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Separation and purification of oligonucleotides using a new bonded-phase packing material

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ABSTRACT

We describe a new bonded-phase packing material, based upon surface-stabilised microparticulate silica, suitable for the rapid separation and purification of oligonucleotides. Columns packed with this material were demonstrated to give rapid separations of individual oligonucleotide species of up to 44 base units with high purity; agarose gel electrophoresis showed that the products were essentially single bands, with only trace quantities of the (n − 1)-mer present. Baseline resolution of the desired oligomer from (n ± 1)-mer was achieved under preparative loading conditions, where up to 200–300 μg of oligonucleotide could be separated. The separation was essentially independent of structure or sequence of the oligonucleotides. The retention mechanism of the oligonucleotides was investigated, and the results used to determine the optimum column configuration and separation conditions.

INTRODUCTION

The synthesis of single-stranded oligonucleotides is an area which has grown rapidly over the past few years, due to the need for DNA probes and for use in gene synthesis. Purification of the synthetic oligonucleotides is of obvious importance and there is a continuing need for methods which are rapid, preferably universal and which result in high yields of high-purity oligomer, free from the shorter-chain-length failure sequences. Probably the most used purification technique for such oliognucleotides is polyacrylamide gel electrophoresis [1]. Although this technique produces homogeneous oligonucleotides, it is time-consuming and often results in very low yields of longer oligonucleotides (greater than 20 bases in length).

Oligonucleotides can also be purified by chromatography. A mixed-mode resin, RPC-5, for low-pressure liquid chromatographic separations, was shown to be very

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successful for separating components of single- and double-stranded nucleic acid fragments [2]. The packing consisted of a resin-based particle, coated with a quaternary ammonium compound. Both hydrophobic and ionic properties are known to be present in this material, thus creating what may be termed a hydrophobic ion-exchange medium. In addition to being unavailable commercially, this packing suffered several shortcomings such as bleed of the amine stationary phase and pressure instability [3].

More recently, high-performance liquid chromatography (HPLC) has been employed for the separation and purification of oligonucleotides. A variety of packing materials such as anion-exchange [4], reversed-phase [5], reversed-phase ion-pair [6,7] and charge transfer [8] have been used for this purpose. One of the more popular techniques is reversed-phase chromatography of the dimethyltrityl-protected oligonucleotides [9]. This suffers to some extent in that the separation is not related to the chain length of the oligonucleotide nor to its sequence, and it is difficult, therefore, to be certain that the correct product has been synthesised, is being collected, and has the desired purity. HPLC techniques are mostly useful for purifying relatively short-chain hetero-oligonucleotides of up to 20–25 bases in length. More recently, reports of the use of ion-exchange materials for the purification of longer chain materials have appeared. Because the separation of oligonucleotides by ion-exchange chromatography is most effective at high pH values (≥ 11), its use is restricted to highly charged, alkali-stable supports like Mono Q (Pharmacia). Under these conditions oligonucleotides up to 30 bases in length can be purified in a single step [8].

During the course of an investigation into potential packing materials for the separation and purification of homo- and hetero-oligonucleotides we have developed a novel, silica-based, microparticulate, bonded-phase packing material which is particularly suited to the task. The surface of the silica particles is stabilised by treatment with zirconium [10]; this enhances its stability to alkaline pH, but does not affect the separation of oligonucleotides. The packing contains both polar and hydrophobic groups in a configuration which is believed to facilitate the chromatographic separation of oligonucleotides [11]. In this report we illustrate how this packing material can be used for the separation and purification of both homo- and hetero-oligonucleotides up to and exceeding 40 bases in length. This packing material is now available commercially as the Zorbax BioSeries OLIGO column.

EXPERIMENTAL

Chemicals

Silica gel was either Zorbax PSM150 or Zorbax PSM300 spherical silica, 7 μm diameter. 2-Aminoethanol was purchased from Sigma (Poole, UK) and acetonitrile (HPLC grade) was from Rathburn (Walkerburn, UK) or from J. T. Baker (Phillipsburg, NJ, U.S.A). Acrylamide and methylene bisacrylamide (ultra pure) were from Bethesda Research Labs. (Cambridge, UK). Glycidoxypropyltrimethoxysilane was from Silar Labs. (Scotia, NY, U.S.A.) or from Petrarch (Bristol, PA, U.S.A.). [^{32}P]ATP was from Amersham International (Amersham, UK) and homo-oligonucleotide standards were purchased from Pharmacia (Pisataway, NJ, U.S.A.). Other chemicals were of analytical grade or higher and obtained from BDH (Poole, UK) or Sigma. Oligonucleotides were synthesised using an Applied Biosystems Model 380B oligonucleotide synthesiser, employing cyanoamidate chemistry. The crude oligonu-

cleotides were deprotected, lyophilised and redissolved in water to a concentration of about 0.5 mg/ml. Stationary phases were prepared by reacting 2-aminoethanol with γ-glycidoxypropyltrimethoxysilane to give 3-[3′-(2″-hydroxyethylamino)-2′-hydroxypropoxy]propyltrimethoxysilane. This was bonded to the silica as described previously [12].

