Synthesis of 3'-PEG-Modified Oligonucleotides on PS-PEG Tentacle Polymers
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PS-PEG tentacle polymers are modified for the synthesis of oligonucleotides covalently bound to polyethylene glycol (PEG) at their 3'-position by the solid phase method without additional steps. The average stepwise coupling rates and overall yields are in the range of standard oligonucleotide synthesis. Oligonucleotides and phosphorothioates up to 20 nucleotides, modified with hexaethylene glycol (HEG), PEG$_{400}$ and PEG$_{1500}$, are synthesized in this manner. The products are characterized by capillary electrophoresis and electrospray mass spectrometry (ES-MS). These new methods are very efficient for purity control as it is required for potential antisense drugs.

**Introduction**

The increasing interest in oligonucleotide chemistry is mainly caused by the development of the ‘antisense’-technology. This requires chemically modified oligonucleotides to control the expression of genes by means of hybridization of complementary parts of either DNA or mRNA strands with synthetic oligonucleotides. The specificity of hybridization and inhibition respectively is based on Watson-Crick base pairing between the nucleobases of the antisense oligonucleotide and the target region of mRNA or DNA [1, 2].

A basic problem among others is the cellular uptake of the chemically modified oligonucleotides. The cell membrane acts as a natural barrier to large negatively charged molecules. The mechanism and extend of penetration seems to be dependent on the type of oligonucleotide. Active transport takes place in case of natural DNA fragments and several modified oligonucleotides [3, 4]. In order to achieve a maximum effect, the reagents should pass the plasma membranes quickly and in sufficient amounts.

Different efforts have been made to improve the cellular uptake of antisense oligonucleotides [5]. Poly-L-lysine was found to have a stimulating effect on the penetration of oligoribonucleotides into tumor cells [6], but it also shows cytotoxic effects at concentrations of more than 2 µmole [7].

Oligonucleotides with lipophilic modifications have been incorporated in liposomes as carriers through the plasma membrane [8, 9]. Another possibility is the modification with polyethylene glycol [10–12]. PEG has low toxicity and is extremely biocompatible [13]. Based on the successful use of PEG-peptides for in vivo and in vitro formation of antibodies [14], PEG-modifications can be supposed to have a positive influence on transport through cell membranes. Modification in 3'-position should increase stability against exonuclease attack.

In the present work we describe the design of new resins for direct solid phase synthesis of 3'-PEG-modified oligodeoxyribonucleotides on the basis of polystyrene (PS) and polystyrene-polyethylene glycol (PS-PEG) tentacle polymers, and the synthesis of 3'-PEG-modified oligonucleotides as well as phosphorothioates. Due to the high loadings of PS-PEG supports in contrast to commonly used controlled pore glass (CPG) [11] the resins can easily be functionalized even with high-molecular PEG. The resulting loadings of the modified supports are in the range of 60–130 µmol/g, thus these new resins are well suited for oligonucleotide synthesis. 3'-PEG-modified oligonucleotides and phosphorothioates up to a length of 20 nucleotides, which is the range required for the antisense approach, have been synthesized automatically.

The introduction of polydisperse PEG chains requires efficient analytical methods for characterization and purity control of these modified oligonucleotides. Capillary electrophoresis and electrospray mass spectrometry (ES-MS) are very efficient for purity control as it is required for potential antisense drugs.

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spray mass spectrometry have been applied for characterization. The developed analytical methods are also of prime interest for other areas of oligonucleotide research.

**Materials and Methods**

High performance liquid chromatography was carried out with a solvent delivery system S 1000 and low pressure gradient mixer S 8110 obtained from Sykam (Gilching, Germany) and a UV/VIS spectrometer UVIS 204 from Linear (Reno, USA) using a Nucleosil C₁₈ reverse phase column purchased from Grom (Herrenberg, Germany). Capillary electrophoresis was performed with the Capillary electrophoresis system 100 with an uncoated fused silica capillary and on-line detection at 260 nm with a Linear UV/VIS detector. For ES-MS analyses an API III Taga 6000 E ionspray mass spectrometer obtained from Sciex (Toronto, Canada) was used. UV/VIS spectra for DMT asays were recorded on a Perkin-Elmer UV spectrometer Lambda 5.

