Journal of Chromatographic Science, Vol. 37, July 1999

Separation of Nucleotide Oligomers by Unitary Anion-Exchange

C.H. Lochmüller[1]*, **Qicai Liu**[1], **Lingyan Huang**[2], and **Ying Li**[1]

[1]Gross Chemical Laboratory, Duke University, Durham, NC 27708 and [2]Integrated DNA Technologies, Inc., 1710 Commercial Park, Coralville, IA 52241

Abstract

This paper is the first report on the retention behavior of synthetic oligonucleotides and nucleotide oligomers on a continuous-bed-matrix, strong-anion-exchange column. The separation mechanism is predominantly an anion-exchange process, but hydrophobic interaction plays a role as well. The separation is based on the chain length of the oligonucleotide. Both the addition of organic mobile phase modifiers and changes in column temperature affect the retention of oligomers significantly. A volatile buffer system (e.g., triethylamine acetate) could be employed to purify oligonucleotides, and no desalting procedure would be required after the column separation step. The recoveries from the separation are 70% or higher. The maximum loading capacity of an analytical column (35 × 7-mm i.d.) was found to be more than 366 µg.

Introduction

Oligonucleotides are of increasing importance in the rapidly growing field of gene technology. There are now automated synthesizers affording the easy and relatively quick preparation of tailored materials, but for many application purposes, the synthesized products require further purification to be of use. The result is that various separation strategies have been developed for the separation and purification of synthetic polynucleotides. Polyacrylamide gel electrophoresis (PAGE) (1) is the most commonly used method for the purification of nucleotides because of its simplicity and high resolution. It is a labor-intensive process, and its recoveries are generally low. Capillary gel electrophoresis provides the highest resolution among the current methods, but its sample loading capacity is very limited, and its application to the preparative or semipreparative purification of nucleotides is therefore limited. High-performance liquid chromatography (HPLC) has also been employed (2). Reversed-phase HPLC (RP-HPLC) can be used to separate "trityl-on" synthetic nucleotides because of the hydrophobicity of the protecting group, but its resolution is sometimes limited (3). An added complication is that RP-HPLC does not necessarily result in separations according to nucleotide chain length. This can lead to confusion regarding the elution positions of the substrates and products. Reversed-phase ion-pair HPLC has been used in oligonucleotide separations. The resolution for oligomers of up to 15 bases is high, but for longer oligomers, the resolution decreases (4). Anion-exchange HPLC is more widely used (4–7). It is primarily based on the charge of nucleotides, and the longer oligonucleotide possesses a larger net charge and exhibits a longer retention time. Spectral contrast techniques combined with anion-exchange HPLC separation have been used for the analysis of oligomers (8).

Traditional ion-exchange stationary phases are particle-based materials. They can be either porous or nonporous small particles with a typical size in the range of 1.5–10 µm, and only the surface charges of the particles serve as ion-exchange sites. Such materials have limited sample loading capacity. Dionex Corporation (Sunnyvale, CA) introduced a strong anion-exchange column, the NucleoPac PA-100, in the mid-1990s. NucleoPac PA-100 is packed with a pellicular anion-exchange resin (13-µm diameter). The column has a high resolution and can be operated under strongly denaturing conditions, such as high temperature (up to 90°C) or high pH (up to pH 12.4), but its loading capacity is limited (9). Recently, Bio-Rad Laboratories (Hercules, CA) introduced anion-exchange columns that contain a single, advanced polymer matrix. The continuous bed matrix is formed by the polymerization of monomers and ionomers directly into the chromatographic column. The polymer chains form aggregates that coalesce into a dense, homogeneous network of interconnected nodules consisting of microparticles. Because of the high cross-linking of the polymer matrix, the surface of each nodule is nonporous, yet the microparticles-based homogeneous matrix provides higher ion-exchange-site density than traditional particle-based phases. The voids or channels between the nodules are large enough to permit a high hydrodynamic flow, and the backpressure is low. This material has been

* Author to whom correspondence should be addressed: e-mail morgan@psc.sc.edu.

used successfully in protein separations, but no use in polynucleotide separation has been reported. This paper reports the first use of this continuous-bed-matrix UNO Q1 anion-exchange column for the separation of synthetic oligonucleotides and nucleotide oligomers.

