SYNTHESIS AND PURIFICATION OF OLIGODEOXYRIBONUCLEOTIDES: A MODIFIED APPROACH

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ABSTRACT

A reduction in monomer consumption in the solid phase synthesis of oligodeoxyribonucleotides has been achieved by introducing a repeat coupling approach. Additionally, a method of anion exchange HPLC– purification of the synthesized nucleotides at room temperature has been developed in which a highly volatile buffer was used as the eluent. An alternative approach of HPLC purification of oligonucleotides is also reported.

INTRODUCTION

A TYPICAL solid phase synthesis of oligodeoxyribonucleotide, an important ingredient of genetic engineering, involves two essential steps, viz (i) chain building on the solid support through coupling reactions and (ii) HPLC purification of the synthetic product. Although, both these steps are well documented in literature[1–9] very little attention has been paid to economize these procedures. Here we present an economized approach to the synthesis and a convenient and easier method of HPLC purification of the synthesized sequences.

MATERIALS AND METHODS

Apparatus:

The Beckman 342 gradient HPLC system comprising of two 112 pumps, a 340 organizer, 420 system controller, 210 sample injector and 165 variable wavelength detector was used for all studies. Signals for two different wavelengths viz 260 and 280 nm for analytical and 260 and 295 nm for the preparative mode, were simultaneously recorded using a double channel strip chart recorder. The anion exchange (AE) HPLC purification was carried out using ultrasphere AX-10 or Partisil SAX columns (25 cm long, 4.6 mm i.d., 10 μm particle size). A C_{18}-reversed phase (RP) ultrasphere ODS column (25 cm long, 4.6 mm i.d., and 5 μm particle size), in conjunction with a precolumn has been used for the RP–HPLC analysis.

Materials:

Formamide was from Fluka AG. Common reagent grade acetonitrile, triethylamine (TEA), analytical reagent grade acetic acid and ammonium acetate were from BDH, E.Merck and SM chemicals (all of Indian make). Both acetonitrile and TEA were distilled before use. The 2M stock solutions of TEAA (pH 6.8) and TEAB (pH 7.2) were prepared as published elsewhere[10]. Since TEAB buffer tends to change its pH on storage, CO_2 was bubbled just before its use.

Mononucleoside-phosphoramidites were prepared by suitable modifications of the existing methods. Oligonucleotides were synthesized on silica support using phosphoramidite approach. Nucleotide chains were removed from the silica support and subsequent deacylation was carried out by ammonia treatment. Oligomers were dissolved in the elution buffer or formamide for HPLC purification. Denaturation prior to injection of the oligonucleotide solution as such or in the presence of 80% formamide was effected by heating it to 90–95°C for 3–5 min.

Radioactive labelling and electrophoresis:

The oligonucleotides were end-labelled using [γ-^{32}P]-ATP and analysed by polyacrylamide gel electrophoresis according to Maniatis *et al*[11].

RESULTS AND DISCUSSION

Synthesis of oligonucleotides by repeat coupling approach:

Solid phase synthesis of oligodeoxyribonucleotides using a manual open column demands that a large excess of the incoming monomer (10–20 times in excess over the support bound nucleotide chain[1–5] bearing 5′ – protecting group) be used to get a high coupling yield ($\geq$ 95%), of which only 5–10% is actually used in the coupling reaction. We have earlier developed a repeat coupling approach[10] the usefulness of which is further demonstrated here through the synthesis of *d*(*TTAA*), *d*(*TATA*),

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Table 1 *Synthesis of deoxyoligonucleotides by repeat coupling approach using phosphoramidite chemistry*

5′–3′ Sequence	Specific Coupling step[a]	Ratio of incoming monomer & support bound chain[b]	Coupling yield in per cent[c]	Overall yield based on 1st base
d(TTAA)	A2 to A1	5	89	
	T3 to A2	7	91	
	T4 to T3	(5+1)[d]=6	94	76
d(TATA)	T2 to A1	(4+1)[d]=5	95	
	A3 to T2	(6+1)[d]=7	96	
	T4 to A3	(3+1)[d]=4	94	86
d(GGCC)	C2 to C1	(6+1)[d]=7	95	
	G3 to C2	6	81	
	G4 to G3	6	85	65
d(CCGGCCGG)	G2 to G1	7	91	
	C3 to G2	7	88	
	C4 to C3	5	88	
	G5 to C4	10	94	
	G6 to G5	7	90	
	C7 to G6	(5+1)[d]=6	94	
	C8 to C7	(5+1)[d]=6	95	53

