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HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY OF OLIGONUCLEOTIDES*

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SUMMARY

This paper presents techniques for the preparation and use of support materials for the resolution of oligonucleotides by high-performance anion-exchange chromatography. Methodology for both static and *in situ* surface modification of silica using polyethylenimine is outlined with emphasis on the contribution of stationary phase thickness and silica type in resolution and selectivity. Short columns (5.0 × 0.41 cm I.D.) are capable of fractionating both oligonucleotide homologues up to 35 bases in chain length and various nucleoside-5′-mono-, di- and triphosphates. Gradient rate is discussed in terms of resolution *vs.* separation time.

INTRODUCTION

Oligonucleotides are commonly fractionated on various types of soft gel media. Within the ion-exchange mode, polysaccharide-based gels such as DEAE-cellulose[1], DEAE-Sephadex[2], QAE-Sephadex[3], and TEAE-cellulose have been used[4]. The ion-exchange derivative of Spheron, a glycol–methacrylate gel, has also been employed[4]. Classical methods other than ion exchange include uncoated poly-CTFE (Kel F)[5] and RPC-5[6–11] type supports. Polysaccharide-based ion-exchange separations work well but are time consuming[12,13]. RPC-5 is based on polychlorotrifluoroethylene beads (Plascon 2300) coated with a trialkyl quaternary amine (Adogen 464)[11]. While superlative fractionations of oligonucleotides have been reported on RPC-5[11] or RPC-5 ANALOG[14], other reports have indicated that reproducibility is suspect[13,15]. Unlike ion-exchange polysaccharide gels, the ion-exchange groups of RPC-5 erode at low ionic strength. At salt concentrations lower than 0.2 *M* or on addition of organic solvents the organic coating washes off[14].

The advent of high-performance liquid chromatography (HPLC) technology has allowed separation of various molecules in much less time while maintaining stationary phase stability. Recently, HPLC has been applied to oligonucleotide separations. Besides DNA or RNA digest fractionations[12,13] an interesting application has been to expedite construction and purification of oligomers in the chemical synthesis of nucleic acids[16–18]. Synthetic oligonucleotides can act as specific templates to

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prime the transcription of cDNA[19] or initiate *in vitro* site-specific mutagenesis[20,21]. In addition to gene construction[17] and DNA or RNA manipulation[22], oligonucleotides have been used to study nucleic acid–protein interactions[23]. The urgent need for support materials that can routinely fractionate long oligomers in both the analytical and preparative mode has been approached by using various alkylamine anion-exchange supports[12,13,24–28]. Although excellent selectivity for sequence isomers of pentanucleotides has been reported[27] with these columns, separations of oligomers longer than ten bases is rare[12,13]. Various reversed-phase methods have also been reported[12,16,29,30] but separation of homologous oligomers has only been demonstrated to a length of less than twenty bases[29].

In this work, the development of a porous-pellicular anion-exchange column based on polyethylenimine (PEI) is described. Fractionation of homologous oligomers of at least 35 bases in length has been achieved. This anion exchanger is stable and can be coated onto silica under batch or *in situ* conditions.

MATERIALS

LiChrosorb and LiChrospher silicas were purchased from E. Merck (Darmstadt, G.F.R.). Nucleosil 100-5 was obtained from Macherey, Nagel & Co. (Düren, G.F.R.). Hypersyl was purchased from Shandon Southern Instruments (Sewickley, PA, U.S.A.). Spherisorb S5W was obtained from Phase Sep (Hauppauge, NY, U.S.A.). Vydac TP was obtained from The Separations Group (Hesperia, CA, U.S.A.). Polyethylenimine PEI-6 (M_r 600) and ethylene glycol diglycidyl ether (EDGE) were purchased from Polysciences (Warrington, PA, U.S.A.). 1,4-Butanediol diglycidyl ether (BUDGE) was obtained from Aldrich (Milwaukee, WI, U.S.A.). 1,4-Dioxane and methanol were purchased from Fisher Scientific (Springfield, NJ, U.S.A.). Polyadenylate, potassium salt [poly(A)] was obtained from Miles Labs. (Elkhard, IN, U.S.A.). Sodium salts of oligothymidylates and oligodeoxyadenylates were a gift from P-L Biochemicals (Milwaukee, WI, U.S.A.). HPLC-grade methanol was purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). Pre-packed LC-Si (15 × 0.46 cm, I.D. 5 μm) silica columns were obtained from Supelco (Bellefonte, PA, U.S.A.).

EXPERIMENTAL

Support preparation

The preparation of polymeric PEI coatings on microparticulate silica to generate a stable anion-exchange support is based on the work of Alpert and Regnier[31]. A description of the synthetic method has been published by Vanecek and Regnier[32,33] which optimized the anion exchanger for proteins. This study optimizes the support for oligonucleotides.

