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Automated Incorporation of Polyethylene Glycol into Synthetic Oligonucleotides

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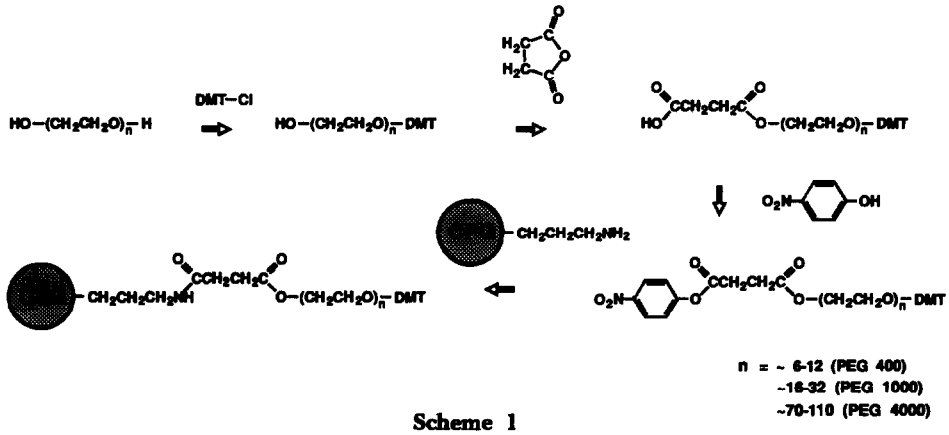
Abstract: (4,4´-Dimethoxytrityl)-polyethylene glycol-(2-cyanoethyl-N,N-diisopropyl)-phosphoramidites and (4,4´-dimethoxytrityl)-polyethylene glycol derivatized glass supports were synthesized and used to introduce PEG with polymerization degrees from about 6 to more than 100 at 3´- and 5´-ends of oligonucleotides by means of automated synthesis. The electrophoretic mobility of the conjugates is altered with respect to the unmodified oligonucleotides. The hydrophobicity of the conjugates increases with increasing length of the PEG chain.

Chemically modified oligodeoxyribonucleotides have found numerous applications for structural investigations of nucleic acids, for the exploration of reaction mechanisms and for the inhibition of cellular functions by antisense oligonucleotides[1]. Besides modification of single bases, sugars or phosphates, for *in vivo*-applications of antisense-oligonucleotides the attention was drawn on the coupling of oligonucleotides to hydrophobic residues or polymers[2]. Thus, the coupling has been described for poly-L-lysine[3], cholesterol[4] and polyamide[5]. Polyethylene glycol (PEG) is known to play an important part in cell fusions and in transport processes through cellular membranes[6]. In addition, PEG is used in protein chemistry for stabilization and shielding of proteins[7]. In nucleic acid chemistry, PEG has been used as a liquid phase for large scale synthesis of oligodeoxyribonucleotides[10]. Oligoethylene glycols have been used as linkers for membrane and glass bound oligonucleotides for hybridisation experiments[11,12]; the incorporation of hexaethylene glycol as a hairpin loop inside an oligonucleotide has been reported[13]. To investigate the influence of covalent coupling of PEG to oligonucleotides on their stability and permeation through cellular membranes, we have developed appropriate synthetic methods. We report here the preparation of synthons for the rapid solid phase synthesis of modified oligodeoxyribonucleotides that carry PEG at their 3´- or 5´- terminal positions.

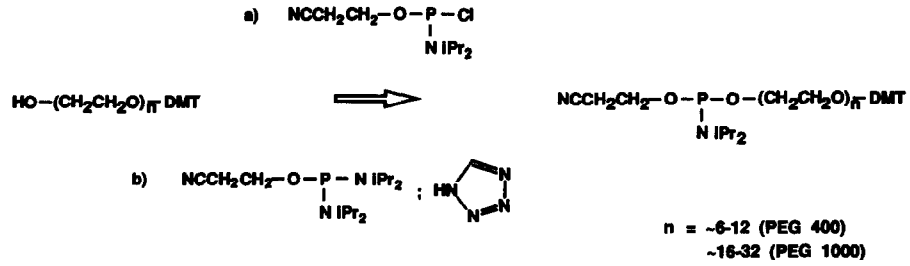
For the synthesis of 3´-PEG-derivatized oligonucleotides, we coupled protected PEG 400, PEG 1000 and PEG 4000 via succinylation and ester activation to an aminopropylated glass support (CPG = Controlled Pore Glass ; Scheme 1). Reaction conditions were adopted from standard protocols[8]; reactions were monitored by TLC. The reaction of 4,4´-dimethoxytrityl chloride (DMT-Cl) with PEG (10mmol = 4g for PEG 400; 10 mmol = 10g for PEG 1000 and 5 mmol = 20g for PEG 4000) in pyridine (1.1 mmol DMT - Cl / mmol PEG) yielded mixtures of the respective PEG, DMT-PEG and Di-DMT-PEG, that were separated after extraction by flash column chromatography on silica gel F 60 using chloroform, 98/2 chloroform / methanol and 9/1 chloroform/methanol (yields of DMT-PEG were 38, 30 and 34 %, for PEG 400, 1000 and 4000, respectively). The DMT-PEGs (1 mmol) were treated with succinic anhydride (1 equivalent) in pyridine containing 4-dimethylaminopyridine (0.5 equiv.). The DMT-PEG-succinates were isolated in yields