[a]Bases are numbered from 3′ to 5′ direction; [b]Support bound chain refers to the sequence having 5′–OH groups after detritylation; [c]Coupling yield for each step of chain elongation was determined on the basis of colour reaction of the di methoxy trityl group at 495 nm[3]; [d]Refers to repeat coupling.

d(GGCC) and *d(CCGGCCGG)* (table 1). The chain building for these oligomers was carried out manually (open column) on a silica support using phosphoramidite chemistry. It is clearly evident from table 1 that the repeat coupling approach enabled us to use only 4–7 fold excess of monomer with practically no concomitant loss of yield. This amounts to a 60–65% saving of the monomer per base addition.

Purification by anion exchange HPLC using volatile buffers:

The detrytilated DNA-octamer *d(CCGGCCGG)* was purified by the AE-HPLC method using TEAB or TEAA buffer. The sample injected was denatured either by heating or by adding a denaturant viz formamide. Figure 1 shows the chromatogram of the oligomer thus purified at 23°C (trace a) and 50°C (trace b). Comparison of these two profiles shows that the analysis can be efficiently carried out at either ambient or elevated temperature.

The efficacy of the AE-HPLC method was checked by injecting the once purified *d(CCGGCCGG)* onto the RP-C_{18} HPLC column (figure 2) as the RP-C_{18} method has been shown to be of comparable sensitivity to the two-dimensional method (homochromatography[12]) of determining sample homogeneity[6]. The observation of only one major peak in figure 2 shows that our AE-HPLC technique affords considerable degree of purification through a single injection of a fairly complicated mixture. The purified octamer, on autoradiography, showed a single spot (figure 3, lane b) which reaffirmed the purity of the compound. The sequence was further characterized by subjecting it to cleavage by the restriction endonuclease *Hae* III (GG$^{\downarrow}$CC) (figure 4). As expected the digested product moved like a tetramer. The applicability of the present AE-HPLC approach to other self-complementary G-containing oligomers was tested by employing this technique for purifying the DNA tetramer, *d(CCGG)*. Autoradiography of the once purified product (figure 3, lane a) showed the product to be homogeneous.

