Rapid synthesis of oligodeoxyribonucleotides on a grafted polymer support

V.K.Potapov, V.P.Veiko, O.N.Koroleva and Z.A.Shabarova

Chemistry Department and A.N.Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow, USSR

Received 29 March 1979

ABSTRACT

Rapid synthesis of oligodeoxyribonucleotides is described by a solid phase method. Polystyrene grafted on the surface of polytetrafluoroethylene has been found to be an adequate support. The properties of the support were studied in the synthesis of a number of oligonucleotides 6 to 15 nucleotides long.

A flow-type semi-automatic synthesizer has been used. Each nucleotide addition involves seven steps and takes 8 hours. Oligonucleotide isolated and purified by ion-exchange chromatography amounts to 1-3 μmol per gram of support.

INTRODUCTION

The major factors in the solid phase synthesis of oligonucleotides is the nature and structure of the polymer support. For example, the use of soluble polystyrene supports [1] allowed short oligonucleotides to be synthesized in over 90% yields at each step of synthesis. On macroporous [2,3] and isotactic [3] supports, internucleotidic coupling amounted in some cases to 75-80%. The use of a polyacrylamide support instead of a nonpolar styrene matrix suggested by Gait and Sheppard [4,5] and Narang [6], allowed one not only to obtain high and stable yields at the initial steps, but also to maintain 65-75% in the synthesis of 7-8 membered oligonucleotides. The main drawback of this type of supports is excessive (more than 10-fold) swelling that necessitates a large (16 to 20-fold) excess of the nucleotide to keep its concentration sufficient in the reaction mixture and a great amount of solvents for washing the polymer.

Recently we have demonstrated that in solid phase synthesis of oligonucleotides use may be made of a grafted support [7] on which a nonadeoxyribonucleotide $d(T_5GTG_2)$ was synthesized during 10 days. In this work we describe in more detail the properties of the new polymer support revealed in the synthesis of hepta- and pentadecanucleotides. Five of them are capable of self-assembly into a DNA-like complex that is a structural gene sequence of peptide enkephalin (comp 1-5 in the Table) others can be primers for transcription of mRNA.

DISCUSSION

a) Polymeric support. The drawback that is common for all the types of supports containing an anchor group distributed all over the granule volume is the significant role of diffusion (reagents into the granule, and the reaction products out of it), which hinders mass exchange. If the support is a polymer with a high degree of cross-linking, the role of steric hindrances increases and the efficiency of the reaction in macro- and micropores is different. If a polymer has a low degree of cross-linking, it markedly changes its parameters when solvents are changed, requires a large excess of reagents and is, therefore, not so good technologically. Finally, supports of these types, owing to the relatively low content of anchor groups (0.1-0.4 mmole/g), hardly change their chemical nature as the oligonucleotide chain grows and remain either nonpolar (styrenes) [2] or polar (acrylamides) [5], which tells on the efficiency of some stages of synthesis. Thus polystyrene supports are less fit for the diester method of synthesis of oligonucleotides, and their treatment with an alkaline agent on deprotection of the 3'-end always requires special conditions. At the same time, acrylamide support [6] required the conditions of acidic deprotection to be changed, since the polar phase of the support proved hardly compatible with such solvents as chloroform or benzene.

As the present study demonstrated, the support prepared by chemical modification of polystyrene grafted on the surface

of an inert polytetrafluoroethylene carrier combines the advantages of all types of supports. Low thickness of a polystyrene layer increases the rate of mass exchange, minimizing the role of diffusion. The absence of cross-linking eliminates steric hindrances on modification and during synthesis. Owing to the presence in the polymeric support of an inert teflon nucleus, it hardly ever swells in any types of solvents, displays low sorptional activity and is readily washed from reagents and solvents. The major feature of the support is that it can be extensively modified (30-50%) on introduction of anchor groups. This results in the support losing the hydrophobicity of polystyrene after it is added to the first nucleoside, and especially after the synthesis of dinucleosidephosphate. Later, as the polynucleotide chain grows and the number of internucleotide phosphate groups increases, the support acquires the features of a polar polymer. Changes in the chemical nature of soluble polystyrene when used in the synthesis of oligonucleotides were reported by us previously [8]. Thus a grafted support with a high specific loading is devoid of the principal drawback of styrene supports, e.g., incongruence between the hydrophobic character of the support and the polar nature of oligonucleotides synthesized on it, which was only too justly mentioned in [4], and is fit for both the diester and triester methods of syntheses of oligonucleotides.

