Tetrahedron Letters,Vol.24,No.45,pp 4963-4966,1983 0040-4039/83 $3.00 + .00
Printed in Great Britain

AN IMPROVED SYRINGE TECHNIQUE FOR THE PREPARATION OF OLIGONUCLEOTIDES OF DEFINED SEQUENCE [1]

H. Seliger *, C. Scalfi and F. Eisenbeiss +

Universität Ulm, Sektion Polymere, Oberer Eselsberg, D-7900 Ulm, W. Germany, and +) Fa. E. Merck, Zentrallaboratorium Reagenzienforschung, Frankfurter Str. 250, D 6100 Darmstadt, W. Germany

ABSTRACT

Resulting from further development of the technique described by Tanaka and Letsinger a simple syringe-based reaction system was assembled for efficient routine synthesis of oligonucleotides, and its applicability exemplified.

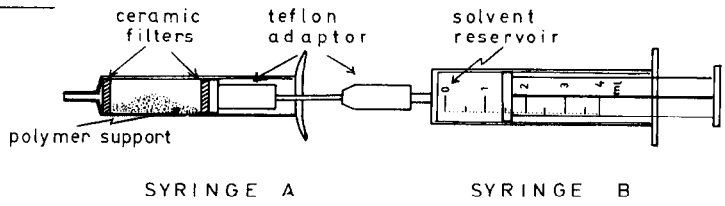
INTRODUCTION

During the last years the chemical synthesis of oligonucleotides has been significantly improved by the elaboration of new preparative methods, among which the phosphite approach originally introduced by R.L. Letsinger et al. [2] and further developed by M.H. Caruthers and coworkers [3] has become especially attractive. It is most efficiently combined with the solid-phase technique, where the use of the highly reactive phosphoramidites and the reduction of purification steps to washing and filtration lead to very short periods for each chain extension. Another interesting feature of polymer support syntheses is that they lend themselves to different types of apparatus. Automatic [3, 4] and manually controlled [5] machines have been described, but good results may as well be obtained with simple shaking vessels made by modification of sintered glass funnels [6, 7]. An interesting variant was recently forwarded by T. Tanaka and R.L. Letsinger [8], namely the use of syringes for this purpose. Experimenting with this technique we found that syringes, as such, are less suitable reaction vessels, especially since frequent motion of the piston causes the formation of debris from the support material and leads to difficulties in the filtration procedures. Therefore, we have further elaborated on this technique and wish here to describe a new type of syringe-based synthesis system and examples of its use for the preparation of oligonucleotides of defined sequence.

APPARATUS

A schematic representation of our apparatus is given in Figure 1 . It is assembled, essentially, by connecting two commercial polypropylene syringes. Syringe A is the reactor itself. It is equipped with two tightly fitting ceramic filters, between which the support material is introduced. The piston of syringe A is replaced by a teflon adapter, which fits to the outlet of syringe B. Both syringes have a LUER lock and can be used with any commercial needle. During the oligonucleotide synthesis all reagents sensitive to air and moisture are directly sucked into the reaction chamber in syringe A from septum cap

Figure 1



bottles and after reaction pressed out using the piston of syringe B. Washing solutions, however, are directly taken up in syringe B and pressed through syringe A.

RESULTS AND DISCUSSION

As an example for the application of this apparatus we prepared the two oligonucleotides dGCTAG and dAATTCTAGCTGCA, which can be hybridized to form an Eco RI - Pst I - stop - adaptor useful for recombinant DNA research (Fig. 2)[9]. For the preparation of the pentanucleotide dGCTAG we largely followed the procedure of Tanaka and Letsinger [8], however using nucleoside-3'-(methoxy-N,N-dimethylamino-)phosphoramidites [3] as monomers. Starting with 60 mg polymer support containing 92 µmol/g immobilized N-isobutyryl-deoxyguanosine (preparation as described [7]) four reaction cycles were completed (omitting the capping step in the last cycle). For demethylation syringe A was filled with 1.5 ml thiophenol/triethylamine/dioxane [7] and rotated for 45 min. After washing (methanol, ether) and treatment with 25 % aqueous ammonia overnight the product was released from the support and purified by HPLC [7] to give 0.55 µmol = 9.5 % of dGCTAG, corresponding to 55 % average yield per elongation.

