from Chem-Impex Intl., Wood Dale (USA).

Synthesis of DMT-PEG-1,2linker-NH_2: The monoprotected DMT-PEG-OH (M.W. = 6000 Da) was obtained following a published procedure.[22]

Synthesis of DMT-PEG-*p*NO_2phenyl Carbonate: DMT-PEG-OH (1.00 g, 0.133 mmol of free OH groups), was dehydrated by a double co-evaporation with anhydrous CH_2Cl_2. The polymer was dissolved in anhydrous CH_2Cl_2 (5 mL) and 2 equivalents each of TEA and *p*NO_2-phenylchloroformate, were added whilst stirring. A pH of 8 was maintained over a 4 hour addition of TEA. The reaction was stopped by precipitating the PEG derivative by a slow addition of MTBE, in an ice bath. The product was filtered and washed with diethyl ether and *i*PrOH. Recrystallization was achieved from EtOH and the product dried under vacuum over KOH pellets. Removal of any excess reagents was confirmed by TLC on Silica Gel 60 F_{254}, eluting with a 9:1 mixture of $CHCl_3$ and EtOH. The degree of modification was evaluated by ^1H NMR spectroscopy and was found to be nearly quantitative, with a DMT/*p*NO_2Ph ratio of 0.96.

Synthesis of DMT-PEG-1,2-linker-NH_2: DMT-PEG-*p*NO_2phenyl carbonate (1.00 g) was dehydrated by co-evaporation with anhydrous CH_2Cl_2. The polymer was dissolved in CH_2Cl_2 (3 mL) to which three equivalents of 1,2-diaminopropane had been added. The mixture was left to react under an argon atmosphere for two

hours, whilst stirring at room temperature. The PEG derivative was precipitated by slow addition of MTBE in an ice bath, filtered, washed with *i*PrOH and diethyl ether, recrystallized from EtOH and dried under vacuum over KOH pellets. The degree of functionalization was estimated by TNBS analysis of the free NH_2 group, and compared with the DMT, as measured from its absorbance at 498 nm. A DMT/1,2-linker-NH_2 ratio of 0.93 was observed and confirmed by NMR spectroscopy.

An almost quantitative recovery of the starting amount of DMT-PEG-OH was observed after the overall synthetic process.

Synthesis of DMT-PEG-tripeptide-Z

Synthesis of DMT-PEG-Gly-NH_2: DMT-PEG-1,2-linker-NH_2 (0.5g) was dehydrated by co-evaporation with CH_2Cl_2, and redissolved in anhydrous CH_2Cl_2 (6 mL). In a separate vessel a threefold excess (with respect to the total amount of free amino groups) of Fmoc-Gly-OH was dissolved in CH_2Cl_2 (3 mL), and an equivalent amount of HOBT, EDC and TEA was added, whilst stirring in an ice bath. This solution was added to the PEG solution and left stirring overnight. Upon completion the product was filtered and washed with diethyl ether and *i*PrOH. This product was recrystallized from EtOH and dried under vacuum over KOH pellets. The reaction yield was determined by the TNBS test, i.e. the amount of free amino groups still present. Only traces of free amino groups were observed, as confirmed by the NMR spectrum, indicating an almost quantitative coupling.

Removal of the Fmoc N-protecting group was achieved by dissolving the PEG derivative in piperidine solution (20 mL of a 20% solution in DMF). After stirring for one hour the product was recovered and purified as in the previous step. A new TNBS test gave a DMT/NH_2 ratio of 1.03, confirming the complete removal of the Fmoc group and the quantitative yield of the previous condensation. Integration of the appropriate NMR signals confirmed this value. Yield: 0.49 g (98%).

Synthesis of DMT-PEG-Gly-L-Phe-NH_2: The previously synthesized DMT-PEG-Gly-NH_2 was coupled with a threefold excess of Fmoc-L-Phe-OH under the same conditions. Less than 2% of the free amino group was observed in the final purified derivative after Fmoc deprotection. Yield: 0.48 g (98%) with the correct ratio of DMT and free amino group.

Synthesis of DMT-PEG-Gly-L-Phe-D-Phe-Z: The previous DMT-PEG-dipeptide was reacted under identical conditions with a threefold excess of Z-D-Phe-OH. No measurable amount of free NH_2 group was found by the TNBS test. The NMR spectrum supported the efficacy of the reaction giving a DMT/Z ratio of about 0.95, as judged from the integral values. Yield of DMT-PEG-tripeptide: 0.48g (99%).