Other bonded-phase materials

Other packings similar in nature to that synthesised from 2-aminoethanol were prepared by analogous procedures, using 2-amino-2-methyl-1,3-propanediol, 2-amino-2-ethyl-1,3-propanediol, 2-amino-2-hydroxymethyl-1,3-propanediol and 2-mercaptoethanol (see Table I).

Polyacrylamide gel electrophoresis

Purified oligonucleotides (50 ng) were end-labelled with [^{32}P]ATP using T4 polynucleotide kinase and then analysed by electrophoresis on 20% polyacrylamide gels using an aqueous 4 m*M* Tris, 1 m*M* borate and 0.1 *M* EDTA buffer adjusted to a pH 8.3. Bands were visualised by autoradiography (X-Omat autoradiograph film, Kodak, France).

Chromatographic procedures

Stainless-steel columns (50 mm × 4.6 mm I.D., 80 mm × 6.2 mm I.D. and 250 mm × 4.6 mm I.D.) were packed by a downward slurry technique. Separations were carried out at ambient temperatures (18 to 24°C) using either a Waters LC system (Model 510) (Waters Assoc., Hartford, UK) or an LKB gradient pumping system (Model 2150/2152, LKB Gaithersburg, MD, U.S.A.)

The solvent systems used were as follows. pH 6: 20 m*M* dipotassium phosphate in 20% (v/v) aqueous acetonitrile, adjusted to pH 6.0 with 1 *M* potassium dihydrogenphosphate. The elution buffer was prepared by adding KCl (1 or 2 *M*) to this buffer. pH 7: 20 m*M* disodium phosphate in 20% (v/v) aqueous acetonitrile adjusted to pH 7.0 with 1 *M* sodium dihydrogenphosphate. The elution buffer was prepared by adding 1 *M* NaCl to this buffer.

The gradients used were as follows. (A) 0.15 *M* NaCl, 20% acetonitrile, pH 7.0 to 0.75 *M* NaCl, 20% acetonitrile, pH 7.0 over 40 min at 1 ml/min: (B) 20% acetonitrile, pH 6.0 to 1 *M* KCl, 20% acetonitrile, pH 6.0 over 40 min at 1 ml/min. (C) 0.35 *M* NaCl, 20% acetonitrile, pH 7.0 to 0.6 *M* NaCl, 20% acetonitrile, pH 6.0 over 90 min at 1 ml/min. (D) 20% acetonitrile, pH 6.0 to 2 *M* KCl, 20% acetonitrile, pH 6.0 over 120 min at 1 ml/min.

The capacity factor, k', defined as $(V_e - V_0)/V_0$, where V_e is the elution volume of the peak and V_0 is the void volume of the column. V_0 was determined by injecting a sample of acetonitrile and measuring its retention time.

Oligonucleotides eluting from the columns were collected manually at the detector outlet. Before analysis by electrophoresis, the fractions were desalted by gel filtration on Sephadex G25 and lyophilised.

RESULTS AND DISCUSSION

Influence of bonded phase

The retention of oligonucleotides increased with the increasing number of hydroxyl groups on the bonded phase. Table I shows the retention times of the homo-oligonucleotides dA_{14} and dA_{15} on the different stationary phases prepared. Increasing the number of hydroxymethyl groups on the carbon atom adjacent to the amino group increased retention. It is not clear whether this is due to the enhancement of the ion-exchange character of the secondary amine, or if there are secondary interactions between the hydroxyl group and the phosphate ester or other polar groups in the oligonucleotide. Increasing the hydrophobic nature of the bonded phase also increases the retention time; substituting an ethyl group for a methyl group leads to a slightly longer retention time. This substitution would obviously increase any reversed-phase interactions, but would also be expected to reduce the ionic nature of the amino group due to the increase in electron-releasing character of the alkyl functionality. The presence of the amino function is essential, as was demonstrated by the use of 2-mercaptoethanol, when no retention of the oligonucleotides was observed.