Experimental

The chromatography system consisted of an LKB 2150 HPLC pump (LKBProdkter AB, Bromma, Sweden) controlled by an LKB 2152 HPLC controller and an LKB 2158 Uvicord SD detector operated at 254 nm or an LC 85 ultraviolet (UV) detector from Perkin-Elmer (Norwalk, CT). Chromatography data were acquired by a computer using a PE-Nelson chromatography package (Perkin-Elmer, Norwalk, CT). The column was a UNO Q1 anion-exchange column from Bio-Rad Laboratories.

HPLC-grade acetonitrile, HPLC-grade water, analytical-grade triethylamine, sodium chloride, and acetic acid were purchased from Mallinckrodt Chemical (Paris, KY). Synthetic polynucleotide samples were from Integrated DNA Technologies (Coralville, IA). Amounts of individual injected oligomers were in the range of 5–30 g (unless stated otherwise). The sequences are as follows:

60 mer: 5' CTT GAC TGT GAC AAG CTG CAG TGG ATC TGA GAA CTT CAG GCT CCT GGG CAA CGT GCT GGT 3'

45 mer: 5' CTG CAG TGG ATC TGA GAA CTT CAG GCT CCT GGG CAA CGT GCT GGT 3'

30 mer: 5' GAA CTT CAG GCT CCT GGG CAA CGT GCT GGT 3'

15 mer: 5' GGG CAA CGT GCT GGT 3'

All recovery estimates were based on UV absorption, and recovered peaks were confirmed (identity and purity) by conventional capillary gel electrophoresis. Capacity was determined using both benzoate and 40 mer. The results are displayed in Table I. Compared to a conventional column of similar size, a load capacity for 40 mer of 14 mg/column is quite high.

The buffer solution was made by slowly titrating an appropriate amount of acetic acid with triethylamine (TEA) to the required pH and then adding solvent to the appropriate volume. The buffer solution was then filtered through a 0.45-µm pore membrane. The pH was measured and adjusted with acetic acid or triethylamine if required. For solvent gradient conditions, see Results and Discussion.

The capacity factor k', defined as $(V_r - V_0)/V_0$, where V_r is the retention volume and V_0 is the column void volume, was calculated using the equation $k' = (t_r - t_0)t_0$, where t_r is the retention time and t_0 is the column void time. The flow rate was monitored by connecting a burette to the detector outlet. It was confirmed that the flow rate did not fluctuate significantly, so the computation of the capacity factor using the equation $k' = (t_r - t_0)t_0$ is acceptable.

Table I. Results from the Loading Capacity Test

Tested samples	Molecular weight	Charge	mmol	mg
Sodium benzoate	144.11	–1	0.3	43
40 mer DNA	12369	–40	1.1×10^{-3}	14

Results and Discussion

Figure 1 shows the purification of a synthetic nucleotide oligomer (30 mer). Separation conditions are shown in the figure caption. The sample had been purified by PAGE and was regarded as a highly pure 30 mer. As can be seen in Figure 1, minor impurities of shorter oligomer chains were resolved by the procedure reported here. The earliest peaks closest to the column void time might be inorganic salt peaks or even shorter oligomer peaks. The identity of these peaks is currently under investigation. Changing the solvent gradient procedure could separate the leading shoulder from the main peak, as shown in

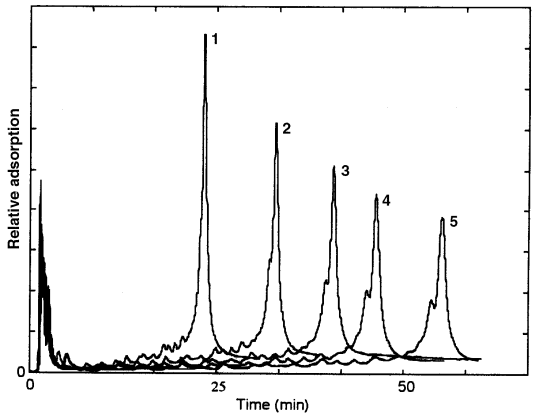

Figure 1. Purification of a synthetic nucleotide oligomer (30 mer). Gradient conditions: Solvent A, 0.025M TEAA, pH 7.40; solvent C, 0.025M TEAA–1M NaCl, pH 7.40. Gradient was 80:20 to 50:50 (A:C), then held at 50:50 (A:C). The flow rate was 1 mL/min. Gradient times: 1, 20 min; 2, 30 min; 3, 40 min; 4, 50 min; 5, 60 min.

