0273-2289/95/5401-03—0001$07.00

Polyethylene Glycol

A High-Efficiency Liquid Phase (HELP) for the Large-Scale Synthesis of the Oligonucleotides

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ABSTRACT

The increasing demand of synthetic oligonucleotides for therapeutic and diagnostic purposes can be hardly satisfied by simply upscaling the commercial synthesizers. The introduction of a liquid-phase method that utilizes a polymeric support soluble into the reaction media can overcome the shortcomings related to the heterogeneity of the solid phase and allow a convenient large-scale process. Recently we have proposed a new synthetic approach for the oligonucleotide production that utilizes polyethylene glycol or PEG as soluble supporting polymer. We call this method High-Efficiency Liquid Phase or HELP. This approach preserves the advantages of a homogeneous synthesis in solution and adds an easy purification step of all the intermediates, mimicking the solid-phase procedure. In fact, reagent excess and byproducts can be eliminated by a simple precipitation-and-filtration step at the end of each synthetic cycle. Since all the reactions take place in solution, the scale-up of the process is easily predictable. Various synthetic protocols have been tested and optimized for the oligonucleotide production, up to the antisense-size level. After the phosphotriester and the phosphoramidite chemistry, the H-phosphonate approach is now under development. The possibility of an efficient automation of the whole process is also investigated.

Index Entries: Oligonucleotides, synthesis of; solid-phase synthesis; liquid-phase synthesis; polyethylene glycol; PEG; polymer-supported chemistry; synthesizers; phosphotriesters; phosphoramidites; H-phosphonates.

INTRODUCTION

Large-Scale Synthesis of Oligonucleotides

As recently stated by Caruthers (*1*), "As we learn more about using DNA (or RNA)...the synthesis of kilogram amounts of oligonucleotides will become necessary." In fact, recent medical and biotechnological applications of synthetic oligonucleotides have produced an expanding demand of these products. In particular, their use in the antisense approach (*2*) is providing a new wave of therapeutically active compounds that may revolutionize the treatment of many viral and oncological diseases. This possibility has introduced a need to scale up the production level of these compounds.

It is easily predictable that for grams of material required for the first clinical tests, the synthesis will have to be scaled up to kilograms or more for pharmaceutical production. The short-term needs can be satisfied by the last generation of automated DNA synthesizers capable of a higher scale of synthesis. However, the eventual commercialization will require a many thousand-fold scaling up of the processes for the synthesis and purification of oligonucleotides. The synthetic problems can be approached and resolved by two different methods, i.e., the solution and the solid-phase procedures. Both methods offer a balance of drawbacks and benefits, yet neither is capable of satisfying future production demands.

Solution Synthesis

The traditional synthesis in solution appears more convenient for unlimited large-scale production of oligonucleotides, since it does not require a large excess of reagents, in contrast to solid-phase methods, and can be scaled up like any organic chemical reaction. However, it suffers two disadvantages: very tedious and laborious purification procedures are required after each condensation step and a consistent amount of starting material is necessary to assure a sufficient quantity of the final oligonucleotide. Additionally, a serious drawback can occur during the synthesis of long oligomers if their solubility properties hamper dissolution of the reaction mixture. Many interesting attempts have been made in the past to overcome some of these undesirable features, such as the "filtration" method (*3*), the three-phase synthesis (*4*), or the 5'-phosphorylated dimer addition (*5*). Yet neither of these improvements is easily scaled up.

Solid-Phase Synthesis

The development of solid-phase synthesis, which uses an insoluble polymeric support as a handle for the growing oligonucleotide chain, has allowed the widespread availability of oligonucleotides for a number of scientific investigations. This approach, together with a reliable

chemistry, such as the phosphoramidite method (*6*), has offered the great advantage of convenient automation of the synthetic process, which resulted in increased speed and reduced labor. However, it must be emphasized that this procedure has been developed for the synthesis of micro- to milligram quantities of product, and it is founded on the use of a very large excess of reagents. Utilization of an automatic synthesizer by even a "trained monkey" is made expensive by the high production cost owing to the reagents and support consumption, and owing to the instrumental depreciation allowance. In addition, scale-up is hampered by the use of heterogeneous chemistry that avoids a linear increase in the parameters designed for smaller scales.

Recently, a different synthetic approach has been investigated, based on the H-phosphonate chemistry (*7,8*). Proposed for oligonucleotide synthesis more than 30 years ago, it has been now improved, but overall yields and quality of products have yet to match those attained by the phosphoramidites. Its main advantage is the possible recovery and recycling of the excess reagent (*9*), resulting in cost saving.