Influence of column dimensions

The effect of column length on the gradient separation of macromolecules by reversed-phase chromatography has been shown to be relatively small, once a certain molecular size is exceeded. In the case of both polystyrene [13] and peptides and proteins [14], the slopes of plots of log k' against solvent composition increase rapidly with molecular size. With these molecules the peak width is more a function of the gradient profile rather than of the column length, as is the case with small molecules. Similar effects have been observed under ion-exchange conditions [15]. Since it was anticipated that oligonucleotides would behave similarly, a range of columns of differing lengths was studied. As shown in Fig. 1, the separation of a 10-mer hetero-oligonucleotide from its associated impurities was improved on changing from a 50-mm column through an 80-mm column to a 250-mm column. The improvement was small for the transition to the longest column, so the 80 mm × 6.2 mm I.D. format was chosen for subsequent investigations, as it had sufficient capacity for the

TABLE I

INFLUENCE OF BONDED PHASE ON THE RETENTION TIME OF THE OLIGONUCLEOTIDES dA_{14} AND dA_{15}

The oligonucleotides were chromatographed using gradient B.

Bonded phase	Structure	k'	
		dA_{14}	dA_{15}
2-Aminoethanol	$H_2NCH_2CH_2OH$	28.8	30.6
2-Amino-2-methyl-1,3-propanediol	$HOCH_2CNH_2(CH_3)CH_2OH$	41.2	43.8
2-Amino-2-ethyl-1,3-propanediol	$HOCH_2CNH_2(C_2H_5)CH_2OH$	43.3	45.2
2-Amino-2-hydroxymethyl-1,3-propanediol	$HOCH_2CNH_2(CH_2OH)CH_2OH$	Not eluted	
2-Mercaptoethanol	$HSCH_2CH_2OH$	0	0

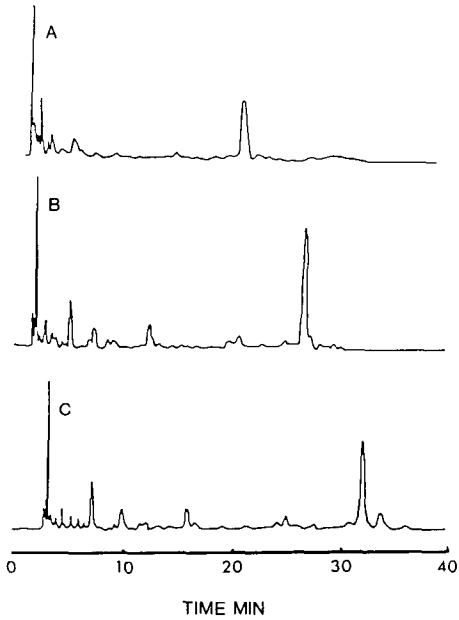

Fig. 1. Effect of column dimensions on the chromatography of an 18-mer. A 10-μg amount of the oligonucleotide d(TCTAATACTTCGGGATGGTG) was loaded and eluted using gradient A; a.u.f.s. = 0.1.

quantities of oligonucleotide generally purified and showed adequate resolution for the oligonucleotides of interest.

Solvent system parameters

The effects of varying the pH of the buffer, the nature and concentration of the organic modifier, the buffer concentration and the temperature at which the separation was carried out were investigated.

The influence of pH on the separation was investigated using a buffer of constant ionic strength over the pH range 3–8. This was prepared from dipotassium hydrogenphosphate–citric acid mixtures and the ionic strength adjusted by the addition of potassium chloride to a final value of 0.2 *M*. A short oligonucleotide (dA_4) was chromatographed isocratically using this mobile phase. A plot of k' against pH is shown in Fig. 2. The reason for the apparent increase in retention time at about pH 7 is not clear; there is, however, a general trend of reduced retention at higher pH values for both small and large oligonucleotides. The oligonucleotide dA_4 was not eluted at pH values below 4.