Chloromethylated polystyrene (2% cross-linked with divinylbenzene) was obtained from Bio-Rad Laboratories (München, Germany) and amino-functionalized [15]. PS-PEG copolymers (TentaGel S, TentaGel N) were obtained from Rapp Polymere (Tübingen, Germany). 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) was purchased from Novabiochem (Bad Soden, Germany), 4-methylmorpholine (NMM), 4-dimethylamino pyridine (DMAP), polyethylene glycol (MG = 400, 1500) and hexaethylene glycol were purchased from Fluka (Buchs, Switzerland). PEG₄₀₀ was purified by crystallization from methylene chloride/diethyl ether and dried under vacuum. PEG₃₀₀₀ and HEG were dried by coevaporation with dry pyridine. Dry solvents were stored over molecular sieves.

**General procedure for preparation of PEG-modified solid supports**

PS-PEG₃₀₀₀-NH-Suc-O-PEG₁₅₀₀-ODMT (2): 10 g (6.6 mmol) PEG₁₅₀₀ were coevaporated twice with anhydrous pyridine, dissolved in 25 ml pyridine and 2.5 g (7.4 mmol) dimethoxytrityl chloride (DMT-Cl) in 25 ml pyridine were added dropwise. After stirring at room temperature for 12 h the mixture was evaporated to dryness, redissolved in 50 ml CH₂Cl₂, washed twice with 10% NaHCO₃ and once with saturated NaCl solution and dried over Na₂SO₄. The DMT-PEG₁₅₀₀ product was separated by liquid chromatography on silica gel F₆₀ using CH₂Cl₂/methanol/triethylamine (93:5:2). DMT-PEG₁₅₀₀ was dissolved in 30 ml CH₂Cl₂/pyridine (5:1), 1.2 g (12 mmol) succinic anhydride and 0.37 g (3 mmol) DMAP and redissolved in 5 ml DMF were added and the mixture was stirred overnight. The solvents were evaporated, the residue redissolved in CH₂Cl₂ and thoroughly washed with 10% NaHCO₃ and saturated NaCl solution to separate from unreacted succinic anhydride. The organic solution was dried over Na₂SO₄ and evaporated.

3.2 g (1.68 mmol) DMT-PEG₁₅₀₀-succinate were dissolved in 10 ml CH₂Cl₂ and coupled on 0.7 g PS-PEG resin (loading 240 μmol/g) in 3 ml DMF by adding 0.11 g (0.37 mmol) TBTU and 75 mg (0.74 mmol) NMM and shaking for 24 h. The PEG-modified resin was filtered, washed three times with each DMF, CH₂Cl₂ and methanol and dried under vacuum. Resins 1, 3 and 4 were prepared by analogous procedure.

**Oligonucleotide synthesis:**

Phosphorothioate and standard oligodeoxyribonucleotides were synthesized on Applied Biosystems DNA Synthesizer 380B and DNA/RNA Synthesizer 394 respectively using the phosphoramidite chemistry. Tetraethylthiuram disulfide obtained from Applied Biosystems (Weiterstadt, Germany) was used as a sulfurization reagent. All other chemicals for solid phase synthesis were purchased from Applied Biosystems, MWG Biotech (Ebersberg, Germany) and Roth (Karlsruhe, Germany). Before synthesis unreacted amino groups of the support were capped with a slightly prolonged capping step using standard capping reagents. For all further steps modified protocols adapted for the synthesis on PS-PEG tentacle polymers were used [16]. The cleavage of the succinyl group was found to be incomplete under standard conditions (1 h, 25% NH₃) and for this reason has to be carried out manually within the deprotection procedure of the bases (8–12 h, 55 °C, 25% NH₃).

**Results and Discussion**

**Synthesis of 3'-modified oligonucleotides**

To prepare oligonucleotides with hexamethylene glycol or polyethylene glycol residues bound as phosphate esters at their 3'-terminus, a suitable resin had to be constructed first. The commonly used succinyl group was selected as an anchor between the PEG side chain and the insoluble ma-
The synthesis of 3’-PEG oligonucleotides was achieved analogous to the standard phosphoramidite method [23–25]. The first nucleotide of the desired sequence is coupled as a phosphoramidite on the deprotected hydroxyl group of the PEG chain during automated synthesis (Fig. 1), in contrast to the usual method where a succinyl nucleoside is coupled to the aminofunctionalized resin manually.

Table I gives a summary of the different resins designed for synthesis of the 3’-PEG modified oligonucleotides presented in this report. The loading of the modified resins was determined by DMT assays.

Several oligonucleotides have been synthesized, covalently attached to PEG chains of different lengths at their 3’-terminus (Table II). The average stepwise yields and the overall yields of the syntheses, calculated from DMT assay, HPLC and/or capillary electrophoresis, were comparable to those of standard oligonucleotide synthesis, if the PS-PEG copolymers were used as basic supports. In case of the polystyrene support (4) the coupling rates were insufficient for solid phase synthesis. This is in general agreement with the reported advantages of PS-PEG polymers over PS polymers in automated solid phase synthesis of peptides and nucleotides.