b) Modification and coupling of nucleosides. As an anchor group of the support, we used an p-methoxytrityl group introduced by a standard scheme [1,3]. All the reactions procedures and the degree of modification were easily followed by IR-spectroscopy with the support being pressed into a tablet.

The spectrum of the polystyrene film of the starting support is from 1400 to 1700 cm^{-1} and from 2800 to 3600 cm^{-1} . The ratio between the intensities of 1590 cm^{-1} of the benzene band and the 1550 cm^{-1} band of teflon as an internal standard characterizes the degree of grafting; it can be easily varied from 70 to 300 mg/g. After benzylation of the support,

a 1665 cm^{-1} band ($\nu_{\text{C=O}}$) is observed in the spectrum, and the band quantitatively disappears after treatment of the polymerketone by p-methoxyphenylmagnesiumbromide. Thereby in the high frequency range of the spectrum there appears a 3580 cm^{-1} band (ν_{OH}) characteristic of a weakly associated hydroxy group. The quantity of the anchor groups introduced in this way depends on the time of the Friedel-Crafts reaction and amounts to 0.1-0.15 mmol/g of the support on acylation during 5 to 15 min at 30°C . The polymer is chlorinated by treatment with acetyl chloride in benzene [2,3] or thionyl chloride in dry pyridine at -10° .

Addition of the first nucleoside (dT, danC, dbzA) is achieved by treating the polymer with a 3-4 fold excess of a respective protected nucleoside in dry pyridine for 16-20 hours at 20°C . The degree of utilization of the anchor groups varies between 60 to 90% and decreases in the following order $\text{T} \gg \text{C} \gg \text{A} = \text{G}$. The total capacity of the support with respect to the nucleoside can be 70-150 $\mu\text{mol/g}$.

c) Synthesis of oligonucleotides. To study in detail the characteristics of the polymeric support, the diester method of nucleotide addition was used. The majority of studies on the solid phase synthesis of oligonucleotides makes use of this approach; therefore it will be feasible to compare our results with the reported data [5,6,9].

The synthesis is machine-aided, with a column type reactor; the reagents are pumped through a layer of the polymeric support and then recycled (synthesis) or discharged (washing

and deprotection).

A. General scheme of synthesizer: 1 - column type reactor with polymer; 2 - pump; 3 - conductometric cell; 4 - discharge vessel, 5-10 - vessels for reagents and solvents.

At the first four steps of the synthesis, a 5 to 6-fold excess of the mononucleotide is used, which is determined by the total volume of the system (3.5-4 ml) at a standard concentration of 0.15 mmol/ml. At each successive stage the excess is increased until a 10-fold excess is achieved (taking into consideration the addition at the internucleotide phosphate groups). The TPS-nucleotide ratio is 1.7 to 1 at all steps of the synthesis. The volume of reactor (1) that has two movable teflon filters can be different depending on the scale of the synthesis; for a standard amount of the support (0.5 g) the volume is 1.5-2 ml. To follow the supply of the activated nucleotide at the initial steps of the condensation and to the washing of the polymer at every successive step of the synthesis, we used a 100 μ l conductometric cell (3) which is highly sensitive and reliable. Table 1 shows the complete cycle of the operations required for the addition on one nucleotide.

d) Separation and analysis. When the synthesis is completed and when samples are analyzed at the intermediate steps for all the protective groups to be removed, the support is treated with a solution of concentrated ammonium in a closed