The concentration of acetonitrile in the mobile phase was investigated by the chromatography of dA_4 in 20 m*M* phosphate, pH 6.0, over a range of solvent compositions from 5% (v/v) to 50% (v/v). A plot of log k' against the concentration of organic modifier (not shown) was linear, showing classical reversed-phase behaviour. A concentration of 20% was chosen, as representing a reasonable compromise between retention and peak shape. The concentration of the buffer system was

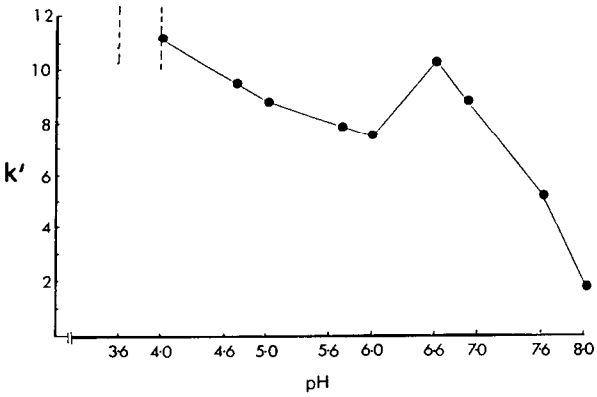

Fig. 2. Effect of pH on the chromatography of dA_4. The column was 80 mm × 6.2 mm I.D. and was eluted isocratically at a flow-rate of 1 ml/min using potassium phosphate–citric acid buffers of constant ionic strength (0.2 *M*). k' = Capacity factor; a.u.f.s. = 0.2.

investigated similarly, using dA_4 over a range of buffer concentrations from 10 to 50 m*M*. A plot of k' against the inverse of buffer concentration (not shown) was linear, demonstrating the ionic nature of the separation.

Acetonitrile, methanol and tetrahydrofuran were chosen as examples of solvents exhibiting quite different selectivities [16]. These were used in the mobile phase at concentrations of 20, 30 and 10% (v/v) respectively, values which approximately reflect their relative solvent strengths in reversed-phase chromatography [16]. A sample of dA_{15} which contained appreciable concentrations of the lower oligomers was used in the gradient separation. Fig. 3 shows the plot of k' against the number of bases in the oligonucleotide. There was little difference between the solvents, apart

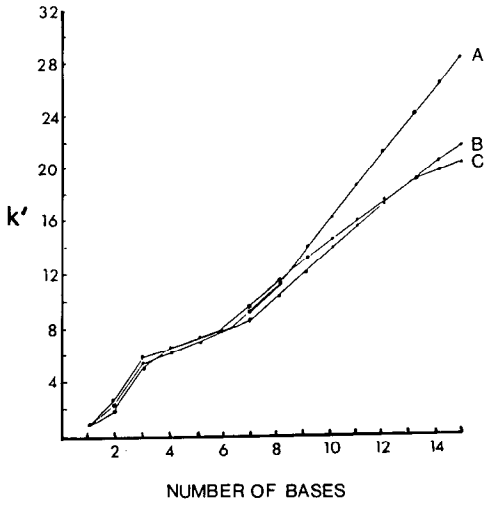

Fig. 3. Effect of organic modifier on the chromatography of oligo-dA_n (n = 1–15). The organic modifiers were (a) methanol at 30% (v/v), (b) acetonitrile at 20% (v/v) and (c) tetrahydrofuran at 10% (v/v). The column was eluted using gradient B; a.u.f.s. = 0.2.

from the greater retention seen with methanol because of its relatively lower solvent strength in this system. As the nature of the organic modifier was not critical, 20% (v/v) acetonitrile was used for the remainer of this work.

The effect of temperature on the retention of dA_{15} was also investigated by running the column in an oven over a range of temperatures from 30 to 75°C. As shown in Fig. 4, retention fell rapidly with increasing temperature.

Loading capacity and recovery

The capacity of the packing for oligonucleotides was investigated by increasing the sample load and observing the effect on the chromatography of a pure oligonucleotide. Using the 80 mm × 6.2 mm I.D. column the amount of pure 18-mer loaded was increased to 140 μg, with no appreciable change in the elution profile. With amounts greater than this, the UV absorbance was too great to monitor the separation; such 100-μg quantities, however, are more than sufficient for hybridisation experiments. In order to assess the recovery of oligonucleotides approximately 10-μg amounts were chromatographed. The eluting peak was collected, and the total amount present was estimated from the UV absorbance at 260 nm. The recoveries of all oligonucleotides, up to 38 bases in length, were in excess of 95%.