To improve the amount of material produced by a single synthetic run, a new high-capacity support has been recently proposed (*10*): a low crosslinked polystyrene has been grafted with ethylene oxide to give a polystyrene–polyethylene glycol (PEG) copolymer. This so-called TentaGel polymer can be functionalized up to 200 μmol/g, and multigram quantities of synthetic oligonucleotides may be prepared in a single automated operation (*11*).

Synthetic efficiency can be further increased by proper development of new instrumental apparatus, based on different reactors and reagent distribution systems. Lately, large-scale synthesizers have been commercialized with operating capacities ranging up to 1 mmol scale (*12*). Further instrumental improvements are directed toward reducing reagent consumption and minimize the chemical waste generation (*13*).

Up to now, commercial equipment adapted for large scale synthesis appears able to satisfy the need for gram-scale production. The synthesis of kilograms, or more, of oligonucleotides still seems far from the current potential of the solid-phase technique, and the production cost based on the above-mentioned technologies would be unaffordable for widespread therapeutic utilization.

Liquid-Phase Synthesis

To overcome the disadvantages of the synthetic procedures so far described, a different approach can be envisioned that integrates or couples the advantages of the solution and the solid-phase methods. The idea, initially proposed for peptide production (*14,15*), retains a polymer as support for the growing oligonucleotide chain, but it uses a "synthetic assistant" fully soluble in all the reaction conditions. The term "liquid phase" has been utilized to describe this synthesis, in analogy with the

solid phase. The result is that all the reactions take place under homogeneous conditions, thereby preserving some attributes of the classical solution procedure, such as the easy scaling up of the process and the ease of reagent diffusion.

The presence of a polymeric support is desired to obtain a fast and reliable purification step. In principle, any soluble byproduct and residual excess of reagents can be easily eliminated from the reaction mixture by exploiting the higher molecular weight of the polymer-bound product. For example, an ultrafiltration process through a size-exclusion membrane or a "desalting" with gel-filtration chromatography can be effective. A rapid alternative to these procedures is the precipitation of the product with a nonsolvent for the polymer followed by filtration and washing (provided that the physical properties of the precipitated polymer do not hamper a practical execution of this procedure). Moreover, the oligonucleotide to support mol-wt ratio has to be carefully evaluated to avoid changes in the solubility properties of the polymer that can occur during the synthesis. This phenomenon could limit the maximum length of the oligomer to be synthesized and dramatically affect yields.

The uniqueness of this method could make its automation difficult or "...practically render an untroublesome automation impossible" (*10*). Nonetheless, the process can be accomplished with ordinary laboratory equipment, and it is suitable for easy scaling up for industrial production with a proper apparatus. Furthermore, the homogeneity of the media will certainly demand a lower excess of reagents, allowing a significant cost saving.

In spite of these advantages, the liquid-phase technique has been only tentatively investigated in the past for oligonucleotide production (*16–18*) without any further application. Only recently has it received renewed attention for its advantages in the large-scale production process (*19*).

THE HELP METHOD

General Overview of the HELP Method

Recently, we have investigated the application of the liquid-phase method for a rapid preparation of a large amount of oligonucleotides. We have called our synthetic procedure High-Efficiency Liquid Phase or HELP. The soluble polymer of choice has been PEG, since it displays a high solubility in the organic solvents used for oligonucleotide synthesis. Its usefulness was demonstrated in the past for the liquid-phase synthesis of peptides (*20*). PEG is commercially available in a wide range of molecular weights, both in the mono- and bifunctional forms. It differs from other similar polymers by its marked tendency to crystallize, which avoids the formation of gelatinous precipitates and inclusions, and allows an efficient precipitation-and-filtration step. Hence, the polymer-bound product can be partitioned from any soluble impurity by a simple process

that mimics the purification step of the solid-phase approach. The complete reaction can be performed in a homogeneous environment and, consequently, a lower excess of reagents (compared to the solid phase) is expected for a maximum yield.