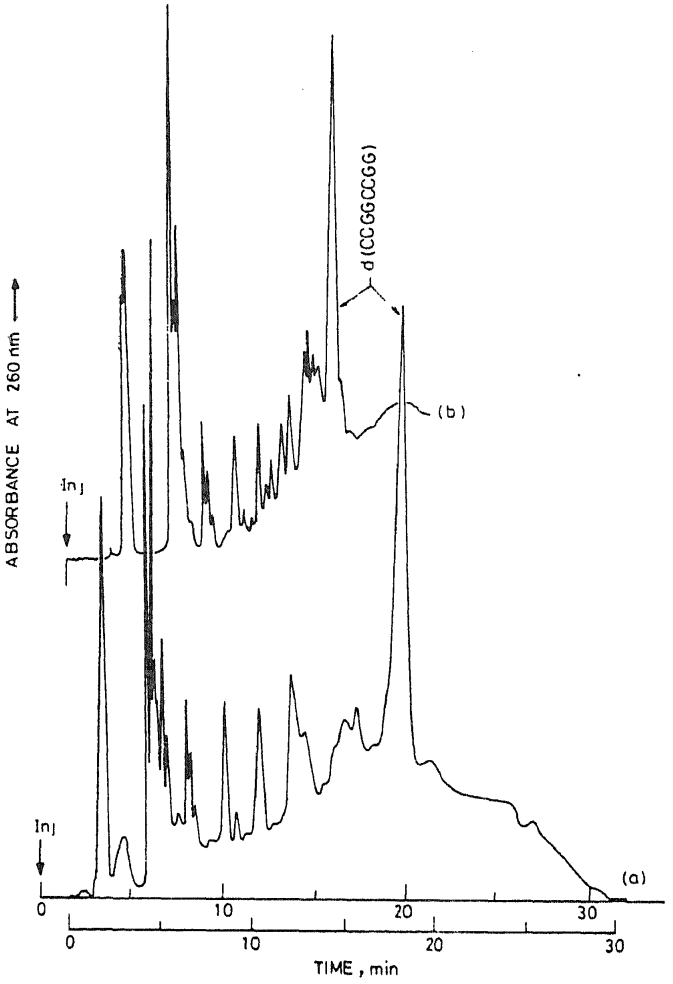


Figure 1. HPLC purification of the denatured *d*(*CCGGCCGG*). Detection: Absorbance at 260 nm. Range: 0.2 AUFS. Flow-rate: 1 ml/min. Column: Anion exchange-Ultrasil AX. For (a) — Eluent: 0–100% of 2M TEAB in water. Temp: 23°C (ambient). For (b) — Eluent: 0–100% of TEAA in water. Temp: 50°C.

Other common volatile buffers, like ammonium acetate, can easily substitute TEAA or TEAB of our method and this is illustrated in figure 5.

Anion-exchange columns are of importance to the purification of the G-rich self-complementary sequences as their recovery from reversed phase (RP) HPLC columns are often unsatisfactory[6]. Conventionally, the GC-rich sequences were purified by AE-HPLC at elevated temperatures (this being necessary to keep the oligonucleotide in the denatured state) using a non-volatile buffer[7,8]. However, the usage of AE-HPLC columns at elevated temperatures with non-volatile buffers makes the recovery of the purified samples, especially of the smaller sequences, troublesome. Our method of injecting heat-denatured samples and purifying using a volatile buffer like TEAA or TEAB overcomes the limitations, otherwise commonly faced in AE-

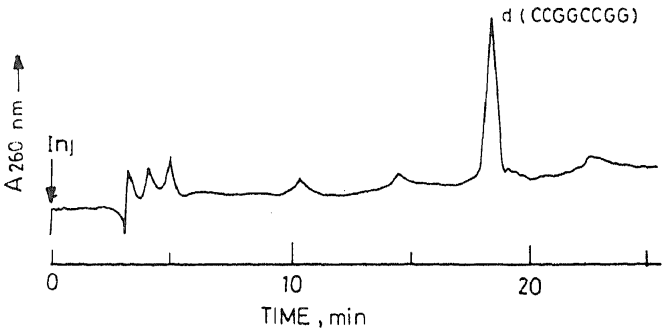
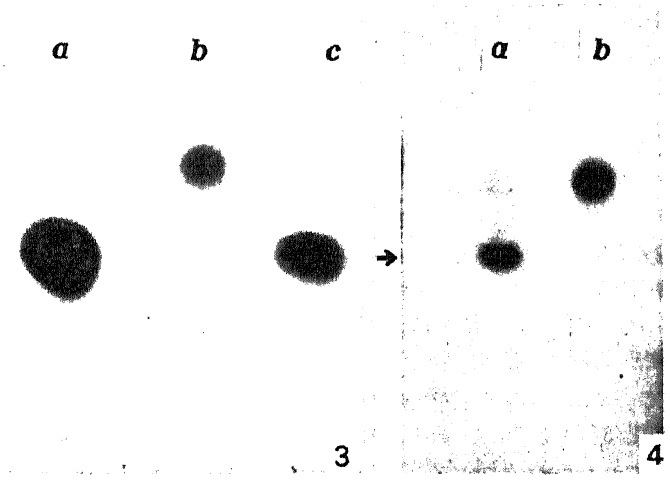


Figure 2. RP-HPLC analysis of AE-HPLC purified *d*(*CCGGCCGG*) Column: C_{18}–Reversed phase ultrasphere ODS. Eluent: Acetonitrile in 0.1M TEAA – 4.5% for 5 min, 4.5–8.5% in 16 min, 8.5–12% in 5 min. Temperature 50°C.

