Large scale, liquid phase synthesis of oligonucleotides by the phosphoramidite approach

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ABSTRACT
A new method for the liquid phase synthesis of oligonucleotides is described which makes use of polyethylene glycol (PEG) as soluble support and phosphoramidite derivatives as synths. The new synthetic protocol was applied to a quite large scale production (about 100 μmole) of such compounds up to the 20mer level. This solution method, called HELP (High Efficiency Liquid Phase) Plus, appears effective in terms of speed and coupling yield and can be evaluated for the production of large amount of oligonucleotides.

INTRODUCTION
Over the past years, the liquid-phase method has been proposed as alternative to the well known solid phase synthesis of oligonucleotides (1), but further applications were hampered by the lack of a convenient synthetic method (2–4). It utilizes as handle for the growing chain a polymeric support soluble in the reaction media; subsequently, the polymer is freed from unreacted reagents and soluble by-products by a diafiltration or a crystallization (5) process.

Since this technique avoids any heterogeneity due to the insolubility of support, the scaling up of the process can be reasonably foreseen. This aspect is nowadays very important, because synthetic oligonucleotides are moving out of the research laboratories and into practical biomedical applications (6). The clinical evaluation and, eventually, their commercialisation will demand a cost-effective production on a scale at least many thousandfold greater than currently available (7). On the other hand, the great potential of the new therapeutic methodologies based on oligonucleotide derivatives justifies any effort aimed at the development of innovative systems of production. Recently, in the light of the oligonucleotide chemistry evolution, we reinvestigated the liquid phase approach in order to realize an economical, large scale synthetic process. A new method, called HELP or High Efficiency Liquid Phase, that utilizes the polyethylene glycol (PEG) as soluble polymeric support and the phosphotriester chemistry for the coupling reaction was at first realized (8–10).

The present paper fully describes the application, disclosed in a preliminary report (11), of the well known phosphoramidite chemistry (12) to the PEG-supported synthesis. This new synthetic procedure, called HELP Plus, clearly improves the former HELP method, since it reduces the time and increases the yield of the overall process, allowing the synthesis of longer oligomers. This method represents, in our knowledge, the first efficient utilization of the phosphoramidite synths, so far widely employed in the solid phase methods, in a solution synthesis of the oligonucleotides.

MATERIALS AND METHODS
Polyethylene glycol monomethylether was purified by crystallization from methylene chloride/diethyl ether and dried under vacuum over KOH pellets. Anhydrous commercial acetonitrile (Pharmacia or Aldrich) was kept under argon over 4 Å molecular sieves. Diethyl ether was passed through a basic alumina column and stored over anhydrous sodium sulfate. All other solvents and reagents, of the highest commercially available purity, were used as such. Nucleoside phosphoramidites were obtained from Pharmacia or ABN; tetrazole was a Pharmacia product. PEG5000 monomethylether was purchased from Fluka and PEG12000 monomethylether was obtained from Union Carbide.

Amidites and tetrazole were dissolved in acetonitrile before using and kept over molecular sieves (amidites at −20°C). All glass equipments were oven-dried and stored in a dessicator over KOH pellets.

UV spectra were recorded on a Perkin Elmer Lambda 5. NMR spectra were recorded on a Bruker 400 AM spectrometer. HPLC analysis were carried out on a Pharmacia System Prep 10. Thin-layer chromatography (TLC) was performed on precoated silica gel sheets 60 F254 (Merck).

Functionalization of PEG
The reported procedure (8) has been marginally modified. In particular, each gram of PEG5000 (0.200 mmole of free OH groups) was dehydrated by coevaporation with anhydrous pyridine and dissolved in 10 ml of 1,2-dichloroethane (DCE). 0.6 mmoles of 5'-O-DMT-2'-deoxynucleoside-3'-O-succinate were dissolved in 5 ml of DCE and 0.33 mmoles of diacyc-hexylcarbodiimide (DCC) were added under stirring at 0°C. After 15 min the solution was filtered, poured into the PEG solution and added with 0.33 mmoles of 4-dimethylaminopyridine. The final solution was concentrated and left to react
under argon atmosphere for 24 hours, at room temperature. The solution was filtered and the modified polymer precipitated under stirring at 0°C with an excess of diethyl ether (about 10 volumes). The product was analyzed by TLC (eluant: ethyl acetate/acetone/water = 5/10/1); if higher migrating compounds are present, it was purified by crystallization from DCE/diethyl ether or from absolute ethanol (100 ml/g).