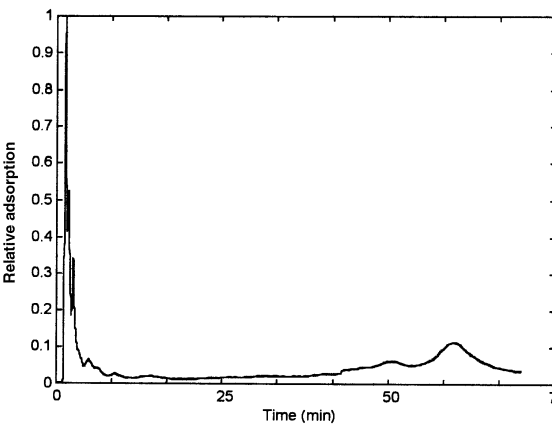

Figure 2. Purification of a synthetic nucleotide oligomer (30 mer). Conditions were the same as in Figure 1, except that the gradient was from 65:35 to 60:40 (A:C) in 60 min, then held at 60:40 (A:C).

Figure 2. Here, the initial ionic strength was stronger, and the shorter oligomer impurities were eluted at an earlier time as expected. In a manner similar to Figure 1, Figure 3 shows the purification of 60 mer, and the shorter length oligomer impurities were separated and detected.

Figure 4 shows the relationship between the concentration of sodium chloride at which an oligomer was eluted and the length of the oligomer chain (base number). This observed relationship is very similar to the one reported earlier in the literature (10). The salt gradient was from 0.3 to 0.5M NaCl in 60 min. The flow rate was 1 mL/min, and the pH was 7.40.

Recovery is one of the most important criteria for judging a purification method. The recovery of the separation was measured by replacing the separation column with plastic tubing. The recovery was calculated by comparing the peak area obtained when using the separation column with that obtained using the plastic tubing; it was found to be greater than 70% in all cases.

The effect of temperature on the retention was found to be significant. Figure 5 shows the retention time of nucleotides as a function of column temperature. It shows that the higher the temperature, the longer the retention time. The column allows an operating temperature up to 60°C. Anion-exchange separations under a high (denaturing) temperature are particularly useful for the separation of oligonucleotides with regions of self-complementary sequence or poly-G tracts. A temperature program would enhance the separation if it were operable and if the sample permitted. Unfortunately, the design of the column prohibits the practice of temperature programming. The thick acrylic outer shield and the pocket of air surrounding the inner separation column result in an extremely low thermal exchange velocity between the inner separation column and the outside heat source.

The addition of organic solvent to the eluent proved to affect the retention significantly. Figure 6 shows the effect of methanol (MeOH) added to the eluent on the retention of oligomers. It was shown that ln k' versus percent MeOH (v/v) was almost linear. It could be concluded that the retention was not controlled by ion-exchange interaction alone; hydrophobic interaction may also play a role in the separation process.

In the process of purifying synthetic nucleotide oligomers, if a volatile buffer can be used to elute oligomers, the desalting procedure after the separation is eliminated, because in the lyophilization process, the volatile buffer salt will be removed from the sample as well. If this could be done, the approach would be very useful for the purification of chemically synthesized fragments, the enzymatic synthesis of nucleotides, or the analysis of nucleic acid digests. Figure 7 shows the potential of using a volatile buffer system to elute oligomers using the UNO Q1 anion-exchange column. The baseline drift was caused by the solvent gradient. By increasing the concentration of TEAA, it may be possible to elute up to 60 mer. The upper-limit concentration of TEAA that can be used is limited by the backpressure of the column. The higher the TEAA concentration, the higher the backpressure. As previously shown, the addition of organic solvent to the mobile phase will shorten the retention time; therefore, it may be possible to elute longer oligomer chains by combining organic solvent and volatile TEAA buffer. The exploration of this possibility is being undertaken.

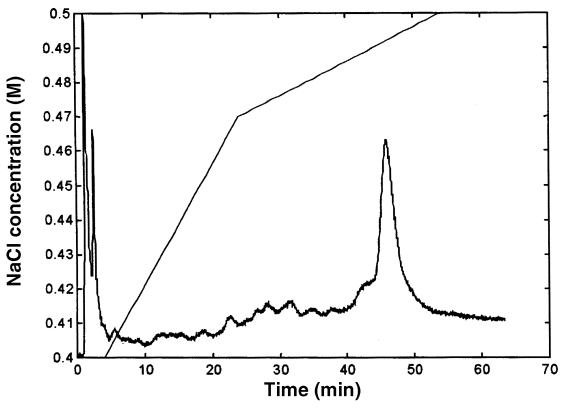

Figure 3. Purification of 60 mer. The flow rate was 1 mL/min.