Scheme 1

of 71, 58 and 60%, respectively. The activated esters were synthesized by coupling p-nitrophenol (1 equiv.) to the respective DMT-PEG succinate using dicyclohexylcarbodiimide. The product was treated with aminopropylated glass support for 48 hours. After capping of the excess amino groups with acetic anhydride in dry pyridine, the support could be used for automated synthesis. The extent of loading of the supports was determined by the DMT cation release[8] to be 30, 8 and 10 μmol/g support.

For preparation of 5'-derivatized oligonucleotides we synthesized the DMT-PEG-(2-cyanoethyl-N,N-diisopropyl)-phosphoramidites by two different approaches (Scheme 2): i) by reaction of DMT-PEG with (2-cyanoethyl-N,N-diisopropyl)-chlorophosphoramidite and diisopropylethylamine[8] (only PEG 400) and ii) by reaction of DMT-PEG with (2-cyanoethyl-N,N,N',N'-tetraisopropyl)-phosphordiamidite and tetrazole[9] (PEG 400 and PEG 1000). The respective PEG 4000-phosphoramidite was not synthesized due to the expected high viscosity of the 0.15 M solution in acetonitrile required for oligonucleotide synthesis. Products were dried after washing with saturated NaCl and 10% $NaCO_3$ solutions, respectively. Yields were about 55%. The low yields compared to standard nucleosides throughout the preparation of synthons are likely caused by the high water solubility of PEG, leading to losses during the washing steps.



Scheme 2

Oligodeoxyribonucleotides were synthesized on a fully automated synthesizer (Applied Biosystems synthesizer 394) by phosphoramidite chemistry. After the last cycle oligonucleotides were decoupled "DMT-On" and purified by Reversed-Phase HPLC. The collected product peaks were lyophilized, the DMT groups were removed by acetic acid treatment and the products were finally purified and characterized by a second Reversed-Phase HPLC.

Table 1 gives a compilation of synthesized short oligonucleotides with PEGs of variing chain length at different positions. All oligonucleotides were purified and characterized. PEG coupled oligonucleotides were stable under the conditions used during synthesis, final deprotection and workup. 3'-Modification using the

derivatized supports worked as expected. No unmodified byproducts could be detected for PEG 400. With PEG 1000 and 4000, some impurities were found that could be removed by HPLC. The coupling to the PEG chains did not influence following synthesis steps; coupling efficiencies were always around 99 %. The coupling efficiency of the DMT-PEG phosphoramidites was 70 and 50 % for PEG 400 and 1000, respectively, as determined by DMT cation release and confirmed by integration of HPLC peak areas. Figure 1A shows the chromatograms of purified, 3'-PEG-modified oligonucleotides. Elution time increases with increasing chain length of the PEG. Since the PEGs are mixtures of different polymerization degree, various peaks appear in the chromatograms, each of them reflecting one discrete molecular size of coupled PEG. Thus, the hydrophobicity of the conjugate can be changed gradually over a wide range using PEG with an appropriate molecular weight distribution.

Table 1. List of Synthesized Oligonucleotides

Nr.	Sequence (5´---3´)
1	TTCGA
2	TTCGA - PEG 400
3	TTCGA - PEG 1000
4	TTCGA - PEG 4000
5	PEG 400 - TTCGA
6	PEG 1000 - TTCGA
7	PEG 400 - TTCGA - PEG 400
8	PEG 1000 - TTCGA - PEG 1000

The modification with PEG leads to an altered electrophoretic mobility (Fig. 2). Different peak fractions from the chromatograms in Figure 1A were collected and phosphorylated by T4-polynucleotide kinase and γ-^{32}P-ATP. The autoradiogram shows that the electrophoretic behavior correlates with the chromatographic behavior in RP chromatography; i. e. the electrophoretic mobility of oligonucleotides is reduced upon increasing length of coupled PEG.

The elution times of 5´-PEG-coupled oligonucleotides differ only slightly from those of the respective 3´-conjugates (Fig. 1). In the chromatograms of the oligonucleotide that carries PEG 400 at both ends more peaks appear due to the permutation of the molecular weight distribution (Fig. 1B). However, compared to oligonucleotides modified at one terminus, the peaks are shifted toward later elution times.