Synthesis of DMT-tetranucleotide-PEG-peptide-Z

Synthesis of DMT(5′)-dT-(3′)-PEG-tripeptide-Z: DMT-PEG-tripeptide-Z (0.45 g) was dissolved in DCE (5 mL) and a 6% (w/v) solution of TCA in DCE (5 mL) was slowly added. The reaction was left at room temperature whilst stirring for 30 min. The product was precipitated with TBME, filtered and washed with diethyl ether. The OH-PEG-tripeptide was twice coevaporated from anhydrous ACN. The residue was dissolved in anhydrous ACN (1 mL) under argon atmosphere in a three-necked vessel and a 3.5-fold excess of dT-phosphoramidite (0.2 M) in ACN and a 14-fold excess of 1(H)-tetrazole (0.45 M) in ACN were injected through the self-sealing septum. After reacting for 5 min the flask was placed in an ice bath and the product was precipitated with TBME (10 times the final volume of the solution), filtered, washed with diethyl ether and recrystallized from EtOH. Completion of the reaction was checked from the DMT absorbance at 498 nm.[26] (If incomplete the sample must be reacted again under the same conditions.) The product was then capped by dissolving it in ACN (5 mL) and adding, in this order, 2,6-lutidine (0.2 mL), *N*-methyl imidazole (0.2 mL) and acetic anhydride (0.2 mL). After 5 min the product was precipitated with TBME, filtered and washed with diethyl ether. The last step of the single nucleotide addition required the oxidation of the phosphite triester obtained by dissolving the capped PEG derivative in ACN (5 mL), and addition of TBHP (0.3 mL) whilst stirring at 0 °C. After 15 min, the product (0.39g, 87% of expected amount) was precipitated with TBME, filtered and washed with diethyl ether, with almost 100% of DMT groups present. The product was confirmed by comparing some significant protons of the terminal DMT (4 protons near the $pOCH_3$: δ = 6.79 ppm) and Z (2 protons of benzylic CH_2: δ = 4.81 ppm) protecting groups of the PEG-conjugate; a ratio very close to 1:1 was measured, within the experimental error.

Synthesis of DMT(5′)-dA(Bz)-dT-(3′)-PEG-tripeptide-Z: The second nucleotide was added following the previous procedure, using a fourfold excess of dA(Bz) phosphoramidite and a 16-fold excess of 1(H)-tetrazole with respect to the evaluated amount of free OH groups [45 μmol of HO-dT-PEG-tripeptide-Z (310mg)]. A 96% yield of DMT was obtained of the final purified conjugate (0.33g, 85% of recovered material).

Synthesis of DMT(5′)-dC(Bz)-dA(Bz)-dT-(3′)-PEG-tripeptide-Z: The same procedure was repeated for the addition of dC(Bz). A 95% DMT yield was obtained of the final purified conjugate (0.30g, 90% of recovered material).

Synthesis of DMT(5′)-dG(iBu)-dC(Bz)-dA(Bz)-dT-(3′)-PEG-tripeptide-Z: The same procedure was repeated for the addition of dC(Bz). A 95% DMT yield was obtained of the final purified conjugate (0.27g, 90% of the recovered material).

Deprotection and Purification

Deprotection of the Oligonucleotide Chain: For fully protected oligonucleotide-PEG-peptide (100mg), a 1:1 (v/v) solution (3 mL) of EDA and EtOH was employed. The reaction was left for 1 hour at room temperature. Solvent was removed under vacuum and the residue precipitated with TBME, filtered and washed with diethyl ether.

Deprotection of Terminal Protecting Groups: The DMT group was removed by treatment with a 1:1 solution of glacial acetic acid and H_2O (25 mL for 100 mg) for 30 min. The aqueous solution was then extracted three times with diethyl ether, the solvents evaporated under vacuum, the residue redissolved in water, the solvents evaporated again, the reside once again redissolved in water and finally lyophilised. The Z group was hydrogenated in a MeOH solution (15 mL for 100 mg) containing Pd/carbon as catalyst (10% of conjugate by weight). The resulting mixture was left under H_2 atmosphere overnight. This solution was then filtered through celite and the solvent removed under vacuum. The product was precipitated with TBME (10 mL for 100 mg), filtered and finally washed with diethyl ether.

Acknowledgments

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