The first nucleoside of the chain is easily bound to the terminal OH group of the PEG unit through a carboxylic ester bond on the 3′-*O*-succinate derivative of the 5′-*O*-protected nucleoside. This bond has been previously demonstrated to be stable during all the reaction conditions, but the synthesized oligonucleotide chain can be easily removed during the final deblocking procedures. Moreover, the general properties of PEG are fully compatible for its chromatographic separation at the end of the synthetic process. A further advantage of PEG is given by its spectral transparency in the absorbance region of the nucleotide unit that allows a nondestructive monitoring of the synthetic process; the PEG-oligonucleotide can also be analyzed in detail by NMR spectroscopy, since the PEG signals can be easily suppressed. The general scheme of the proposed method is reported in Fig. 1. PEG is easily functionalized as described before (*21*) and subjected to the deprotection of the 5′-OH group of the first nucleoside of the chain. Then, it is reacted with the second properly activated and protected nucleotide. These two steps (deprotection and condensation) are repeated until the desired oligonucleotide length is attained. The final deblocking procedure produces a mixture from which the final product can be easily purified by usual chromatographic procedures.

The HELP Method via Phosphotriesters

The first oligonucleotide synthesis conducted with the PEG-based liquid-phase approach utilized the well-known phosphotriester chemistry (*22,23*). Most of the condensing reagents and reaction conditions reported in the literature have been tested, and two synthetic protocols have been set up (Fig. 2). Route A employs a protected phosphotriester as building unit; the condensation step has been optimized by using mesitylenesulfonylnitrotriazole (MSNT) as activating agent and *N*-methylimidazole (NMI) as catalyst, in anhydrous pyridine as solvent. The coupling yield ranges from 90 to 95% with a threefold excess of nucleotide; the reaction time is 60 min. In route B, the starting material is the less-expensive protected nucleoside, phosphorylated *in situ* with the bifunctional reagent 2-chlorophenyl-*O*-*O* bis(1-benzotriazolyl) phosphate; after the activation step, the mixture is left to react for 60 min. Coupling yields are generally higher than 90%, and no side reactions are observed at the guanine moiety, unlike in route A.

During the synthesis, anhydrous conditions are required. After each reaction step, the polymer is precipitated with diethyl ether (*tert*-butyl methyl ether is also effective) at 0°C and washed. A TLC analysis confirms the removal of the reagent excess and soluble byproducts. If necessary, a further recrystallation from ethyl alcohol is suitable, but in that case, a small loss of material (<1–2%) can occur. As for the solid-phase