The supports in Table I were generated by a two-step procedure: (1) adsorption of PEI onto silica, and (2) stabilization of the adsorbed layer by covalent cross-linking. A 30% (w/v) solution of PEI in methanol was made and serially diluted to 10, 5, 1 and 0.37% with addition of methanol. Also 10% (w/v) solutions of BUDGE and EDGE in 1,4-dioxane were prepared. Seven 800-mg samples of Hypersil (3 μm) silica were each added to 8-ml aliquots of PEI-6–methanol solution. Supports were filtered

TABLE I

STATIC PEI COATING AND CROSS-LINKING CONDITIONS

Column No.	*PEI in coating soln. (%)**	*Cross-linking** temp. (°C)*	*Resolution**** R_{s12-16}	*Elemental analysis (%)*		
				C	*N*	*H*
1	0.37	100	Poor	–	–	–
2	1.0	100	1.40	1.23	0.54	0.54
3	1.0	24	1.51	–	–	–
4	5	100	1.25	6.11	1.45	1.23
5	10	100	1.37	7.32	2.45	1.54
6	10	24	1.47	–	–	–
7	30	100	None [§]	18.25	5.08	3.45

* Hypersil 3-μm silica coated and packed in 5.0 × 0.41 cm I.D. column.

** Cross-linking done with BUDGE (see Experimental).

*** Average resolution ($\bar{R}_{s12-16}$ is defined as the average of the sum total individual resolution values for $d(A)_{12-13}$, $d(A)_{13-14}$, $d(A)_{14-15}$ and $d(A)_{15-16}$ combinations. Linear gradient, 0.375%/m.n eluting 0.25 A_{260} O.D. unit of oligodeoxyadenylates (see Experimental).

[§] Heavy PEI-coating filled pores. Column back-pressure more than 300 atm.

on medium Buchner filters and vacuumed to dryness. Supports were then added to 8 ml of BUDGE–1,4-dioxane solution and allowed to stand overnight. Some samples were heated on a steam bath the following day for 20 min. Supports were filtered and washed with water and acetone.

The coating of supports in Table II followed the same procedure. All PEI-6–methanol coating solutions were 1% (w/v). Cross-linking was accomplished overnight in either 10% (w/v) EDGE–methanol or BUDGE–methanol at room temperature. Supports were washed and dried as described.

Four dynamically coated columns were prepared using pre-packed 150 × 4.6 mm I.D. high-efficiency columns and an *in situ* PEI adsorption process. Columns were conditioned with methanol (1 ml/min) for 1 h, after which a 10% (w/v) solution

TABLE II

SILICA TYPE *vs.* RESOLUTION

Column No.	*Silica**	*Stationary phase***	*Particle size (μm)****	*Column back pressure (atm)* [§]	*Pore diameter (Å)****	$\bar{R}_{s12-16}$ [§§]
3	Hypersil	E	3	96	120	1.51
8	Nucleosil 100-5	E	5	100	100	1.44
9	Spherisorb S5W	E	5	38	80	1.13
10	LiChrosorb	E	5	94	100	1.09
11	Hypersil	E	5	40	120	0.99
12	Vydac TP	E	5	54	330	0.69
13	Hypersil	B	3	98	120	1.51

* All silicas are spherical except LiChrosorb which is irregular.

** All supports identically coated except for crosslinker: EDGE for E and BUDGE for B; columns were all 5.0 × 0.41 cm I.D.

*** Particle size and pore diameter are manufacturers values unless otherwise indicated.

[§] Mobile phase: 0.05 *M* KH_2PO_4 with 30% methanol, pH 5.9, 24°C, 0.6 ml/min.

[§§] Same conditions as in Table I.

of PEI-6–methanol was pumped through each column for 30 min at 0.5 ml/min. Three to four column volumes of methanol were then pumped through to wash off excess PEI. A 10% (w/v) solution of EDGE–methanol was pumped for 4 min at 0.5 ml/min. Columns were capped and allowed to stand overnight. Before use, columns were conditioned by washing for 30 min (1 ml/min) with methanol followed by a 30-min water wash.

Column packing

Supports were slurry packed (2%, w/v) into columns with 2-propanol at 8000 p.s.i. by means of a pneumatic pump (Haskel, Burbank, CA, U.S.A.). All columns were 5.0 × 0.41 cm I.D. LiChroma stainless-steel precision tubing (Anspec, Warrenville, IL, U.S.A.); 0.5- and 2.0-μm frits were used for columns packed with 3- and 5-μm silica particles, respectively. A precolumn (4.5 × 0.41 cm I.D.) was fitted to the slurry vessel and connected to the analytical column to channel the microparticulate silica during the packing process. Packing time was 10 min.