Table 1

Nos	Operation	Time	Solvent	Conductivity at the end of the operation (μ S)	Regime
1.	Drying of support with pyridine	0.5-1.0 hour	dry pyridine	0.1-0.5	flow
2.	Addition of preactivated nucleotide	3.5-4.0 hour	same	100 $\pm$ 30	cycling
3.	Washing of excess of nucleotide and condensing agent	30 min	same	5-10	flow
4.	Cleavage of pyrophosphates at internucleotide phosphorus	30-40 min	70% aqueous pyridine	5-10	flow and cycling
5.	Deblocking of 3'-hydroxy group	30 min	alkaline mixture	1500	flow
6.	Washing of support from alkali	30 min	70% aqueous pyridine	20-30	flow
7.	Neutralization of the support	10 min	2% CH ₃ COOH in dry pyridine	5-10	flow

Total time 8 hours

Note. Preactivation of the nucleotide is carried out during operations 4-7

vessel at 50° for 12 hours. The removal of the nucleotide material is carried out by treatment of the polymer by 1% solution of trifluoroacetic acid in methylene chloride at -30° for 10 min. The polymer is washed from the excess of the acid by dry methylene chloride and the mixture of oligonucleotides is extracted first by dry and then 70% aqueous pyridine. Preparative or analytical fractionation was carried out by the Tomlinson and Tener method [16] on DEAE cellulose in linear gradient of sodium chloride, 7 M urea, pH 7.5. The target nucleotide, which is the last to be resolved, is rechromatographed by the same method, pH 3.5. The structure of all the oligonucleotides was substantiated by the modified method of Maxam-Gilbert [9,10] after phosphorylation by γ -³²P-ATP and T-4-polynucleotide kinase.

RESULTS

The results of the synthesis are presented in Table 2.

As can be inferred from the Table, a grafted support allows one to carry out the synthesis of hepta- and pentadeca nucleotides with a good efficiency, i.e. the yield of isola-

Table 2

Nos	Oligonucleotide ($\epsilon \cdot 10^{-3}$)	Quantity		Isolated oligonucleotide		Yield mol. %
		support mg	nucleoside μ mol	A_{260} O.u.	μ mol	
I	CATGTA (67)	310	17	70	1.0	6.1
II	TGGGGGG (81)	200	12	15	0.184	1.54
III	CCCATACA (83)	180	11	30	0.36	3.3
IV	AGGAACCC (92)	200	8	25	0.27	3.5
V	TTCCTTTGA (86)	200	12	60	0.7	5.8
VI	TATAATGCAT (116)	100	5	34	0.3	6
VII	T ₉ CG (98)	125	7.5	110	1.1	14
VIII	T ₁₅ (132)	80	4.5	110	0.83	20

ted and purified target product is 80-300 o.u. ($1\pm 3 \mu$ mol) per gram of the support with the initial load being 50-70 μ mol of the nucleoside. According to the data of the last published work of Gait and Sheppard [5], from 0.4 g of an acrylamide support, 0.2-0.7 μ mol (0.4-2 μ mol/g) of nonanucleotide was obtained with the initial load of the nucleotide being 150-220 μ mol per gram of the support. This indicates that their results and ours are comparable; however, the loss of the nucleotide material in the course of growth of the polynucleotide chain for polar polymers is somewhat greater. This seems to be due to the cleavage of an internucleotide chain under the action of the condensing agents, which was reported previously [11] and is obviously a drawback of the diester method.

In the present work we did not use the protection of the hydroxy group of the oligonucleotide that does not participate in the condensation. Therefore the separation profiles of the reaction mixture obtained in the analysis of each step of the synthesis show not only the peak of the target oligonucleotide but also partial interconversion of each of the oligonucleotides on the support into an oligomer that has acquired another nucleotide.

This does not allow correct determination of the yield

by measuring the ratio of the peak areas on the separation profiles. Besides, the peak ratio reflects the degree of conversion at a certain step without the loss of the nucleotide material due to degradation of the oligonucleotide chain under the action of the condensing agent. Therefore the efficiency of the polymer can be estimated by measuring the ratio of the isolated and purified oligonucleotide of a given length to the quantity of the initial nucleoside bound to the support. This is how the yield of the synthesized oligonucleotides was calculated in this work.