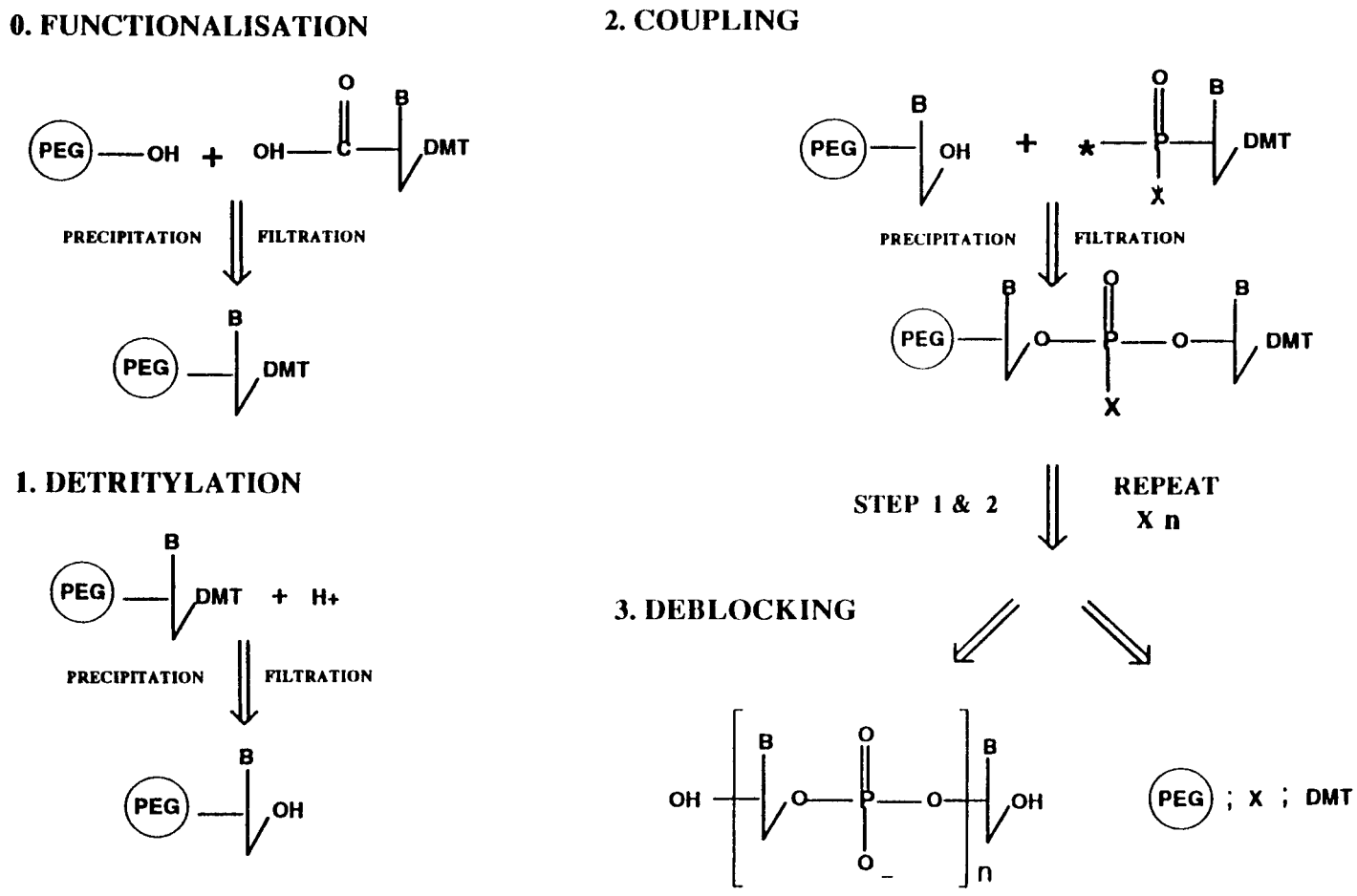

Fig. 1. Scheme of the general process of the HELP method.

