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PURIFICATION OF SYNTHETIC OLIGONUCLEOTIDES ON A WEAK ION-EXCHANGE COLUMN

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SUMMARY

A weak ion-exchange column (PVDI 4000-5) was used to purify oligonucleotides of relatively large size. The purification of polynucleotides was very rapid, they were separated according to size and retained all their properties relevant to genetic engineering experiments.

INTRODUCTION

Molecular biology is developing very rapidly and synthetic oligonucleotides are required for numerous types of experiments. The synthesis of oligonucleotides can now be performed rapidly, owing to automatic apparatus, but although the yield of each reaction is quite good it never approaches 100%. This means that synthetic polynucleotides are generally contaminated by undesired species. In some cases, purification is performed on protected nucleotides, and separation based on hydrophobicity is carried out on reversed-phase columns[1]. However, in other cases the purification must be performed on deprotected polynucleotides, and reversed-phase separation is then difficult for two reasons: first it can be achieved only for small oligonucleotides; secondly, since it proceeds according to the composition as well as the number of bases, the resulting chromatogram is difficult to interpret[2].

It has been shown that ion-exchange chromatography allows the separation of oligonucleotides according to their sizes. However, this method gives poor resolution for oligonucleotides containing more than fifteen nucleotides. On the other hand, it is possible to separate rapidly relatively large oligonucleotides according to size by using a weakly basic ion-exchange column. This property has been exploited successfully by different authors[4–6], using different columns. We have chosen to use a new packing material, designed for protein separation, and developed a procedure that allows the separation of synthetic oligonucleotides according to their sizes. The purification is more rapid than by electrophoresis, the yield is quite good and the biological activity is totally retained.

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EXPERIMENTAL

Materials

All experiments were performed with a Gilson apparatus equipped with two high performance liquid chromatography (HPLC) pumps (Model 302) and an UV detector set at 254 nm (Model 111B). The PVDI 4000-5 columns (100 mm × 4.6 mm; Société Française de Chromato Colonne, Neuilly-Plaisance, France) are weak silica-based ion exchangers, with a large pore size of 4000 Å. The packing was made by the polymerization of poly(vinylimidazole). Oligonucleotides were synthesized on a Model 380B apparatus (Applied Biosystem, Roissy C. de Gaule, France). Ultra pure urea was obtained from Bethesda Research Labs. (Cergy Pontoise, France).

Methods

The columns were eluted with a gradient of salt (ammonium acetate pH 6.5), starting with 0.2 *M* ammonium acetate in 5 *M* urea (eluent A) and increasing to 2 *M* ammonium acetate in 5 *M* urea (eluent B). The gradient slope and time dependency is described in the figure legends. The flow-rate was 1 ml/min, except where stated otherwise.

Analysis of oligonucleotides by polyacrylamide gel electrophoresis was performed on 20% acrylamide in 8 *M* urea[7].

RESULTS

We have studied the efficiency of PVDI 4000 for the separation of oligonucleotides. For this purpose we used poly(A) (A = adenosine), hydrolysed by boiling in water, to obtain a range of oligonucleotides. Elution was performed by increasing the salt concentration linearly from 0 to 40% of eluent B. Under these conditions, sep-

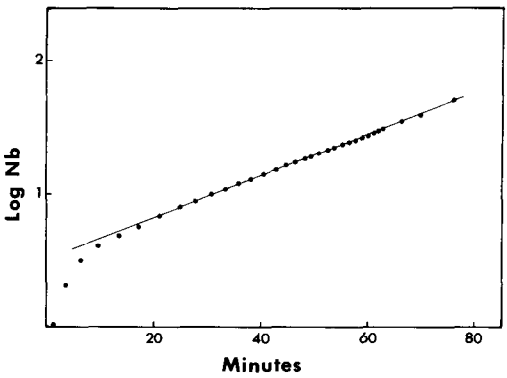


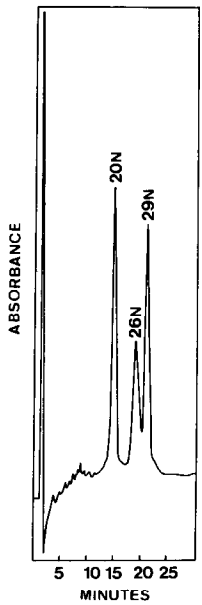
Fig. 1. Relationship between the retention time and the number of nucleotides in an oligonucleotide. Oligonucleotides of different sizes, varying by one nucleotide, were generated by hydrolysis of poly(A). A 150-μg amount of this mixture was analyzed on the PVDI 4000 column (100 mm × 4.6 mm). After equilibration in eluent A (0.2 *M* ammonium acetate in 5 *M* urea), elution was performed by increasing the amount of eluent B (2 *M* ammonium acetate in 5 *M* urea) from 0 to 40% in 90 min at a flow-rate of 1 ml/min. The logarithm of the number of nucleotides is plotted against the elution time. Each individual point represents the top of an elution peak.

aration was obtained for molecules up to the 50-mer. The separation is quite good in the degree of polymerization range 20–35, *i.e.*, the degree of polymerization of the majority of synthetic oligonucleotides. Fig. 1 shows the relationship between the retention time and the number of nucleotides, which is logarithmic within the range of 5–50 nucleotides.