The unreacted OH groups of PEG were capped by reacting with 10 ml of an acetonitrile solution containing 10% of acetic anhydride, 10% of 2,6-lutidine and 10% of N-methylimidazole (v/v). The reaction mixture was kept at room temperature under stirring for 3 minutes. The polymer was precipitated from the ice-cooled solution with diethyl ether, filtered and dried under vacuum over KOH pellets.

The PEG12000 was derivatized by the same procedure.

**General procedure for oligonucleotide assembly**

All the reactions are performed in a three-necks flask. The two small side necks are permanently closed with a self-sealing septum; the larger neck is connected through a three-way stop cock with the argon reservoir.

Disposable plastic or oven-dried glass syringes rinsed with anhydrous acetonitrile were used.

**Dethritylation.** 1.0 g of 5'-DMT-nucleoside 3'-O-PEG was dissolved in 10 ml of DCE. 10 ml of a 6% solution of trichloroacetic acid (TCA) in DCE was added dropwise under stirring, at 0°C with purine nucleosides. After 15 min the polymer was precipitated with ether, washed and filtered. The extent of deblocking was evaluated as previously reported (8). The product was recrystallized from DCE/diethyl ether and dried under vacuum.

**Condensation.** 1.0 g of 5'-OH-nucleoside-3'-O-PEG was co-evaporated 3 times with a few ml of anhydrous acetonitrile and dried under high vacuum. Argon was flushed and the flask was closed to the outer atmosphere. Through the two septa were injected: 1 ml of acetonitrile to dissolve the PEG, 2.5 equivalents of amidite from a 0.1 M solution in acetonitrile and 10 equivalents of tetrazole from a 0.5 M solution in acetonitrile. The solution was stirred under argon atmosphere for 5 minutes. The polymer was precipitated from the ice-bath cooled solution with 10 volumes of ether and recrystallized form acetonitrile/ether. The yield of the reaction was evaluated spectrosocopically from the DMT absorption, after a TLC analysis to ascertain the complete removal of reagents and by-products.

**Capping.** The unreacted 5'-OH groups were acetylated as previously described (see functionalization of PEG).

**Oxidation.** 1.0 g of PEG-phosphite derivative was dissolved in 20 ml of acetonitrile and treated, at 0°C, under stirring, with 1.2 ml of 80% solution of tert-butylhydroperoxide (TBHP) in di-tert-butylperoxide for 15 minutes. The PEG-phosphate derivative was precipitated, filtered and recrystallized as after the condensation step.

**Deprotection and purification**

The final PEG-oligonucleotide was dissolved (25 ml for each gram) in a 30% solution of ammonia in water and the reaction mixture was allowed to stand at 60°C for 24 hours. After cooling, the aqueous solution was evaporated, the residue redissolved in 25 ml of water and extracted three times with 25 ml of ether. To remove the 5'-DMT group, the solution was evaporated to dryness and treated with 25 ml of 80% acetic acid for 30 minutes. After complete evaporation of the acetic acid, the residue was

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**Figure 1.** $^1$H and $^{31}$P NMR spectra of fully protected: A) DMT-d(TpT)-PEG phosphite and B) DMT-d(TpT)-PEG phosphate in CDCl$_3$-10 mg in 0.5 ml.
reddissolved in water and extracted with ether. The oligonucleotide was separated from PEG by a precipitation with an excess of acetone or methanol (where PEG is soluble) and following centrifugation (or filtration).