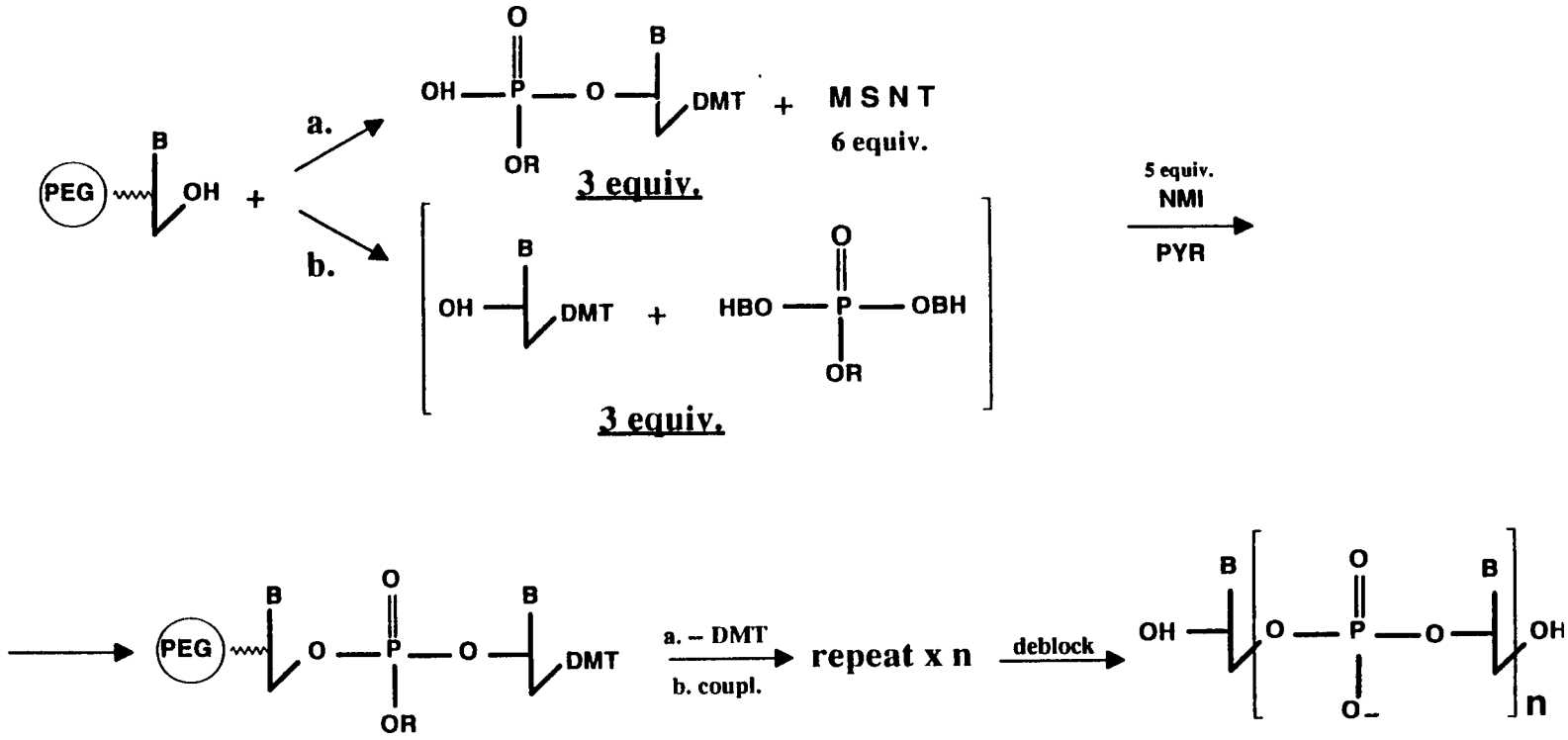

Fig. 2. Reaction process of the application of the phosphotriester chemistry to the HELP method.

process, it is useful to submit the growing PEG-bound oligonucleotide to a capping step, but removal of the 5′-OH blocking group from the last nucleotide of the chain. The chain elongation step takes about 6 h to be completed, and the reagent excess is no more than double that required in the classical solution procedure.

The feasibility of this protocol, optimized at the dinucleotide level, has been tested in the synthesis of an octanucleotide. The product has been released from PEG and deprotected following the standard procedures reported for the solid-phase processes. After purification by ion-exchange chromatography, nearly 100 mg of pure octamer have been obtained from 1.0 g of starting PEG-nucleoside (PEG = monomethylether polyethylene glycol of average mol wt 5000). Lately, this procedure has been extended to the production of cyclic oligonucleotides (*24*); the PEG appears to inhibit to some extent the unwanted intermolecular coupling, and a gel filtration has been introduced as intermediate purification method.

From these results, the application of phosphotriester chemistry to the HELP method has confirmed some of the expected improvements offered by the liquid-phase procedure. The synthesis performed in homogeneous conditions demands a lower excess of reagents than the solid phase, with an appreciable cost saving. Moreover, a large amount of product can be obtained from a single synthetic run, and the reaction pathways are easily monitored by nondestructive spectral analysis. Consequently, with the HELP method, it appears possible to obtain short oligonucleotides in the hundred-milligram scale at a cost lower than with the solid phase and in a time shorter than with the standard solution procedure.

The HELP Method via Phosphoramidites

The phosphoramidites are the most commonly used reagents in the solid-phase synthesis of oligonucleotides, but their application to solution procedures has never been successfully reported. The realization of a new liquid-phase approach based on these synthons appeared very promising, since a further improvement in terms of speed and yield of reaction was expected.

A set of experiments at the dinucleotide level were performed to set up the new synthetic protocol. As expected, the homogeneous coupling conditions required a lower excess of reagents than similar solid-phase procedures; an almost quantitative yield was obtained with only 2.5-fold excess of amidites and 10-fold excess of tetrazole, in anhydrous acetonitrile solution. The reaction was carried out under argon atmosphere.

Many studies have been devoted to the optimization of the intermediate oxidation step from phosphite to phosphate. An anhydrous oxidation with *tert*-butylhydroperoxide, in acetonitrile solution, at 0°C, was found to be preferable to the aqueous iodine solution used in the solid-phase synthesis. From NMR analyses, no side reactions were observed

after the 15-min treatment. A capping treatment was always performed before the oxidation, since this step is known to remove some possible side modification at the base level (25). A scheme of the reaction cycle is depicted in Fig. 3. The complete reaction cycle takes about 20 min, but a couple of hours are required to execute the intermediate purification steps (by precipitation and filtration with ether); 1 h more is required for a final crystallization from ethanol to clean up the PEG-oligonucleotides from residual byproducts. From the spectral and HPLC analyses of the same octanucleotide synthesized before by the HELP method via phosphotriesters, a far better quality of product was obtained by the phosphoramidite approach. The higher average and overall yields observed were verified by a significant 50% increase in the octamer production.

To evaluate fully the capacity of this new HELP method for larger molecules, a 20-mer oligonucleotide was synthesized. To prevent the unfavorable solubility properties of the synthesized chain from outweighing those of the polymeric support, a higher mol-wt PEG (namely the monomethylether PEG 12,000) was used. During the synthesis, the higher number of purification steps required for 20-mer production resulted in an appreciable loss of material: < 50% of the theoretical expected amount was isolated. From each 100 mg of the final polymer-bound oligonucleotide, about 10 mg of pure 20-mer were isolated (1/3 of the crude material determined from the final DMT content) (*26*).