The first five oligonucleotides were synthesized according to the diester scheme and the best results were obtained in the case of polypyrimidine sequence (No. V) and the worst in the synthesis of oligoguanilate (No. II). The fractionation profiles of total products of synthesis II and V are shown in Figs 1 and 2.

Analysis of the reaction mixture at each addition in the first case (II) showed that at none of the steps, a yield exceeding 65-70% was obtained. But in the case of a polypyrimidine sequence (V) all the condensation steps were carried out with a degree of conversion not exceeding 80-85%.

Fig. 1. Fractionation of oligonucleotides on DE-32 in the synthesis of d(T-G-G-G-G-G-G).

Fig. 2. Fractionation of oligonucleotides on DE-32 in the synthesis of d(T-T-C-C-T-T-T-G-A).

Fig. 3.

Fractionation on DE-32 of oligonucleotides in the synthesis d(T-A-T-A-A-T-G-C-A-T)

Fig. 4.

Fractionation on Aminochrom AC-10/100 of oligonucleotides in the synthesis d(T)₁₅

EXPERIMENTAL

Material and Methods

Use was made of deoxynucleoside-5'-phosphates and deoxynucleoside manufactured by Serva. Fully protected deoxynucleoside-5'-phosphates were prepared as described by Khorana et al [14, 15]. TPS was purified by passing the solution in chloroform through burnt silicagel followed by recrystallization from heptane. Dry pyridine was prepared by keeping over P₂O₅ and by two distillations over BaO. Pyridine was kept over molecular sieves 4A in 6x80 cm columns, from which it was fed through teflon tubes to the synthesizer. Chromatographic separation of the oligonucleotide mixtures after their removal from the polymer and the rechromatography of the isolated oligonucleotide was performed as described by Tompkinson and Tener [16] on DE-32 DEAE cellulose (Whatman) in 0.9x20 cm columns. IR and UV spectra were taken in UR-20 and "Spectord" spectrophotometres of Karl Zeiss company (DDR). The column reactor was 16x60 mm.

1. Synthesis of the support

1. The grafting of styrene on the surface of polytetra-

fluorethylene was carried out by the modified method of [17] from styrene vapour in vacuo. The degree of the grafting was regulated by the styrene-teflon ratio. The total irradiation dose from a ^{60}Co source was 0.5-1.5 Mrad. After the grafting was completed, the copolymer was washed by boiling in benzene and then on a filter by dichlorethane, ether and dried in vacuo. The quantity of the grafted polystyrene was determined by the increment in weight and by IR spectrometry. To this end, 50 mg of the copolymer were pressed into a tablet ($d = 10$ mm); then the IR spectrum in the $1400\text{--}1700\text{ cm}^{-1}$ range was measured. The equal intensities of the 1560 cm^{-1} band of the teflon and the 1590 cm^{-1} band of polystyrene corresponded to 100 mg/g of the grafted material.

2. Polymer-ketone. In a 100 ml round bottom flask the acylating complex was prepared by mixing 6.65 g (50 mmol) of AlCl_3 and 5.7 ml (48 mmol) of benzoyl chloride in 80 ml of nitrobenzene. The flask was supplemented with 20 g of the copolymer (90 mg of polystyrene/g), liberated from air by a pump and the mixture was stirred for 15 min. The polymer was filtered out, washed by nitrobenzene (2x30 ml), by a mixture of alcohol, and hydrogen chloride (3:1) (3x30 ml), by alcohol (3x30 ml) and ether (4x30 ml). The polymer was dried in vacuo. In the IR spectrum there appears a band at 1665 cm^{-1} which is approximately equal in intensity to the 1460 cm^{-1} band.

3. Polymer-p-methoxytritylcarbinol. The polymer-ketone from the previous experiment was treated with a solution of p-methoxyphenylmagnesiumbromide obtained from 2.4 g (0.1 g) of magnesium and 12 ml (0.1 mol) of freshly distilled p-anizolbromide in 80 ml dry THF. The mixture was agitated for 1 hour and left to stand for the night. The polymer was washed by THF (3x30) on the filter, with a mixture of acetic acid and ethanol 1:5 (4x30 ml), alcohol (4x20 ml) and ether (4x30 ml) and dried in vacuo. In the IR spectrum of the polymer there disappears the absorption at 1665 cm^{-1} and there appears a new broad band with the maximum at 3580 cm^{-1} .