Microanalysis

Carbon, hydrogen, and nitrogen analyses were performed by C. S. Yeh, Chemistry Department, Purdue University. Accuracy was ±0.2%.

High-performance liquid chromatography

Analyses by HPLC were done using a Varian Vista system fitted with a Varian UV-50 variable-wavelength detector (Varian, Walnut Creek, CA, U.S.A.). The program employed allowed the microprocessor-controlled data system to tabulate retention and resolution measurements directly. The system was fitted with a Valco Model 9080 sample injector (Anspec) with either 100-μl or 1-ml injection loop.

Mobile phase conditions

A binary gradient system was used for all oligonucleotide separations. Buffer A was 0.05 *M* potassium phosphate plus 30% methanol adjusted to pH 5.9. Buffer B was 1 *M* $(NH_4)_2SO_4$ in buffer A. The flow-rate was 0.6 ml/min at ambient temperature for all gradients unless otherwise indicated. Buffers were degassed before use and refrigerated immediately afterward. No detectable deterioration of oligomer resolution occurred with fortnightly aged buffers, but fresh preparations were made every two weeks as a precaution.

Preparation of oligonucleotides

Oligonucleotide fragments from poly(A) were generated by subjecting 24.5 mg of poly(A) to 1 ml of 1.0 *N* potassium hydroxide solution for 20 sec at 60°C. The cleavage process was terminated by addition of 0.2 ml of 10 *M* $HClO_4$ and adjusted to neutral pH with additional $HClO_4$. Oligodeoxyribonucleotides were solubilized in buffer A and used directly. All sample aliquots were kept frozen with minimal freeze–thaw transitions when not in use. Identification of oligodeoxyribonucleotides was determined by coelution with some purified individual oligomers as internal standards.

Oligonucleotide resolution

A standard test mixture of deoxyadenylate oligomers ranging from 12 to 16 bases in length was used to evaluate columns (Fig. 1). The 25-μl injections (0.25 A_{260}

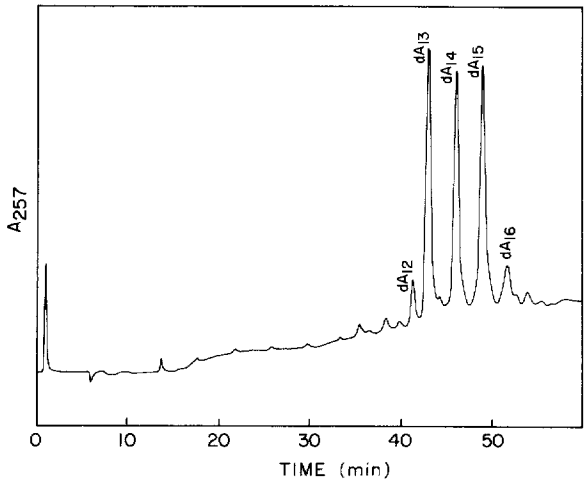

Fig. 1. Fractionation of oligodeoxyadenylates ranging from 12 to 16 bases long on column No. 3 (5.0 × 0.41 cm I.D.). Sample, 0.125 A_{260} O.D. units in 25-μl injection. Buffer A, 0.05 *M* phosphate, pH 5.9 + 30% methanol. Buffer B, 1.0 *M* $(NH_4)_2SO_4$ in buffer A. Gradient, 0–30% in 60 min at 0.6 ml/min, 24°C.

O.D. units) of oligomers were eluted by 0.375%/min linear gradients at ambient temperature. Results were reported as average resolution values ($\overline{R_{s12-16}}$) defined as the average of the sum total individual resolution values for $d(A)_{12-13}$, $d(A)_{13-14}$, $d(A)_{14-15}$, and $d(A)_{15-16}$ combinations.

RESULTS AND DISCUSSION

Silica-based supports with an adsorbed PEI layer were first devised for high-performance anion-exchange chromatography by Alpert and Regnier[31]. Vanecek and Regnier[32,33] found that varying polyamine density on the support surface could enhance anion-exchange capacity and resolution of proteins. This study focuses on the manipulation of adsorbed PEI supports for anion-exchange fractionation of oligonucleotides.

The resolution values in Table I indicated that there is a lower limit (less than 1%, w/v) for the PEI–methanol ratio in the coating solution that will produce a viable support. Presumably, the 0.37% (w/v) solution produced incomplete surface coverage. At the other extreme, a 30% (w/v) PEI–methanol coating solution resulted in a packing that would not allow elution of oligonucleotides. Data from microanalysis and column back-pressure measurements suggested that very heavy coatings filled the pores. Solutions from 1 to 10% (w/v) worked best for preparing packings. It is interesting to note that although resolution values of the test samples varied by only 17% the amount of adsorbed PEI varied about five-fold (Table I). Oxirane cross-linking reactions between primary or secondary amines in the previous studies[31–33] have been carried out at 100°C. For oligonucleotide fractionation, resolution was slightly better when cross-linking was achieved at room temperature (Table I). A chromatogram of the oligodeoxyadenylate test mixture used to evaluate supports in Tables I and II is shown in Fig. 1.