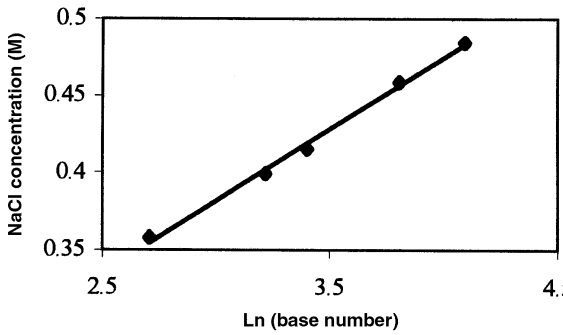

Figure 4. The concentration of NaCl at which an oligomer was eluted versus the logarithm of the chain length of oligomers.

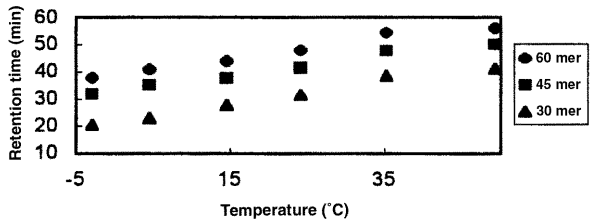

Figure 5. The effect of temperature on the retention of oligomers. The salt gradient was 0.3M to 0.8M NaCl in 120 min. The pH value was 7.40. The flow rate was 1 mL/min.

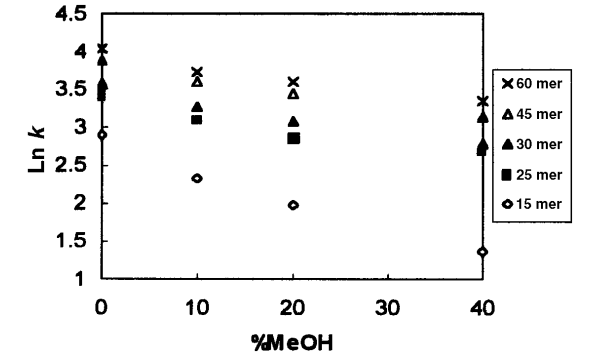

Figure 6. The effect of methanol as a mobile phase additive on the retention of oligomers. The salt gradient was 0.3M to 0.55M NaCl in 60 min. The flow rate was 1 mL/min. The pH value was 7.40.

Loading capacity is one of the most important characteristics of a column. The larger the loading capacity, the better the column performs when other properties are the same. Loading capacity is of particular importance in preparative separations and purification. The loading capacity for oligonucleotides was investigated by increasing the sample load and observing the effect on the chromatography. As shown in Figure 8, the chromatographic peak shape remains symmetric, and the resolution was not degraded when as much as 366 g of 30 mer was injected into the column. It can be concluded that the loading capacity of the analytical column is larger than 366 g. In fact, further work revealed that the capacity for 40 mer is some 14 mg/column.

Conclusion

The primary study has shown the broad potential of the UNO Q1 ion-exchange column for the purification and separation of polynucleotides. It has high selectivity, high recovery, and very high sample loading capacity. It has been shown that a volatile buffer system could be used to purify nucleotide oligomers. The advantage of using a volatile buffer system is that it does not require a stand-alone desalting procedure after separation; the buffer salt can be removed in the lyophilizing process. Other volatile buffer systems are under investigation. The retention capacity can also be adjusted by the addition of an organic solvent to the mobile phase or by the manipulation of column temperature if the sample permits. A temperature gradient instead of a salt gradient might also enhance the separation of nucleotide oligomers if it could be practiced. Unfortunately, the design of the column prevents the practice of temperature gradients. The fact that organic solvent affects the retention implies that hydrophobic interaction plays a relatively important role in the retention process. Therefore, it may be possible to separate isopliths (oligomers having the same chain length but differing minimally in sequence) by a gradient of organic additive. This avenue of investigation is also being undertaken.