The final crude product was afterwards dissolved in the minimum amount of the desired chromatographic buffer and purified by low pressure ion-exchange chromatography on a DEAE-Sephadex column, as reported (8), or by a preparative reverse phase HPLC of the DMT-protected material (C18 column; buffer A: triethylammonium acetate [TEAAc] 0.1 M, pH 7.0; buffer B: TEAAc 0.1 M, pH 7.0, 80% acetonitrile; gradient: from 0 to 50% B in 30 minutes). The products were analyzed by reverse-phase HPLC (column: SuperPac Pep S C2/C18, 250×4 mm, 100 Å, 8 µm; eluent: TEAAc 0.1 M, pH 7.0; gradient: 0–40% acetonitrile in 40 min.) or by ion-exchange HPLC (column: PL5ax, 50×5 mm, 100 Å, 8 µm; buffer A: NaCl 0.1 M, pH 12.0; buffer B: NaCl 1.0 M, pH 12.0; gradient: 0–55% B in 30 min.

**Enzymatic analysis.** The oligonucleotide was treated with snake venom phosphodiesterase and bacterial alkaline phosphatase (13). The hidrolysate was analyzed by reverse-phase HPLC (column: C18 Delta Pak, 150×5 mm, 100 Å, 5 µm; eluent: TEAAc 0.1 M, pH 7.0; gradient: 0–25% acetonitrile in 30 min). The chromatogram was analyzed by comparison with that of a standard nucleoside mixture; from the integrated peak areas the content of each nucleoside was obtained.

**RESULTS AND DISCUSSION**

**Functionalization of PEG**

As previously reported (8), the procedure for the derivatization of PEG is very similar to that utilized in the solid phase technique. We found that a reduced amount of DMAP together with a shorter reaction time allows to obtain a cleaner product, easier to purify. Moreover, a new capping procedure has been introduced leading to a faster acetylation of the residual free OH groups. In the reported conditions, the use of N-methyl imidazole (NMI) as catalyst (14), instead of DMAP, avoids any substitution by acetyl groups at the N6 level of dA and at the N2 level of dG, as judged by 1H NMR investigations (data not shown).

The final loading value of nucleoside corresponds to an almost quantitative yield: 180 µmol/g for lower and 90 µmol/g for higher molecular weight PEG, respectively, were achieved.

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**Table 1. Chain elongation cycle**

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Quantity*</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Destritylation</td>
<td>3% TCA in CH2Cl2</td>
<td>70 ml</td>
<td>15 min</td>
</tr>
<tr>
<td>Condensation</td>
<td>Amidite 0.1 M in CH2CN</td>
<td>2.5 equiv.</td>
<td>5 min</td>
</tr>
<tr>
<td></td>
<td>Tetrazole 0.5 M in CH2CN</td>
<td>10.0 equiv.</td>
<td></td>
</tr>
<tr>
<td>Capping</td>
<td>Acetic anhydride in CH2CN</td>
<td>1 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2,6-lutidine in CH2CN</td>
<td>1 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NMI in CH2CN</td>
<td>1 ml</td>
<td></td>
</tr>
<tr>
<td>Oxidation</td>
<td>TBHP in CH2CN</td>
<td>1 ml</td>
<td></td>
</tr>
<tr>
<td></td>
<td>in CH2CN</td>
<td>20 ml</td>
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*Quantities are for 1.0 g of PEG1500-oligonucleotide.

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**Figure 2.** Reverse phase HPLC profiles of crude DMT-homodimers: A) d(TpT); B) d(CpC); C) d(ApA); d(GpG).

**Figure 3.** Ion-exchange chromatography of crude d(TAGCGCTA). Insert: reverse phase HPLC of purified octamer.
Chain assembly

Synthesis of homodimers. To set up the synthetic protocol for the PEG-supported synthesis of oligonucleotides via phosphoramidites, the four homodimers d(TpT), d(CpC), d(ApA) and d(GpG) were prepared. The influence of the excess, concentration and reciprocal ratio of amidites and tetrazole, and of the time of reaction were carefully investigated; particular attention was paid to the minimum amount of reagents required for the maximum yield. From these studies a 98–99% yield in the phosphite bond formation was observed by using a 2.5 times excess of phosphoramidite (and 10 times of tetrazole), as judged by UV and NMR analysis.

The capping step was always performed before the oxidation reaction to allow the removal of possible side-modification at the base level (15).