Various types of porous silicas were coated under conditions identical to those

producing column No. 3 in Table I. The results (Table II) indicated that resolution could be increased by either of two ways: (1) decreasing particle size and (2) selecting an inherently better silica to modify. The influence of particle size may be seen with the Hypersil support. Resolution was increased by 34% when 3 μm *versus* 5 μm particles were employed. But this reduction in particle size was accompanied by an increase of column head pressure from 40 to 96 atm as expected[34]. On the other hand, even greater variations in resolution were achieved using an identical bonding protocol on different 5-μm silicas. For example, the $\overline{R_{s12-16}}$ values for column Nos. 9, 11, and 12 ranged from a low value of 0.69 for column No. 12 to a value of 1.3 for column No. 9. The increase in resolution with column 8 was even greater, but the high back-pressure leaves a question as to whether the Nucleosil 100-5 was correctly sized by the manufacturer. The high back-pressure for column No. 10 relative to the other 5-μm silicas was expected, as irregular porous-silica particles are known to have a flow resistance factor twice the value of spherical particles[35]. Column Nos. 3 and 13 differed only in the crosslinker employed in the coating process. This variable did not seem to be significant as indicated by resolution values (Table II).

Other oligonucleotide mixtures were also tested on PEI coated Hypersil 3-μm silica. Fig. 2a illustrates the fractionation of thymidine oligomers. An increased gradient rate with a slightly elevated flow-rate was capable of reducing the separation time from 60 min to under 20 min (Fig. 2b). A profile of the partial alkaline hydrolysate of polyadenylate ribonucleotide is shown in Fig. 3. Approximately 2 mg of oligomers were loaded onto the column (5.0 × 0.41 cm I.D.). The small peaks eluting before the A_1–A_8 oligomers were not identified but it is known for partial alkaline hydrolysis that the terminal phosphate occurs on both the 2′- and 3′-ribosyl carbon with the possibility of the less charged 2′,3′ cyclic form also being present. The 2′,3′ cyclic diester would be expected to elute ahead of the monoester. It should be noted

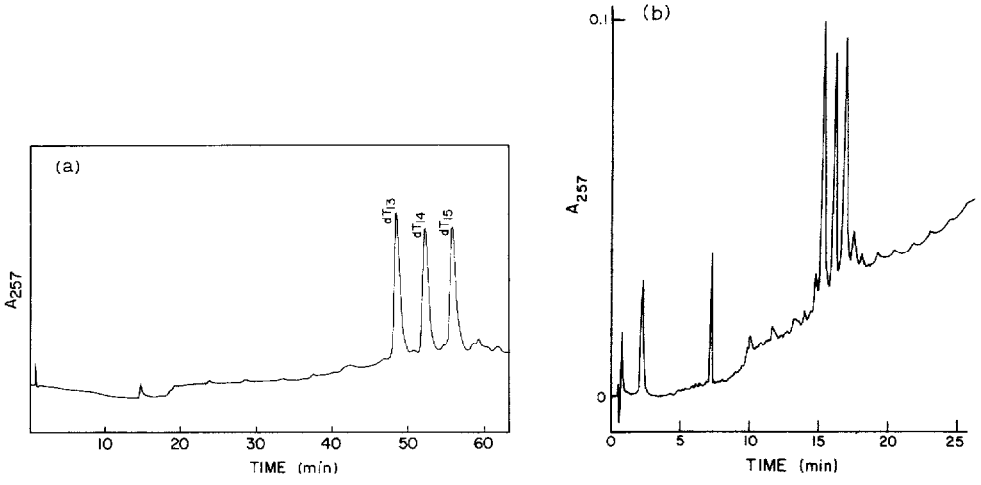

Fig. 2. (a) Fractionation of oligothymidylates ranging from 13 to 15 bases in length on column No. 3. Sample, 0.125 A_{260} O.D. units injected in 25 μl. Gradient rate, 0.5%/min. Other conditions as in Fig. 1. (b) Fractionation of oligothymidylates on column No. 3. Gradient rate, 1.67%/min, flow-rate, 1.0 ml/min; 10-μl injection of 0.10 A_{260} O.D. unit.