The use of the phosphoramidite chemistry in the HELP method has also allowed the production of modified oligonucleotides, such as the phosphorothioate derivatives widely investigated for their therapeutic antisense utilization (27). The substitution of the oxidation with the sulfurization step (a 15-min treatment with an acetonitrile solution of tetraethylthiuram disulfide) allowed the preparation of a fully thioated 20-mer. The production data were similar to those observed during the synthesis of the previous unmodified 20-mer; more than half of the estimated crude material was isolated after the final purification. The solubility of the thioate derivative was fully compatible with the HELP procedure and resulted in a better recovery of the PEG-bound products during the intermediate purification steps.

From these results, the phosphoramidite chemistry seems to be able to give further advantages to the HELP method. In fact, it allows the synthesis of longer oligonucleotide chains, both natural and backbone-modified, useful for their antisense utilization by demanding a lower amount of reagents in comparison with a similar solid-phase procedure.

The HELP Method via H-Phosphonates

The application of the H-phosphonate chemistry to the HELP method is now under investigation. The use of these reagents suggests a further improvement over the prior procedure, since the H-phosphate nucleosides offer a reactivity comparable with the phosphoramidites, but appear

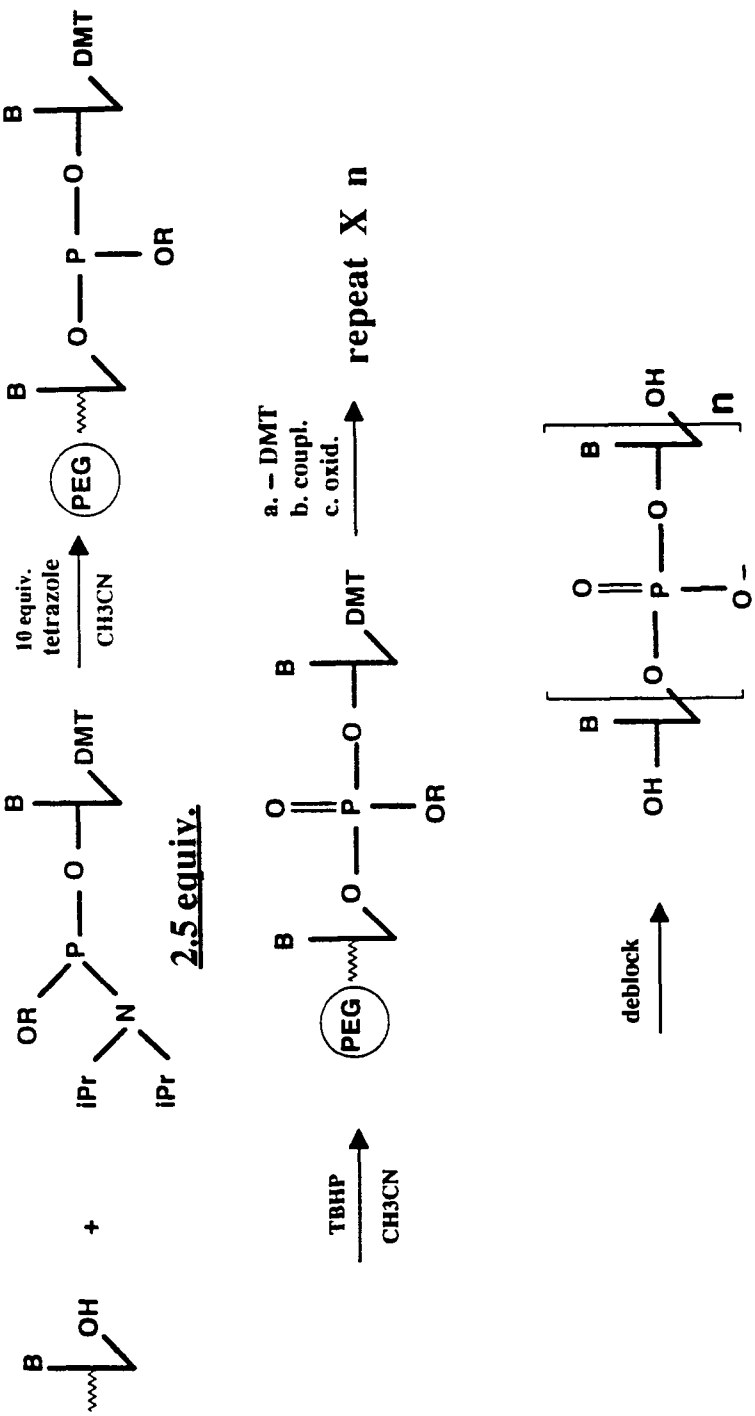

Fig. 3. Reaction process of the application of the phosphoramidite chemistry to the HELP method.