As a possible source of impairment we found in the course of this preparation, that, although internucleotide bond formation proceeded readily, solvent washes alone seemed not to be effective enough to exclude moisture completely and to prevent traces of adsorbed reagents to be carried on into the next reaction step. Therefore, the reaction cycle for the preparation of the tridecanucleotide dAATTCTAGCTGCA was modified as shown in Table 1 . Drying procedures were included before the coupling, capping and detritylation steps, which consisted of passing a stream of dry argon through syringe A via the adaptor for several minutes. Other features are the replacement of viscous

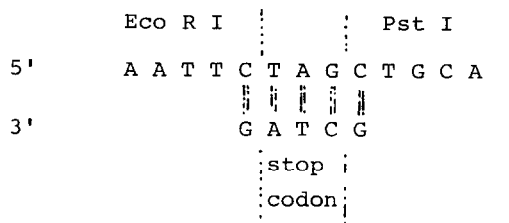


Fig. 2

and less volatile solvents and the use of nucleoside-3'-phosphoromorpholidites [10] as intermediates. In this way, the sequence dAATTCTAGCTGCA was prepared, starting from 100 mg support (56 µmol dA^{bz}/g) in an overall yield of 9.4 %, which averages 82 % of internucleotide bond formation per coupling step. After workup, done as described above, the oligonucleotide was isolated by RP-HPLC (Fig. 3). The product (peak P in Fig. 3) was found to be pure material, giving rise, after detritylation and phosphorylation with γ-^{32}P-ATP/polynucleotide kinase, to a single spot on polyacrylamide gel electrophoresis (Fig. 4a). The correct sequence was established by two-dimensional fingerprint analysis [11] (Fig. 4b).

Table 1 : Reaction cycle used for the synthesis of the tridecanucleotide dAATTCTAGCTGCA

Step	Reagent	Quantity	Time
Detritylation:	2% Cl_3CCOOH in CH_2Cl_2	2 x 1 ml	1'
Washing :	CH_2Cl_2	3 x 2 ml	
	CH_2Cl_2/pyridine 4 : 1	1 ml	
	dry CH_2Cl_2	5 x 2 ml	
Drying :	dry argon		ca. 5'
Coupling :	20fold nucleoside-phosphoramidite in 1 ml of a saturated solution of tetrazole in abs. acetonitrile		10'
Washing :	acetonitrile	3 x 2 ml	
	THF/pyridine/H_2O 40:20:1	1 ml	
Oxidation :	0.1 M I_2 in THF/pyridine/H_2O 40:20:1	1 ml	1'
Washing :	THF/pyridine/H_2O 40:20:1	3 x 2 ml	
	THF		
Drying :	dry argon		ca. 2'
Capping :	CH_2Cl_2 600 µl + $(CH_3CO)_2O$ 100 µl + $N(C_2H_5)_3$ 100 µl + N-methyl-imidazole 30 µl		1'
Washing :	CH_2Cl_2	3 x 2 ml	
Drying :	dry argon		ca. 2'

While equalling the previously described syringe method [8] in ease of handling, the syringe-based apparatus described here avoids difficulties due to mechanical disruption of the carrier, minimizes the access of moisture and oxygen during the coupling steps and simplifies the washing procedures allowing deliberate quantities of solvents to be passed through syringe A using different sizes of syringes B as reservoirs. Through optimization of the chain elongation steps (Table 1) yields of oligonucleotides are comparable to those obtained with automatically or manually controlled synthesizers [3 - 5] . The apparatus shown in Fig. 1, however, is assembled from cheap commercial materials. It, therefore,

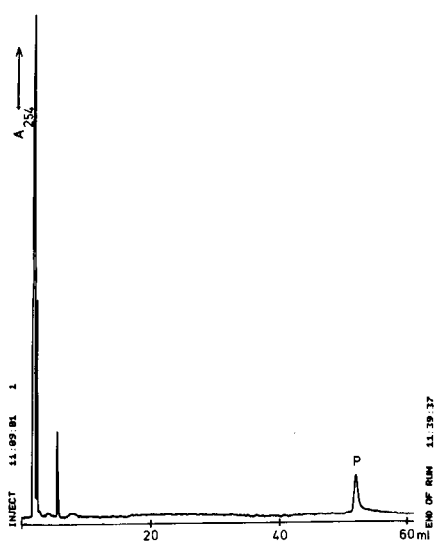
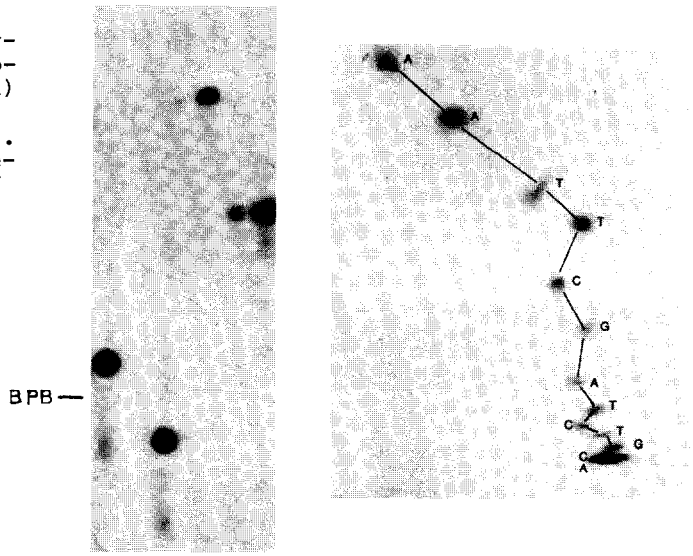