Separation of homo-oligonucleotides

In order to demonstrate the separation of the n−1 oligonucleotide, the homo-oligonucleotides dA_{14}, dA_{15}, dT_{24} and dT_{25} were synthesised, and the separation of each oligomer pair was examined. As shown in Fig. 5A and B, baseline

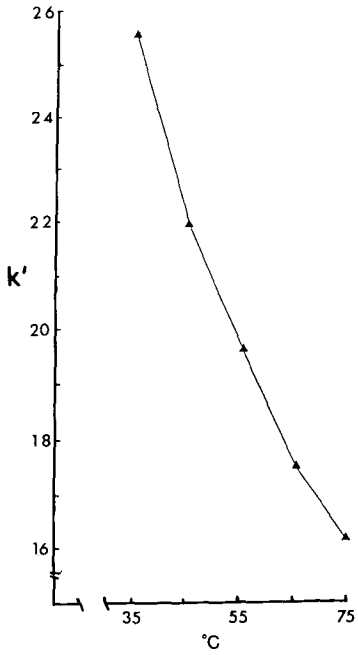

Fig. 4. Effect of temperature on the chromatography of dA_{15}. The column was eluted using gradient B; a.u.f.s. = 0.2.

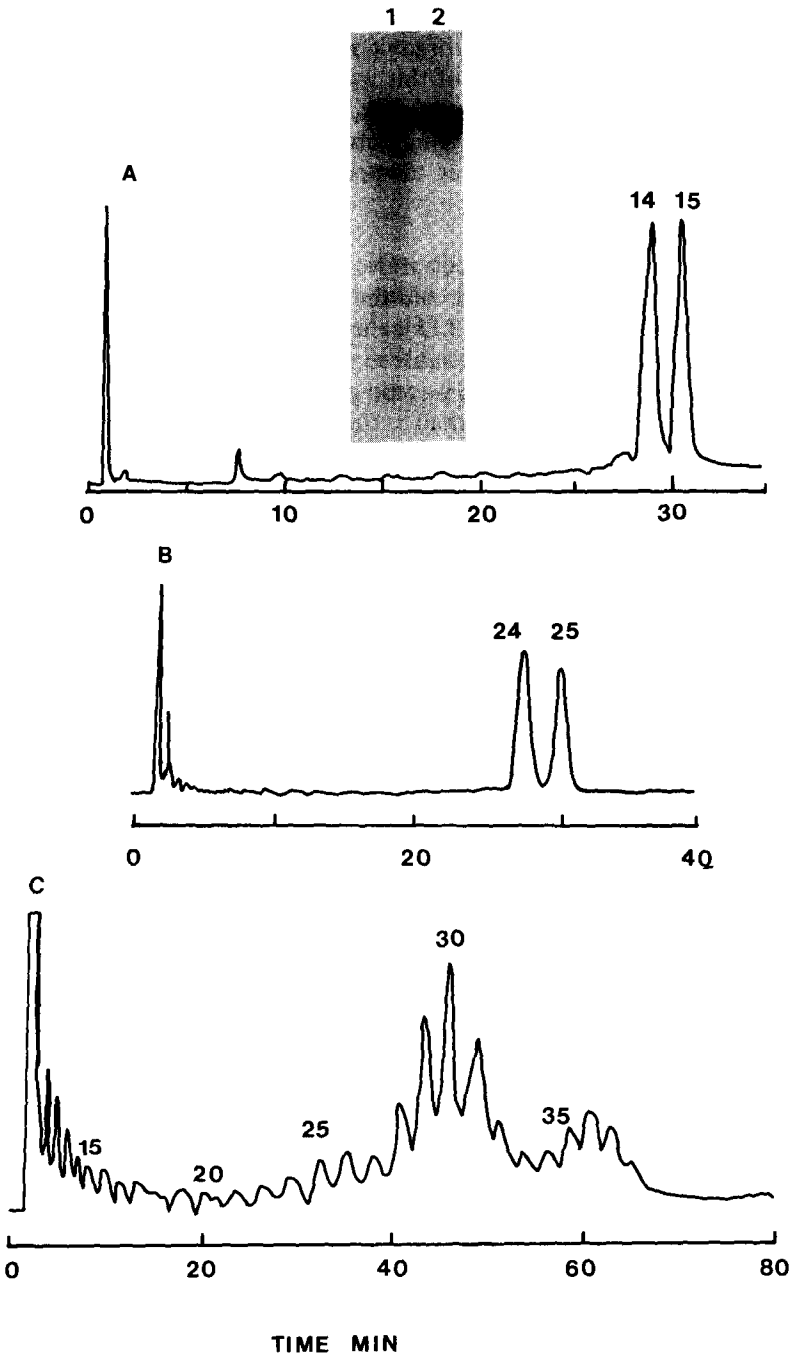

Fig. 5. Chromatography of mixed homo-oligonucleotides. (A) Separation of dA_{14} and dA_{15} using gradient B; (B) separation of dA_{24} and dA_{25} using gradient C; (C) chromatography of a failed synthesis of dT_{40} using gradient C; a.u.f.s. = 0.2. The inset in (A) shows the electropherogram of dA_{15} (1) before and (2) after chromatography.