more stable and less expensive to produce. Their solid-phase utilization did not succeed over the corresponding amidites, since the condensation yields are reported to reach a lower average value, but the use of these reagents in the liquid-phase approach could result in a better behavior, owing to the homogeneous reaction conditions. This appeared to be confirmed from the first experiments performed, as usual, at the dinucleotide level for the optimization of the synthetic protocol. A nearly quantitative condensation yield was obtained in a preliminary test by using <2 Eq of H-phosphonates. The scheme of the proposed reactions is reported in Fig. 4.

Moreover, as reported in the literature (*28*), this chemistry avoids any waste of expensive reagents, since it is possible to collect and recycle the unused monomer excess. A further advantage is provided by the lack of a compulsory oxidation step after each coupling step; a single oxidation of the final product is sufficient. As a consequence, a reduced number of precipitation-and-filtration steps is required and an increase in the recovery of the final PEG-oligonucleotide can be obtained.

The final oxidation step can also be substituted by other chemical treatments to produce a number of modified oligonucleotides, as thioates, amidites, esters, and so forth (*27*). The liquid-phase method can be particularly useful for these final chemical modifications, since it operates in solution and not (as the solid phase) in heterogeneous conditions. In addition, the wide availability of spectroscopic techniques allows the reaction proceeding to be followed in detail and both the extent of the chemical modification and the best operating conditions to be precisely defined.

For all these reasons, the extension of the H-phosphonate chemistry to the HELP method is receiving a careful evaluation to verify the expected advantages mentioned above.

Automation of the HELP Method

The final objective of the HELP method, which utilizes PEG as soluble polymeric support for the liquid-phase synthesis of oligonucleotides, is undoubtedly its automation. The realization of an automatic synthesizer operating in the previously reported conditions could provide a wider availability of this method. On the other hand, the characteristics of this process could, as stated before, render automation very difficult, if not impossible.

To resolve this problem, it is necessary to design a proper reactor system able to operate efficiently both with homogeneous and heterogeneous phases. This reactor should be able to retain a stirred, thermoregulated solution, allow the introduction of precise aliquots of solvent and reagents, separate the oligonucleotide bound to the polymer from the solution containing excess reagents and byproducts, and permit a new dissolution of the solid PEG-oligonucleotide to continue the reaction cycle.