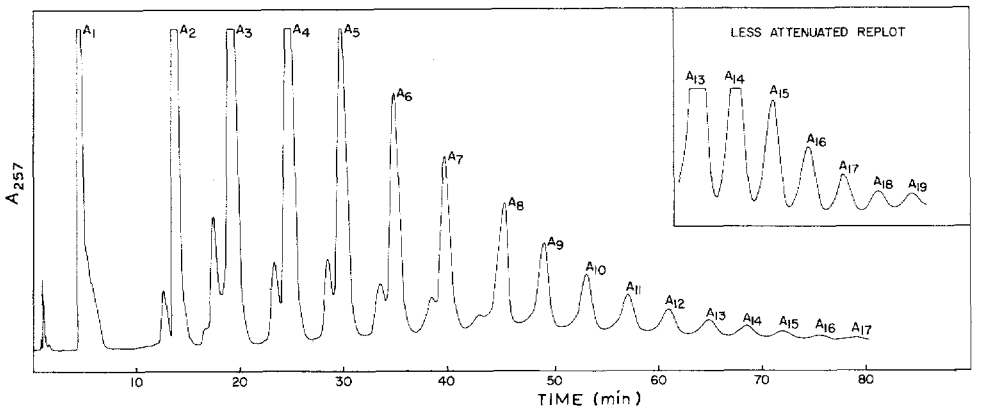

Fig. 3. Poly(A) partial alkaline hydrolysis profile on column No. 5, using 100-μl injection of *ca.* 2 mg of poly(A) oligomers. Gradient, 0–55% in 90 min. Other conditions as in Fig. 1.

here that the mobile phase employed was capable of separating both RNA and various DNA type homologues without adjustment. Column No. 3 generated only 166 plates (3320 plates/m) determined by injection of acetone into an aqueous mobile phase at room temprature and a flow-rate of 0.5 ml/min. Although it was by no means a high-efficiency column *per se*, enhanced selectivity allowed oligomers longer than 35 to be fractionated (Fig. 4).

Efficiently packed columns obtained from a commerical source were coated *in situ* (see Experimental) for comparison. Each Supelco LC-Si column (15 × 0.46 cm I.D., 5 μm) had at least 10,500 plates (70,000 plates/m) determined by the manufacturer. The separation of twelve mononucleotides of various charge and nitrogenous

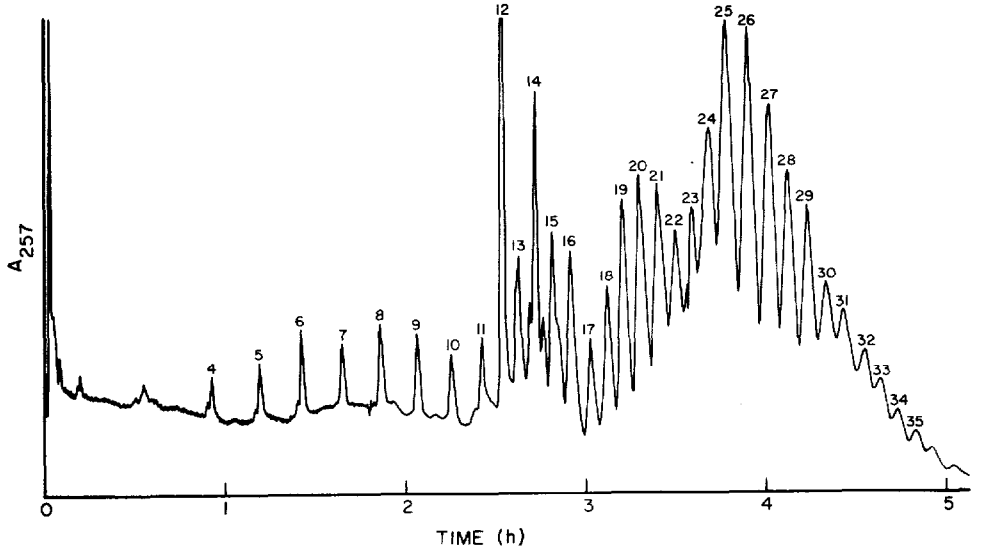

Fig. 4. Fractionation of 2.3 A_{260} O.D. units oligodeoxyadenylates on column No. 13, using 230-μl injection. Gradient rate, 0.1%/min. Other conditions as in Fig. 1.

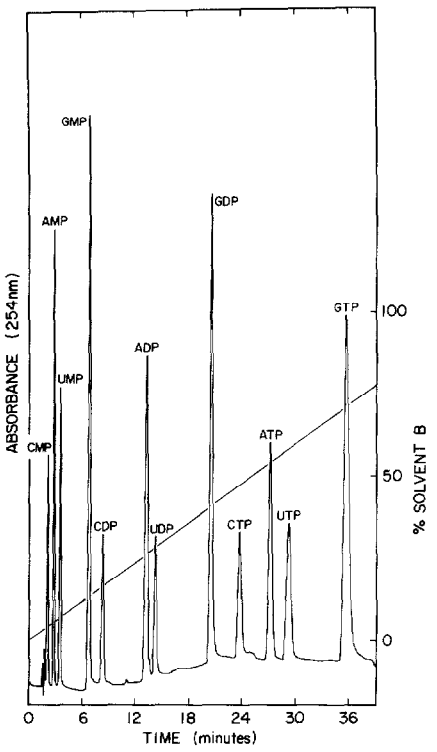

Fig. 5. Nucleotide separations on Supelco LC-Si (15 × 0.46 cm I.D.) column coated *in situ*. Buffer A, 0.1 *M* NaH_2PO_4, pH 3.0; buffer B, 0.1 *M* NaH_2PO_4 + 2.0 *M* NaCl, pH 2.0. Linear gradient, 0–100% in 50 min at 1.0 ml/min, 24°C.