Addition of nucleosides

3 g of the support were applied on the column reactor and washed by slowly passing through it 10-15 ml of dry pyridine. Then 6 ml of a mixture of pyridine and thionyl chloride (5:1) obtained at -10°C were applied to the column and recycled for 5 min. The polymer was washed by passing dry pyridine until the conductivity was 1-2 μ s and a solution of 1.5 mmol of the corresponding N-protected nucleoside in 6-8 ml of dry pyridine were applied to the column. The nucleoside was predried by a 3-fold evaporation of the pyridine in vacuo. After the synthesizer was filled with the solution of the nucleoside, the system was switched over to the recycling which is carried out for 16-20 hours at 20°C. The synthesizer was supplemented with 0.5 ml of dry methanol and the mixture was recycled for 1 hour. The polymer was washed by pyridine, alcohol and ether.

Removal of N-protecting groups

To a weighed amount of the support (20-300 mg) with the coupled nucleoside (or oligonucleotide) wetted with pyridine, 5-10 ml of concentrated ammonia were added. The reactor was sealed and kept at 50° for 12 hours. The polymer was washed on a filter with a solution of ammonia, water, alcohol, ether and dried in vacuo.

Determination of the support capacity

A thoroughly dried amount (50 mg) of the support with the deprotected nucleoside was placed in a column (6x50 mm) with a filter and treated (moisture excluded) with 10 ml of 1% solution of trifluoroacetic acid in methylene chloride cooled to -40°C. The polymer was washed with 10 ml of pure methylene chloride and the pooled filtrate was evaporated to dryness. The polymer was extracted with dry (5-7 ml) and then with 70% aqueous pyridine (10 ml); the filtrate was collected into the same vessel. The pyridine was evaporated to dryness and the precipitate was evaporated with concentrated ammonia. The content of the vessel was dissolved in a certain

amount of water and the quantity of the nucleoside was measured in a spectrophotometre. The calculations were carried out according to the following formula:

quantity of nucleoside mmol/g = $\frac{V \cdot D \cdot 1000}{E \cdot a}$, where D is the

optical density; V - is dilution; (ml) E is the molar extinction coefficient; a is a weighed amount (mg) of the polymer. The quantity of the added nucleoside per gram of the support used in the present work was: dT 75 μ mol, danC 56 μ mol, dbzA 45 μ mol.

The removal of the mixture of oligonucleotides from the support after termination of the synthesis was carried out in the same way.

Condensation. 0.5 g of the polymer with the bound nucleoside (25-35 μ mol) was placed in a column of a synthesizer and dried by passing pyridine until the conductivity at the output of the system was 0.3 μ S (operation 1, Table 1). 0.3-0.5 mmol of the respective protected nucleotide were dried in a flask by evaporating dry pyridine (3-4 ml portions); the flask was filled with dry argon. The flask was quickly filled with 0.5-0.8 mmol of TPS and preactivated was carried out for 1.5-2.0 hour. The solution of activated nucleotide was pumped, under the pressure of dry argon, to the synthesizer until the conductivity at the output was 5 μ S, after which the device was switched over to recycling for 3.5-4 hours (operation 2). The polymer was washed consecutively by dry pyridine (operation 3), 70% aqueous pyridine (operation 4). To remove the 3'-acetyl group, 1 M solution of tetraethylammonium hydroxide in 70% aqueous pyridine was passed through the system for 30 min (operation 5). The support was washed from alkali with 70% aqueous pyridine until the conductivity was 20 μ S (operation 6) and then 2% solution of acetic acid in dry pyridine (operation 7).

Purification and analysis

After preparative fractionation in a column packed with DEAE cellulose as described by Tomlinson and Tener [16] (Figs 1-4) of a mixture of oligonucleotides cleaved from the

Fig. 5 d(θ-C-C-A-T-A-C-A).