We have used this column to purify synthetic oligonucleotides. Each oligonucleotide was purified in less than 15 min (not shown). Although the desired nucleotide represents *ca.* 85% of the starting mixture, purification is necessary if it is to be used for sequencing. It is noteworthy that electrophoresis, the method generally employed in molecular biology, requires at least 24 h for migration and elution. The preparation presented here was completed in 1 h, including ethanol precipitation, giving a purified oligonucleotide suitable for further applications. For example, these oligonucleotides were successfully used for sequence determinations.

In order to perform library screening, we have synthesized oligonucleotides containing 6-methyladenine and inosine. These rare bases decreased the yield of the reaction, resulting in a very small amount of the desired oligonucleotides. Under these conditions the desired oligonucleotides represent less than 5% of the total nucleic acids. Fig. 2 shows their separation obtained in a few minutes. Because the separation is achieved according to the sizes of the molecules, identification of each nucleotide is very easy. The desired oligonucleotide is always the longest species. The purified oligonucleotides were used for library screening.

Fig. 2. Purification of a complex mixture of oligonucleotides. Two oligonucleotides were synthesized containing the rare bases 6-methyladenine and inosine (TCIGGIGCIAmGITAmGTCIGGIGTICC and TCIGGIGCIAmGITAmATCIGGIGTICC). Purification was performed on the weak ion-exchanger PVDI 4000 (100 mm × 4.6 mm, pore size 400 Å) by gradient elution, increasing the amount of eluent B (see Fig. 1) from 0 to 50% in 20 min at a flow-rate of 1 ml/min. A 50-μg amount of oligonucleotides was chromatographed. After separation the different components were analysed by acrylamide gel electrophoresis. The expected oligonucleotide was the longest (arrow) and is purified in one step.



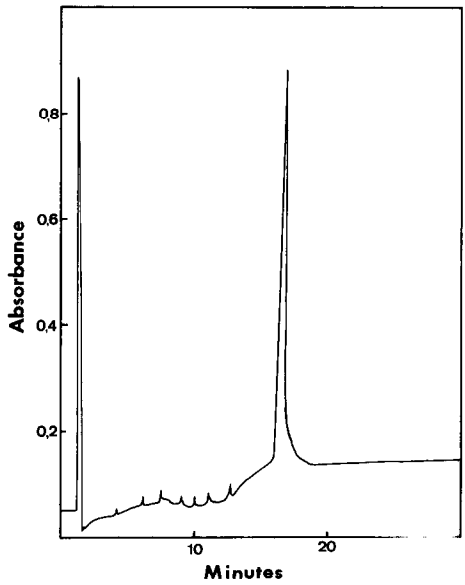


Fig. 3. Separation of three oligonucleotides having chain lengths of 20, 26 and 29 nucleotides. A 2-μg amount of each oligonucleotide was mixed and analysed on a PVDI 4000 column. Gradient elution was performed by increasing the amount of eluent B (see Fig. 1) from 0 to 25% in 35 min at a flow-rate of 1 ml/min.

Fig. 4. Analysis of a self-associating 12-mer oligonucleotide (CCCGAATTCGGG). Gradient elution was performed by increasing the amount of eluent B to 50% in 30 min. For other conditions see Fig. 1 and the Experimental section.

The resolution can be further improved by changing the elution gradient. Fig. 3 shows an example of a total separation of three different oligonucleotides ranging in size from 20 to 29 residues. The recovery of oligonucleotides is excellent, *ca.* 90%, in each case. They precipitate readily after the addition of two volumes of ethanol and can be used for sequence determination, library screening, linker addition, radioactive labelling or site-directed mutagenesis.

In the course of analysis and purification of the oligonucleotides we have used self-complementary oligonucleotides containing the recognition sequence of EcoRI restriction enzyme: GAATTC. The behaviour of this molecule is depicted in Fig. 4. This oligonucleotide having twelve residues exhibits the retention time observed for a 24-mer. This result implies that, in spite of the presence of 5 *M* urea, the two strands of the DNA were still hybridized and the number of phosphates was therefore twice as high.

CONCLUSIONS

A poly(vinylimidazole)-based column (PVDI 4000) originally designed for protein separation is very useful for the purification of oligonucleotides used in genetic engineering. Although other materials achieve approximately the same degree of

separation[4–6] they are generally more expensive. The biological activity of the purified products was also tested. The use of high pH[4] may result in the degradation of oligoribonucleotides, although oligodeoxynucleotides are not affected. Conversely, the use of low pH may result in a partial depurination of the nucleic acids. We have employed a nearly neutral solution (pH between 6.5 and 7.0) containing urea. These conditions are generally used for the preparation of large cellular nucleic acids[7].

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REFERENCES

1 F. Chow, T. Kempe and G. Palm, *Nucleic Acids Res.*, 9 (1981) 2807–2816.
2 J. Crowther, in P. Brown (Editor), *HPLC in Nucleic Acid Research*, Marcel Dekker; New York, Basel, 1984.
3 C. Newton, A. Greene, G. Heathcliffe, T. Atkinson, D. Holland, A. Markham and M. Edge, *Anal. Biochem.*, 129 (1983) 22–30.
4 Y. Kato, T. Kitamura, A. Mitsui, Y. Yamasaki, T. Hashimoto, T. Murotsu, S. Fukushige and K. Matsubara, *J. Chromatogr.*, 447 (1988) 212–220.
5 D. Drager and F. Regnier, *Anal. Biochem.*, 45 (1985) 47–56.
6 M. Colpan, J. Schumacher, W. Bruggemann, H. Sanger and D. Riesner, *Anal. Biochem.*, 131 (1983) 257–263.
7 T. Maniatis, E. Fritsch and J. Sambrook, *Molecular Cloning a Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982.