base composition was achieved (Fig. 5). Oligomers longer than twenty bases were fractionated by four *in situ*-coated columns connected in series in *ca.* 3 h. (Fig. 6a). Although relatively a long run time for HPLC gradients, the elution of the 25-base oligomer in 3 h is ten-fold faster than the best equivalent fractionation achieved by classical column chromatography[11]. With fewer peaks preceding a 25-base oligomer, elution has been achieved in less than 1 h[36]. Decreasing the gradient rate from 0.375%/min to 0.1%/min (Fig. 6b) increased the separation time, but also increased the resolution (Table III). Comparison of resolution values for column Nos. 14 and 15 indicate no apparent advantage (other than probably loading capacity) in using *in situ*-coated columns in series as opposed to a single column. From plots of peak width *vs.* base number and resolution *vs.* base numbers derived from Fig. 6b, it is interesting to note that the region of sharpest peak width increase (oligomers longer than 25 bases) (Fig. 7a) corresponds to nearly constant R_s values (Fig. 7b).

Decreased linear gradient slopes improved resolution (Tables III and IV) but sometimes at the cost of lengthy retention times. A resolution *vs.* gradient rate study indicated that 0.375%/min was best in terms of enhanced resolution with a minimal amount of time expenditure (Table IV). This was determined by dividing resolution values by retention times.

Both adenylate (Fig. 8a) and deoxyadenylate (Fig. 8b) homologues longer than

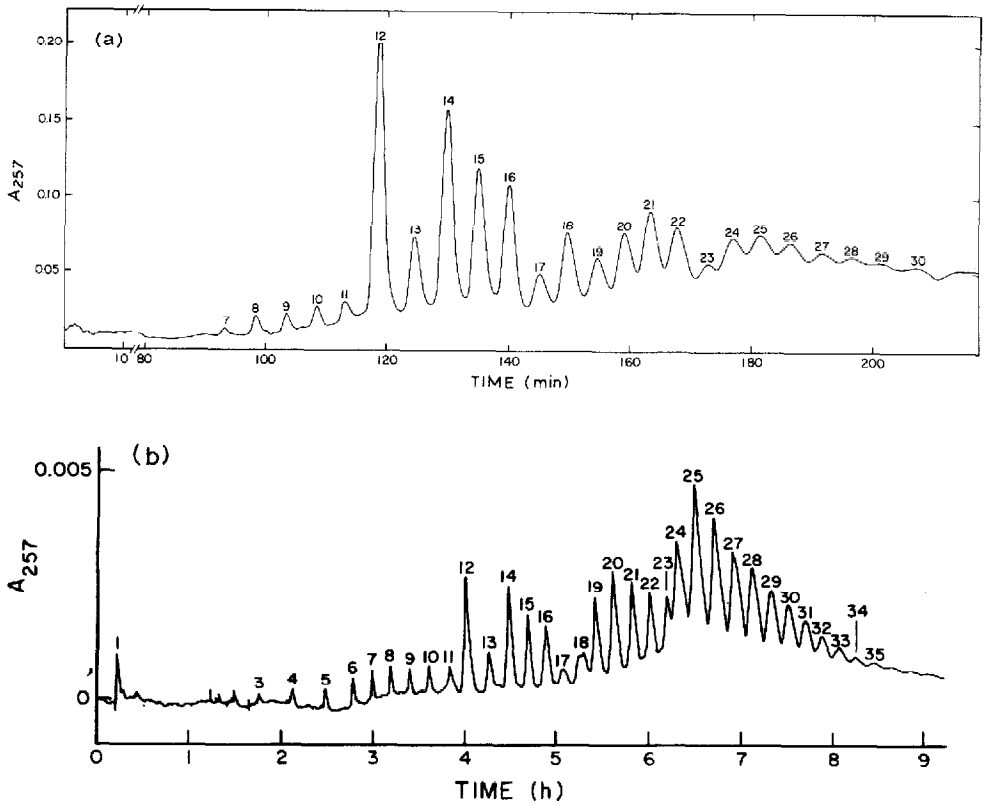

Fig. 6. (a) Fractionation of 1.3 A_{260} O.D. units of oligodeoxyadenylates on four Supelco LC-Si (15 × 0.46 cm I.D.) columns in series coated *in situ*, using 100-μl injection. Gradient rate, 0.375%/min. Other conditions as in Fig. 1. (b) Fractionation of oligodeoxyadenylates. Gradient rate, 0.1%/min. Same load as in Fig. 6a plus 0.65 A_{260} O.D. units of $d(A)_{19-30}$ for a total injection volume of 170 μl. Other conditions as in Fig. 6a.