Fig. 6 d(A-G-G-A-A-C-C-C).

Fig 7.d(T-G-G-G-G-G-G).

Fig 8.d(T-T-C-C-T-T-T-G-A).

Fig. 5-8. Modifications are; 1. A→C 2. G 3. A+G 4. T+C 5. C
 Autoradiographs of polyacrylamide gels from sequence
 analyses of oligonucleotides II-V.

Fig. 9. Autoradiograph from sequence analyses of d(T-A-T-A-A-T-G-C-A-T). Dimensions; 1.electrophoresis on cellulose acetate 2.homochromatography on DEAE-cellulose.

polymeric support; the zone of the target product was rechromatographed by the same method. A sample of each oligonucleotide was treated with γ - ^{32}P -ATP and T4 polynucleotide kinase; the homogeneity of the preparation was checked by electrophoresis in polyacrylamide gel. The preparation isolated in this way was used to determine the base sequence by the method of Maxam-Gilbert [10]. After chemical modification of the respective bases and degradation of the polynucleotide chain, the products were fractionated by electrophoresis in polyacrylamide gel. The structure is read off from the lower edge of the phoregram (Figs 5-8). For V1 structure was checked by the method Sanger [18]. (Fig. 9).

REFERENCES AND NOTES

- 1 H.Hayatsu, H.G. Khorana (1966) J.Amer.Chem.Soc., 88, 3182-3183
- 2 H.Koster, A. Pollak, F. Cramer (1974) Liebigs Ann. Chem., 959-966.
- 3 V.K. Potapov, O.G. Chakhmakhcheva, Z.A. Shabarova, M.A. Prokofiev (1971) Dokl.Acad.Nauk SSSR, 196, 360-363.

- 4 M.J.Gait, R.C. Sheppard (1977) Nucleic Acids Research 4, 1135-1158.
 - 5 M.J. Gait, R.C. Sheppard (1977) Nucleic Acids Research 4, 4391-4410.
 - 6 Ch.K. Narang, K. Brunfeldt, K.E. Norris (1977) Tetrahedron Letts, 21,1819-1822.
 - 7 V.K. Potapov, S.I. Turkin, V.P. Veiko, Z.A. Shabarova, (1978) Dokl.Acad.Nauk SSSR 241, 1352-1354.
 - 8 V.K. Potapov, S.I. Turkin, Z.A. Shabarova (1972) Zhur.Ob-schei Khim., 42, 2349.
 - 9 Abbreviation used in this paper: p - polymeric support; pN(Ac) N-blocked 3'-O-acetyl-2'-deoxynucleoside-5-phosphate; TPS-triisopropylbenzenesulfonyl chloride, us-microsims.
- The method of [10] was modified for analysis of short oligonucleotides in the laboratory of Dr. A.A. Baev of the Institute of Molecular Biology of the USSR Academy of Sciences by Dr. K.G. Skryabin et al. to whom we are very grateful for the analysis.
- 10 W. Haseltine, A. Maxam, W. Gilbert (1977) Proc.Natl.Acad. Sci. USA 74, 989-993
 - 11 J. Hashmann, H.G. Khorana (1968) J.Amer.Chem.Soc. 91, 2749-2757.
 - 12 G.F. Mishenina, V.V. Samukov, L.N. Semenova, T.N. Shubina, (1978) Bioorgan. Khim. 4, 735-739.
 - 13 E. Ohtsuka, S. Morioka, M. Ikehara (1972) J.Amer.Chem. Soc. 94, 3229-3233.
 - 14 H. Weber, H.G. Khorana (1972) J.Mol.Biol. 72, 219-249.
 - 15 H. Buchi, H.G. Khorana (1972) J.Mol.Biol. 72, 251-288.
 - 16 R.V. Tomplinson and G.M. Tener (1963) Biochemistry 2, 697-702.
 - 17 D.S. Ballontine, A. Glines, D.J. Metz (1956) J.Polym.Sci. 19, 219
 - 18 G.G. Brownlee and F. Sanger (1969) Eur.J.Biochem. 11, 395-399