HPLC purifications using nonvolatile buffers. Since this method is equally effective at ambient temperature as well, the problems that might arise due to the use of high concentrations of buffers like TEAA at elevated temperatures are also overcome. We have found that use of high concentrations of TEAA at elevated temperature (≥50°C) can be detrimental to the silica-based columns. Further, we find that for dilute solutions of the oligomers where intermolecular interactions are minimal, addition of denaturant to the sample prior to injection is generally not necessary.

A similar method of using volatile buffer at ambient temperature for RP-HPLC has been reported by us earlier[10].



Figures 3 and 4. 3. Autoradiogram of ^{32}P-labelled PAGE-urea analysed oligonucleotides. Lanes: **(a)** *d*(*CCGG*); **(b)** *d*(*CCGGCCGG*) and **(c)** *d*(*GGCC*); **4.** Autoradiogram of *Hae* III digested ^{32}P-labelled *d*(*CCGGCCGG*) Lanes **(a)**–octamer digested with *Hae* III, **(b)**–octamer control. Arrow indicates the position of the tetramer marker.

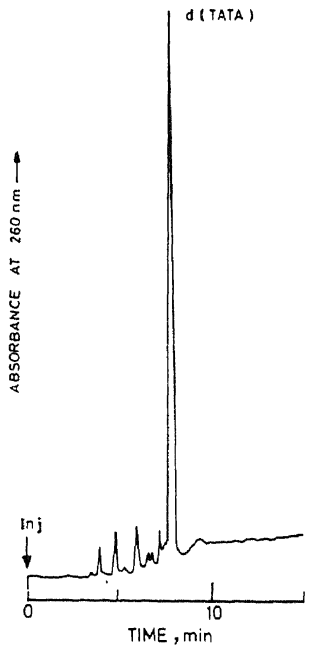


Figure 5. AE-HPLC analysis of *d(TATA)*. Column: Partisil SAX. Eluent: A 0–100% gradient (in 15 min.) of 2M ammonium acetate (pH 6.5) in water. Temperature: 23°C (ambient).

An alternative HPLC approach:

The tetramer *d(GGCC)* was purified by using a combination of RP-HPLC and AE-HPLC. The nucleotide chains after removal from the silica support was injected on to an RP column and the elution carried out using a mixture of acetonitrile and TEAA. This initial purification takes advantage of the lipophilicity of the DMTr group which in a synthetic mixture is present only in the final sequence of the synthesis. The sequence of interest, therefore, becomes the longest retained species and hence easily identifiable. The once purified sample after detritylation with 80% acetic acid was subjected to ion-exchange HPLC following the method described earlier in this paper. The tetramer *d(GGCC)* thus purified was found to be very pure by autoradiography (figure 3, lane c). It may be pointed out that although the purification of detritylated oligonucleotides by AE-HPLC followed by RP-HPLC has been reported earlier[8,13–16], the reverse order of columns i.e. RP-followed by AE-HPLC, was considered to be difficult or even often impossible[8,14–16]. This problem has been overcome in the present study by resorting to the RP-HPLC prior to the detritylation and then carrying out the AE-HPLC analysis.

CONCLUSION

In summary, a practical approach for the cost-efficient synthesis of oligodeoxyribonucleotides, especially for large scale batches of smaller sequences, has been developed. The method of injecting samples in the denatured state and their elution with volatile buffers not only overcomes the earlier mentioned limitations of the AE-HPLC method, but also makes this approach attractive for the purification of self-complementary oligomers. The alternative HPLC approach reported here was designed to exploit the known advantages of both the RP– and AE-HPLC methods.

ACKNOWLEDGEMENTS

The authors are grateful to Prof. H. Seliger, University of Ulm, FRG, for introducing them to the phosphoramidite chemistry. Thanks are also due to Rajani Srinivasan and K. Usha for the technical assistance received. LPK thanks CSIR, New Delhi, for a fellowship. Financial assistance from CSIR, New Delhi through a grant to SKB is gratefully acknowledged.

15 December 1986; Revised 13 March 1987

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