TABLE III

RESOLUTION ON *IN SITU*-COATED PEI COLUMNS

Column No.	*Column length (μm)*	*Gradient rate (%/min)*	$\bar{R}_{s12-16}$**	*N***	*Retention $d(A)_{14}$ (min)*	*Column back-pressure (atm)*
14	15	0.375	1.47	11,442	89.7	44
15	60*	0.375	1.48	42,885	129.3	130
15	60*	0.1	2.18	42,885	269	130

* Four Supelco LC-Si, 5 μm (15 × 0.46 cm I.D.) columns were coated *in situ*. Columns connected in series equaled 60 cm.

** Same calculation as in Table I.

*** Column efficiency determined by manufacturer using acetanilide with mobile phase dichloromethane–methanol–water (99.4:0.5:0.1) at 1.0 ml/min, ambient temperature.

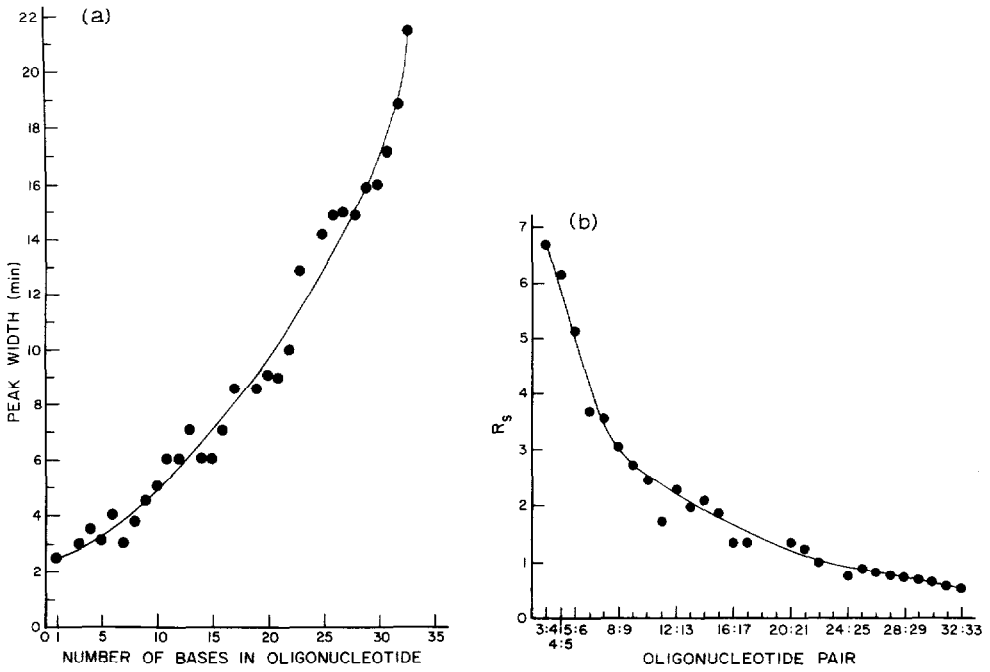

Fig. 7. (a) Plot of peak width *vs.* homologue length from Fig. 6b data. (b) Plot of resolution *vs.* homologue pair from Fig. 6b data.

about a decamer had elution times directly proportional to the number of bases. Curves with continuous positive slope change would have implicated a synergistic type of electrostatic association between increasingly longer oligomers and the stationary phase, resulting in tenacious retention of lengthy homologues. Since the relationship was linear it appeared that pore diffusivity could be the limiting factor for oligonucleotide fractionation, although test probes were apparently not long enough in this study to evaluate this hypothesis. Oligomers of up to only 37 residues were available. An interesting point can be made about the conformation of a 37-base oligomer in this buffer system. Assuming a helical rodlike structure, the length of the oligomer is calculated to be 128 Å. Considering the pore-diameter of Hypersil is less than 120 Å after coating[33], the structure of long oligodeoxyadenylates in buffer A

TABLE IV

RESOLUTION *vs.* GRADIENT RATE

*Gradient rate (%/min)**	$\bar{R}_{s12-16}$	*Retention* $d(A)_{14}$ *(min)*
0.625	0.74	59.9
0.5	1.11	64.9
0.375	1.51	72.7
0.25	1.74	101.4
0.1	1.76	161.2

* Column No. 6 (Hypersil, 3 μm, 10% PEI) 5.0 × 0.41 cm I.D.