![Fig. 1. Structure of the modified resins for the synthesis of 3’-PEG oligonucleotides. Basic supports: a) polystyrene; b) PS-PEG copolymer. PEG chains: (1) PEG400; (2) PEG1500; (3) HEG.](image-url)
Analytical results

Analyses of the products with capillary electrophoresis show the expected gaussian-distribution of PEG\(_{400}\) and PEG\(_{1500}\)-modified oligonucleotides. Fig. 2 depicts the separations of crude (AA TAT)-PEG\(_{400}\) on PS-PEG based resin 1 and PS-based resin 4 by micellar electrokinetic capillary chromatography (MECC). The relatively pure oligonucleotide 5a synthesized with resin 1 shows mainly one gaussian profile set of signals caused by the molecular mass distribution of PEG from 3 to 14 ethylene oxide units (Fig. 2a). The oligonucleotide 5b synthesized with relatively poor yields on resin 4 (Fig. 2b) shows at least two sets of signals representing failure sequences, which are additionally superposed.

The same separation effect appears in Fig. 3a. The MECC electropherogram of crude 6 shows a homogenous gaussian profile of the PEG chains from 25 to 45 ethylene oxide units. However slight variations of the buffer composition result in a separation only dominated by the oligonucleotide part of the molecule (Fig. 3b). In this case the failure sequences can be distinguished, which is more difficult if the separation is dominated by the PEG moiety.

The soft ionization conditions of electrospray mass spectrometry open new possibilities for the investigation of large and labile biomolecules, es-

![Fig. 2. MECC separation of the crude oligomers: a) d(5'-AA TAT-3')-PEG\(_{400}\) (5a), synthesis on PS-PEG-copolymer based resin; b) d(5'-AA TAT-3')-PEG\(_{400}\) (5b) synthesis on polystyrene based resin. Capillary: fused silica, 50 mm I.D., 0.7 m length; det.: 0.5 m (260 nm); buffer: 5 mmole phosphate, 10 mmole Na\(_2\)B\(_4\)O\(_7\), 50 mmole SDS, pH 6.3; cond.: a) 17.5 KV, 21 mA; b) 17 KV, 15 \(\mu\)A.](image)

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![Fig. 3. MECC separation of the crude d(5'-AA TAT-3')-PEG\(_{1500}\) oligomer: capillary: fused silica, 50 \(\mu\)m I.D., 0.7 m length; det.: 0.5 m (260 nm); a) buffer: 5 mmole Tris, 5 mmole Na\(_2\)B\(_4\)O\(_7\), 50 mmole SDS, 7 mole urea, pH 8.7 cond.: 15 KV, 15 mA, b) buffer: 30 mmole Na\(_2\)B\(_4\)O\(_7\), 10 mmole SDS, pH 9.3; cond.: 15 KV, 20 \(\mu\)A.](image)

![Fig. 4. IS-MS spectrum of the crude d(5'-AA TAT-3')-PEG\(_{400}\) oligomer.](image)
especially for peptides and oligonucleotides [26–29]. The spectra of crude PEG400-5mer (5a) and purified PEG1500-15mer (7) also show the distribution of the molecular masses mentioned above. As depicted in Fig. 4 and Fig. 5, the different negatively charged quasimolecular ions are splitted each due to the distribution of the molecular mass of PEG.

In case of 8 (20mer modified with monodisperse hexaethylene glycol) only one main product can be expected in both MECC and IS-MS analysis (Fig. 6). The peak series in the mass spectrum is caused by the different mass to charge ratios.

**Conclusion**

The direct synthesis of 3'-PEG-modified oligonucleotides and phosphorothioates is possible by a new resin based on PS-PEG graft copolymers. In comparison to polystyrene polymers better yields could be obtained. The resins are suitable for automated solid phase synthesis of oligonucleotides with phosphoramidite chemistry using special protocols. The high coupling rates are comparable to synthesis on CPG supports and allow the facile preparation of oligonucleotides up to a length of 20–30 nucleotides. However the higher, up to ten fold, loadings in comparison to CPG material [11] are interesting for large scale synthesis as it is desirable for the antisense approach. Among the PEG-modified phosphorothioates we also synthesized antisense oligonucleotides, which are active in inhibition of pancreatic cancer cell proliferation [30,31]. Capillary electrophoresis and electrospray mass spectrometry have been shown to be powerful methods for characterization and purity control of oligonucleotides.
[31] E. Bayer, H. Kalthoff, M. Maier, K. Bleicher, in progress.