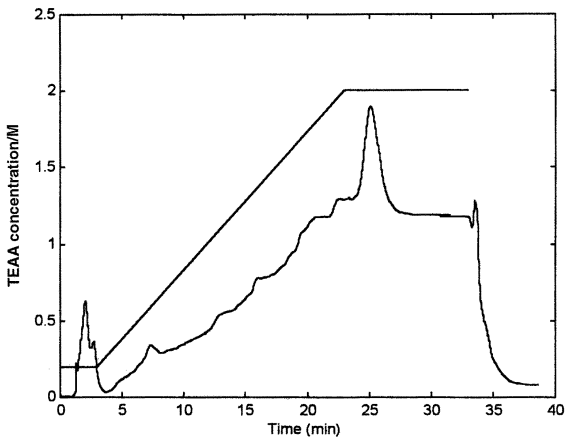

Figure 7. The elution of 30 mer by a volatile buffer (TEAA only). The flow rate was 1 mL/min.

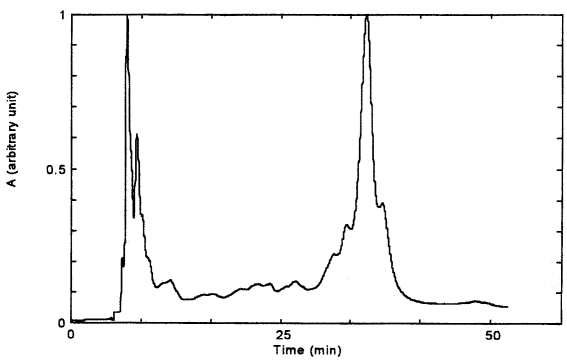

Figure 8. Column loading capacity test. 0.366 mg PAGE purified 30 mer was injected. Solvent A was 20mM TEAA (pH 7.40). Solvent B was 20mM TEAA–1.0M NaCl (pH 7.40). The gradient was 70:30 to 45:55 (A:B) in 60 min. The column was an UNO Q1. The flow rate was 1 mL/min.

Acknowledgments

Q. Liu would like to thank the Society of Analytical Chemists of Pittsburgh for sponsoring the American Chemical Society, Division of Analytical Chemistry Fellowship. The authors thank Bio-Rad Laboratories for the courtesy use of the UNO Q1 column used in this study.

References

1. D. Rickwood and B.D. Hames. *Gel Electrophoresis of Nucleic Acids: a Practical Approach*, 2nd ed. IRL Press, New York, NY, 1990.
2. A.M. Delort, R. Derbyshire, A.M. Duplaa, A. Guy, D. Molko, and R. Teoula. Use of reversed-phase high-performance liquid chromatography for the purificaiton of 5'-phosphorylated oligonucleotides. *J. Chromatogr.* **283:** 462–67 (1984).
3. C.R. Becker, J.W. Efcavitch, C.R. Heiner, and N.F. Kaiser. Use of C_4 column for reversed-phase high-performance liquid chromatographic purification of synthetic oligonucleotides. *J. Chromatogr.* **326:** 293–99 (1985).
4. W. Haupt and A. Pingoud. Comparison of several high-performance liquid chromatography techniques for the separation of oligodeoxynucleotides according to their chain length. *J. Chromatogr.* **260:** 419–27 (1983).
5. Y. Kato, T. Kitamura, A. Mitsui, Y. Yamasaki, and T. Hashimoto. Separation of oligonucleotides by high-performance ion-exchange chromatography on a non-porous ion exchanger. *J. Chromatogr.* **447:** 212–20 (1988).
6. A.A. Kurganov, V.A. Davankov, and K.K. Unger. Ion-exchange high-performance liquid chromatography of nucleotides and polypeptides on new types of ion-exchange sorbents, based on polystyrene-coated silica. *J. Chromatogr.* **548:** 207–14 (1991).
7. T. Lu and H.B. Gray. Combined pH gradient and anion-exchange high-performance liquid chromatographic separation of oligodeoxyribonucleotides. *J. Chromatogr. A* **686:** 333–38 (1994).
8. W.J. Warren, W.A. Stanick, M.V. Gorenstein, and P.M. Young. HPLC analysis of synthetic oligonucleotides using spectral contrast techniques. *Biotechniques* **19:** 282–87 (1995).
9. W.A. Ausserer and M.L. Biros. High-resolution analysis and purification of synthetic oligonucleotides with strong anion-exchange HPLC. *Biotechniques* **19:** 136–39 (1995).
10. J.L. Vaerman, P. Moureau, Y. Larondelle, P. Martiat, and M. Philippe. Prediction of oligodeoxyribonucleotides eluting salt conditions on anion exchange column. *Nucleic Acids Research* **20:** 3522 (1992).

Manuscript accepted May 28, 1999.