The oxidation step was minutely investigated. The iodine aqueous solution employed in the solid phase synthesis (16) was successfully tested: a complete oxidation of the internucleoside bond to phosphate was observed after 5 minutes of reaction, as indicated by 31P NMR spectra. However the high hygroscopicity of PEG and its affinity to I2 molecules hampers, during the following steps, the complete removal of these molecules. To overcome this problem an organic, anhydrous oxidation agent was introduced. Best results were obtained with TBHP in acetonitrile (17): from NMR analysis no side reactions were observed with the reported amounts after 15 minutes at 0°C.

From all these studies a standard synthetic protocol was set up (Table 1). Each elongation cycle takes at least four hours to be completed, since a couple of hours are requested to execute all the precipitation-and-filtration steps within the synthetic protocol and about one hour more is demanding for the final crystallization from ethanol, usually required for the cleanup of the PEG-bound oligonucleotide from residual, less soluble by-products.

An example of a 1H NMR and 31P NMR spectra for a PEG-bound phosphite and phosphate dinucleoside is shown in Figure 1. The HPLC analysis of crude deprotected dimers, reported in Figure 2, reveals a far better quality of the products respect to that previously obtained by the phosphotriester-based procedure (8).

Synthesis of d(TAGCGCTA). The feasibility of the method was tested in the synthesis of the same octanucleotide previously obtained by the phosphotriesters-based HELP method. The same PEG5000 monomethylether was employed. From the DMT absorption value of the crude 5'-protected PEG-8mer an overall yield of 93% and an average yield of 99% were calculated.

The fully deprotected oligomer was purified by low pressure ion-exchange chromatography on DEAE–Sephacel. The elution profile is shown in Figure 3, together with the HPLC of the purified product.

From 1.0 g of starting PEG, 51 μmoles (140 mg) of pure (≥95% from HPLC), final octamer were obtained as TEA salt; this amount signifies more than a 50% increase as to the former PEG-supported synthesis via phosphotriesters.

Synthesis of a 20mer. To evaluate the capacity of the new HELP Plus method in the production of longer oligonucleotides, an higher molecular weight PEG, namely the PEG12000 monomethylether, was adopted. This choice was dictated by the necessity to avoid that the unfavorable solubility properties of the growing oligonucleotide chain take over those of the polymeric support. The oligomer to be synthesized was a 20mer, as example of an antisense-size molecule.

From the DMT absorption value of the fully protected PEG-20mer, an overall yield of 85% and an average yield of 99% were observed.

After the usual deblocking procedure, purification of the DMT-protected oligomer by preparative reverse phase HPLC and detritylation, about 10 mg of pure lyophilized product were isolated from a 100 mg batch of the PEG-20mer. In Figure 4 the chromatographic patterns of purified oligonucleotide are reported.

To prove the absence of wrong linkages or modified bases, a purified sample was subjected to a total enzymatic digestion. The reverse phase HPLC analysis of the hydrolytic mixture and its nucleoside composition is reported in Figure 5. The expected nucleoside composition was observed, with no detectable base modifications.
During the synthesis a general drawback was observed: a constant loss of material, that likely occurs during the repeated precipitation-and-filtration steps. The weight reduction after each step was usually less than 1% of the total amount; some higher value was noticed toward the end of the process, when the size of the oligonucleotide chain becomes comparable to that of the supporting polymer.

CONCLUSIONS

From all the reported experiments the advantages offered by the new HELP Plus in comparison to the previous method based on the phosphotriester chemistry appear quite remarkable.

The utilization, in a solution procedure, of the phosphoramidite synthons offers strong improvements in terms of speed of reaction and coupling yield. These achievements and the use of an higher molecular weight PEG, allow to produce oligonucleotides of the size suitable for the antisense approach (18). In this context, the production by the HELP technique of chemically modified oligonucleotides (e.g. phosphorothioates), as demanded for their in vivo stabilization (19), is currently under way and will be reported in a forthcoming paper (20). Moreover, new synthetic strategies are under development aimed at the preparation of oligonucleotides covalently bound to the PEG. It is conceivable that this will supply new bioactive derivatives that, besides the increased ability to cross the cellular membrane, could be stabilized under assay condition and therapeutic use (21). Preliminary experiments indicate a feasibility of the process up to the millimolar scale (22). A larger production scale can be reasonably envisaged.

The practical value of the proposed method will be completely estimated with the automation of the overall synthetic process, now in progress.

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