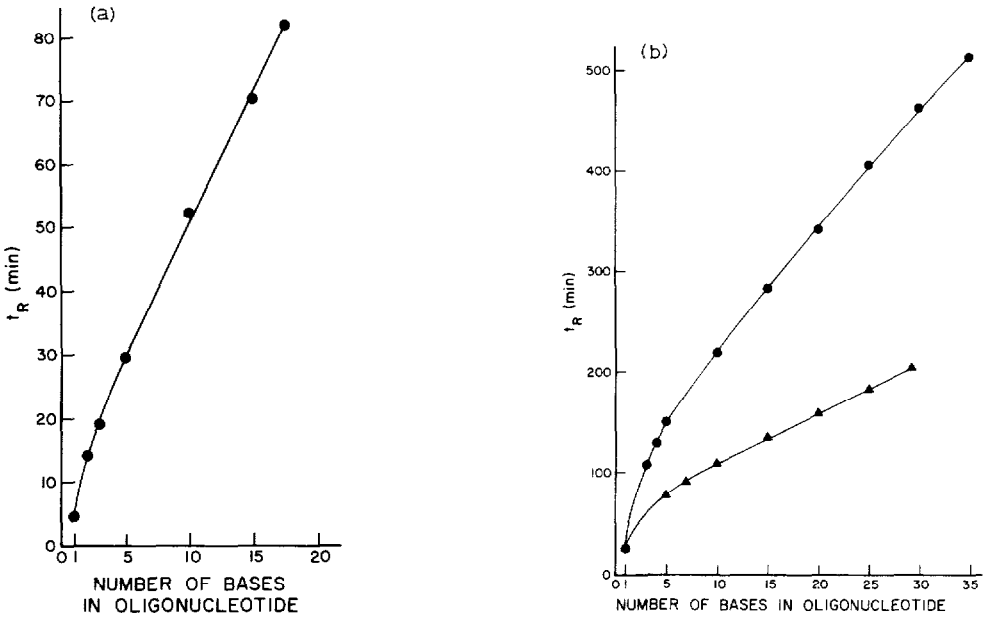

Fig. 8. (a) Data from poly(A) fragment elution profile in Fig. 3. Gradient rate, 0.6%/min. (b) Oligodeoxyadenylate elution data: ●, from Fig. 6b (gradient rate 0.1%/min); ▲, same conditions as Fig. 6b, but 0.375%/min gradient. t_R = Retention time.

must deviate from a pure rod-like structure. Random coil, single- and double-helix contributions have all been reported for polyadenylic acid[37].

A curious observation was that the macroporous silica (330 Å) in Table II did not fractionate oligomers as well as the 80–120 Å pore-diameter silicas. Vanecek and Regnier[32,33] have reported enhanced ion-exchange capacity and resolution of proteins on large-pore-diameter PEI-based anion exchangers. Similar results have been found for reversed-phase chromatography of proteins[38,39] and peptides[39–41]. In general, peptides longer than *ca.* twenty residues are better separated on 300 Å as opposed to 100 Å pores. Assuming an extended β-conformation[42] this translates into peptides longer than 72 Å. A detailed report by Pfannkoch *et al.*[43] on size-exclusion chromatography of proteins implicates this steric explanation: as solute size approaches the support pore diameter, diffusion of that molecule in the support is retarded, which in turn results in poor efficiency of separation. In addition to pore diffusivity, Pearson *et al.*[40] have reported for reversed-phase separation of proteins that siliceous matrix selectivity, independent of coating, varied when several macroporous silicas were compared.

The modest resolution obtained for the macroporous column No. 12 in Table II was therefore probably due to either: (1) a disproportionate, comparative pore diameter *vs.* solute diameter ratio, or (2) an inferior selectivity. A recent study has shown the utility of this macroporous silica when nucleic acid constituents (tRNAs) larger than those employed in this work are used[44].

It is concluded that oligonucleotides eluted in this study fractionated well employing anion exchangers based on 100 Å pore diameter microparticulate silica.

Adsorbed polyamine layers resulting in 0.5 to 2.5% *N* supports worked the best. Columns were capable of resolving homologues of oligodeoxyadenylates, oligothymidylates, oligoadenylates, and various mononucleotides. The support may be prepared both statistically and *in situ*. Short 5-cm columns proved to be very effective.

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NOTE ADDED IN PROOF

Lawson *et al.*[45] have used similar *in situ*-coated anion-exchange columns for fractionation of synthetic, heterogeneous oligodeoxyribonucleotides. Less than 1 μg to several milligrams have been loaded with greater than 90% recovery and purity. Column reproducibility was demonstrated, and lifetime ranged from 600 h with and 450 h without a silica guard column.

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