Fig. 3: HPLC purification of DMTrdAATTCTAGCTGCA
Column: LiChrosorb SupeRP (Merck; 7 µ; 5.0 x 250 mm)
Eluant: Lin. gradient (30') of 20 - 35 % CH_3CN in 0.1 M triethylammonium acetate, pH 7
Flow: 2 ml/min; Range: 0.20; Detection: $UV_{254\ nm}$

Figure 4 :

a) (left side) : 16 % polyacrylamide gel electrophoresis (0.2 mm denat. gel) of the tridecanucleotide dAATTCTAGCTGCA (lane 3). Lanes 1, 2 and 4/5 : Reference oligonucleotides of chain length 7, 5 and 10.

b) (right side) : two-dimensional fingerprint[11] of dAATTCTAGCTGCA .



should be most suitable for low-cost oligonucleotide preparations, a proposition, which is now being corroborated during routine use of this apparatus in our laboratory.

ACKNOWLEDGEMENT

Financial aid of this project by the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

REFERENCES

1. Polymer Support Synthesis. X. Part IX : H. Seliger and H.-H. Görtz, Angew. Chem. 93, 709 (1981); Angew. Chem., Intern. Ed. Engl. 20, 683 (1981).
2. R.L. Letsinger and W.B. Lunsford, J. Amer. Chem. Soc. 98, 3655 (1978).
3. M.D. Matteucci and M.H. Caruthers, J. Amer. Chem. Soc. 103, 3185 (1981).
4. G. Alvarado-Urbina, G.M. Sathe, W.-C. Liu, M.F. Gillen, P.D. Duck, R. Bender and K.K. Ogilvie, Science 214, 270 (1981); F. Chow, T. Kempe and G. Palm, Nucleic Acids Res. 9, 2807 (1981).
5. K. Ballas and H. Seliger, Communications, Conference on "Synthetic Oligonucleotides in Molecular Biology", Uppsala, 1982, 84; J.E. Davies and H.G. Gassen, Angew. Chem. 95, 26 (1983); Angew. Chem., Intern. Ed. Engl. 22, 13 (1983); M.J. Gait, H.W.D. Matthes, M. Singh, B.S. Sproat and R.C. Titmas, in: **Chemical and Enzymatic Synthesis of Gene Fragments, H.G. Gassen and A. Lang, eds., Verlag Chemie, 1982, 1 ff .**
6. J.A. Nickoloff and R.B. Hallick, Nucleic Acids Res. 10, 8191 (1982).
7. H. Seliger, S. Klein, C.K. Narang, B. Seemann-Preising, J. Eiband and N. Hauel, in: Chemical and Enzymatic Synthesis of Gene Fragments, H.G. Gassen and A. Lang, eds., Verlag Chemie, 1982, 81 ff .
8. T. Tanaka and R.L. Letsinger, Nucleic Acids Res. 10, 3249 (1982).
9. H. Seliger, T.C. Bach, E. Happ, M. Holupirek and E.H. Teufel, Hoppe-Seyler's Z. Physiol. Chem. 360, 1044 (1979); S.A. Narang, R. Brousseau, H.M. Hsiung, W. Sung, R. Scarpulla, G. Ghangas, L. Lau, B. Hess and R. Wu, Nucleic Acids Res. Symp. Ser. 7, 377 (1980).
10. L.J. Mc Bride and M.H. Caruthers, Tetrahedron Lett. 24, 245 (1983); T. Dörper and E.-L. Winnacker, Nucleic Acids Res. 11, 2575 (1983).
11. C.-P.D. Tu and R. Wu, in: Methods in Enzymology, L. Grossman and K. Moldave, eds., Academic Press, 1980, 620 ff.

(Received in Germany 1 August 1983)