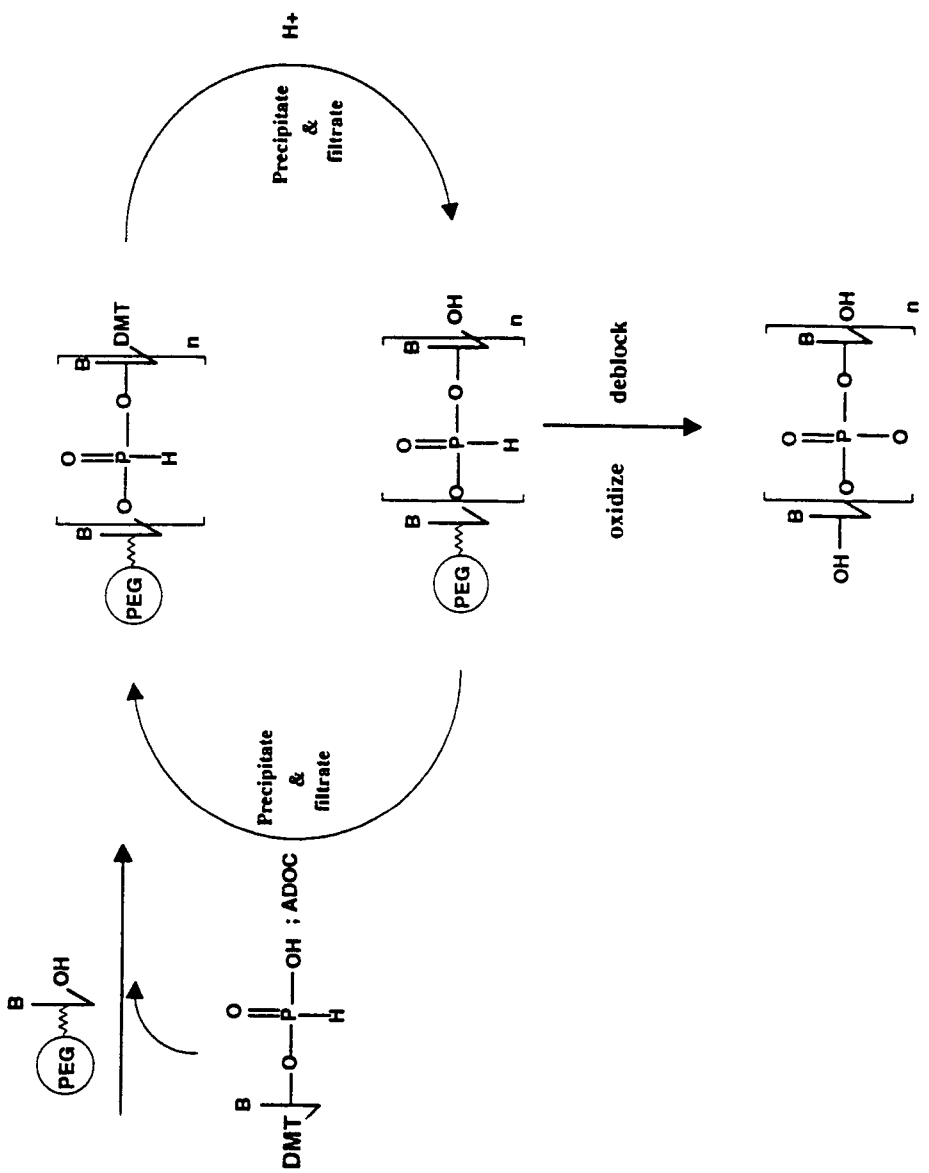

Fig. 4. Reactions and scheme of the process of the application of the H-phosphonate chemistry to the HELP method.

The system must be connected to a computer-driven apparatus, so that all the operations are exactly calculated, monitored, executed, and verified. A separate device for the waste management could be planned to allow a separate collection of reagents and solvents and, when possible, their recycling. An automatic, final deblocking of the product from the support can eventually be envisaged.

The main improvement derived from the automation of the HELP process is that the amount of the final product is likely to be increased; in fact, if manual operation is avoided, the loss of material during the purification step is expected to be reduced. If such instrumentation is to be realized, an interchangeable reactor could be considered so that it is possible to perform a set of operations ranging from the optimization of the process to the production of the final product.

CLOSING REMARKS

In conclusion, the utilization of a soluble polymeric support, like PEG, in the liquid-phase synthesis opens new favorable prospects. This approach appears particularly convenient for a large-scale synthesis, as demanded for the commercialization of these derivatives. Since all the chemical reactions are performed in solution, the scaling up of the process can be reasonably achieved. Up to now, the use of phosphoramidite reagents offered the best results in terms of speed of reaction, coupling yield, and oligonucleotide length. The application of the H-phosphonate chemistry is very promising in terms of cost saving and production of modified derivatives, but it needs further investigation to ascertain its real efficiency. New synthetic procedures are also under investigation, aimed at obtaining oligonucleotides permanently bound to the supporting PEG. It has been suggested that the presence of this moiety, known to be nontoxic, nonantigenic, and biocompatible (*30*), could supply new bioactive derivatives more able to cross the cellular membranes and less subjected to the nuclease degradation under in vivo conditions (*31*). Finally, the true potential of the HELP method will be fully evaluated when an automation of the overall process is realized. This possibility could give an ultimate boost to the exhaustive evaluation of the pharmacological properties of selected oligonucleotide chains and their therapeutic utilization as life-saving drugs.

ACKNOWLEDGMENTS

This work was in part supported by funds from MURST—Rome (Italy) and Progetto Finalizzato "Chimica Fine II"—CNR (Italy). The contribution of Drs. F. P. Colonna and A. Garbesi, I.Co.C.E.A.—CNR (Italy) to

the development of the phosphotriester-based HELP method is gratefully acknowledged.

The author wishes to thank Drs. G. Biancotto and M. Maffin, University of Padova (Italy) for their invaluable activity in the application of the phosphoramidite chemistry. He is also particularly indebted to Dr. C. L. Scremin, University of Padova (Italy), without whose collaboration most of the subject here reported, and his Ph.D. thesis, would not have been realized. The precious contribution of Dr. S. Beaucage, Bethesda, MD (USA), to the development of the PEG-based phosphorothioate synthesis is also acknowledged.

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