Since the described strategy is compatible to standard solid phase synthesis of oligonucleotides, it may be combined with other modification methods, such as incorporating thiophosphates. Moreover, the procedure is applicable to the synthesis of PEG derivatized ribooligonucleotides (data not shown).

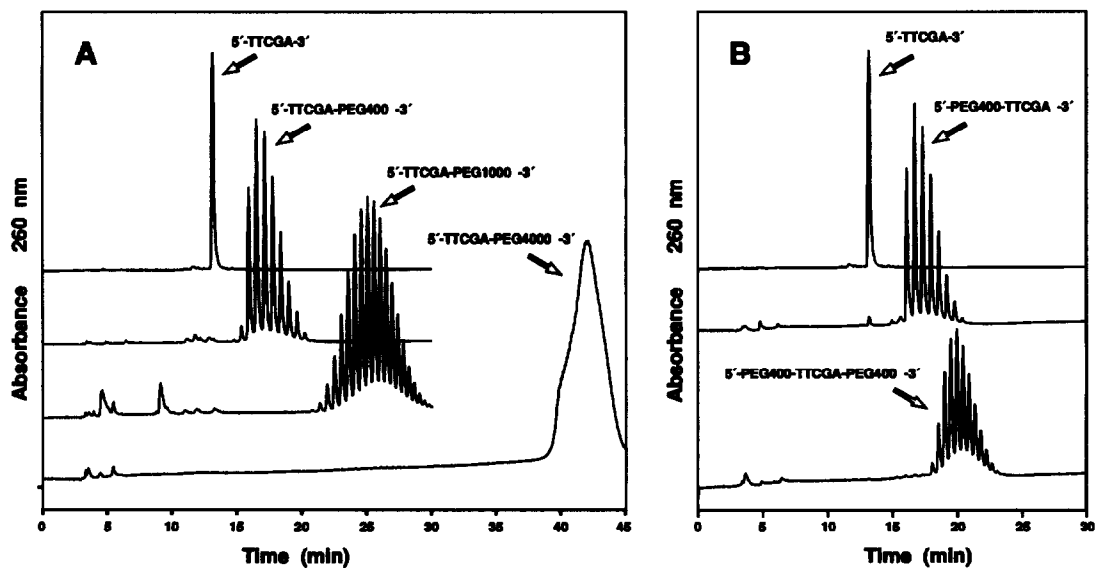


Figure 1. HPLC chromatograms of oligonucleotides

20 μl sample volumes were separated on a C_4 reversed phase column (Nucleosil 300/5) in a gradient from 0.8 to 32.0 % (0.8 to 47.6 %) acetonitrile in 100 mM triethylammonium acetate , pH 7.0 in 30 min (45 min) .

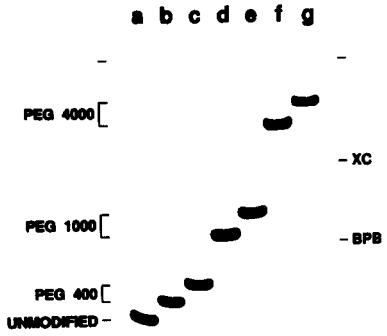


Figure 2. PAGE of ^{32}P-labelled 3´-PEG-Oligonucleotide-conjugates
Peak fractions from the chromatograms in Fig. 1A were collected and phosphorylated by using T4 polynucleotide kinase and γ-^{32}P-ATP. 20 μl sample volumes were loaded onto a native 20 % polyacrylamide gel. Lane a, unmodified oligo; lanes b and c, PEG400-conjugate (elution times at 15.9 and 17.7 min); lanes d and e, PEG1000-conjugate (elution times at 23.4 and 26.4 min); lanes f and g: PEG4000-conjugate (elution times at 38.5 and 42.0 min). XC = xylene cyanol, BPB= bromophenol blue.

Furthermore, the DMT-protected hydroxyl group of the PEG phosphoramidite allows chain elongation after PEG incorporation and therefore permits the synthesis of oligonucleotides carrying PEG at internal positions (data not shown).

One of the major limitations for *in vivo* applications of oligonucleotides is the low permeability of cell membranes to large polyanionic molecules. Cellular uptake of oligonucleotides has been improved by the attachment of terminal hydrophobic groups, e. g. cholesterol and polyalkyl groups[4,14], poly-L-lysine[3] or the 1,2-di-O-hexadecyl-3-glyceryl group[15]. However, it is conceivable that modified oligonucleotides with reduced water solubility might be trapped within the cellular membrane. The automated coupling of PEG allows to specifically adjust the hydrophobicity of a given oligonucleotide. Thus, the influence of hydrophobicity on cellular uptake can now systematically be evaluated. First experiments indicate a significant increase in cellular uptake of PEG-modified oligonucleotides (data not shown). In addition, the terminal blocking of oligonucleotides with PEG could lead to an increased exonuclease stability. Further experiments are under way to test these possibilities.

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