resolution was obtained in each case. The inset to Fig. 5A shows the electropherogram obtained from dA_{15} before and after chromatography. Fig. 5C shows the chromatogram obtained from a failed synthesis of dT_{40}. The large number of peaks are indicative of the truncated sequences present in the synthesis product. Peaks can be discerned up to dT_{38}, although the complex nature of the mixture reduces the resolving power of the system.

Separation of hetero-oligonucleotides

To investigate the effect of length and nucleotide sequence on the resolution of hetero-oligonucleotides a number of 17- and 18-mer oligomers were synthesised and chromatographed. The retention times and structures of some of these are given in Table II. Although the majority of the oligonucleotides eluted with retention times close to the mean value, some were retained much longer. This may be due to the presence of sequences which allow self-hybridisation of the oligonucleotides, leading to a change in the apparent molecular size of the chromatographing species, which results in an increased retention time and peak distortion. Fig. 6 illustrates the elution profiles of some 18-mer hetero-oligonucleotides; Fig. 6D shows clearly the typical increased retention and skewed peak thought to be due to self-hybridisation of the

TABLE II

RETENTION TIMES OF 17-MER AND 18-MER OLIGONUCLEOTIDES

Oligonucleotide sequence	*n*	Retention time (min)	Mean retention time (min)
ATGGTCTTGTGTGATAA	17	30.5	
CCTTTGTAAGTGCTAAA	17	30.4	
CTTCGATAATGTCTTGA	17	30.3	
AAGTGAGCGGACAAATA	17	33.8	30.8 (S.D. = 1.65)
TGAACTTCAGAAGTAGA	17	33.8	
TAGAATAGTGCTTCACA	17	29.9	
TCTACTGGTGTATAAAC	17	30.1	
TGGATAAGTACAAGTTA	17	29.8	
TTTCTTATGGGTAGCAAG	18	31.2	
ACCGGAAAGATTAATCAG	18	36.9	
CACCATCGAAGTATTAGA	18	32.5	
AAATTTCGAACTGACGCA	18	33.8	
CTTCAAACAGGGTGTTT	18	31.4	
CTTGCAGGAACAAAATGA	18	31.4	
ACTTCCTATCTAGAAGGA	18	33.5	
GCGATTATGAGAGTGAAT	18	29.8	
AATCGTCTCGTCTTATTA	18	29.8	31.8 (S.D. = 1.82)
GATTAAACCCAGCACATA	18	32.3	
TCTAATACTTCGATGGTG	18	31.5	
TCATTTTGTTCCTGCAAG	18	30.3	
CAGGAACAACATGAAGAA	18	32.1	
CCCTTCGTTCAACAAAAT	18	30.5	
CGCTTGGTTCTGGTTCAC	18	31.5	
AATCGTCTCGTCTTATTA	18	31.5	
TCCTTCTAGATAGGAAGT	18	31.0	

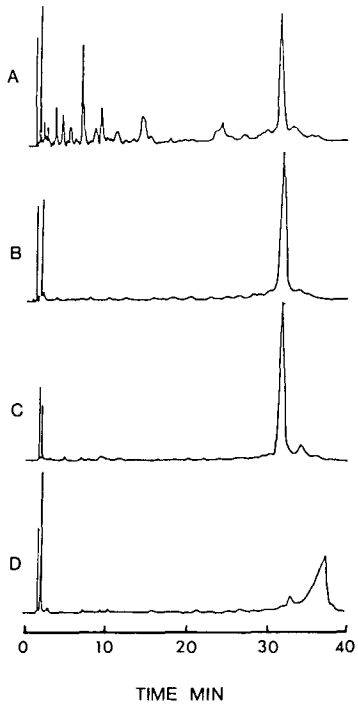

Fig. 6. Chromatography of 18-mer hetero-oligonucleotides. (A) d(TCTAATACTTCGATGGTG); (B) d(CTTGCAGGAACAAAATGA); (C) d(ATTTTGTTGAACGAAGGG); (D) d(ACCGGAAAGATTA-ATCAG). The column was eluted using gradient C; a.u.f.s. = 0.2.

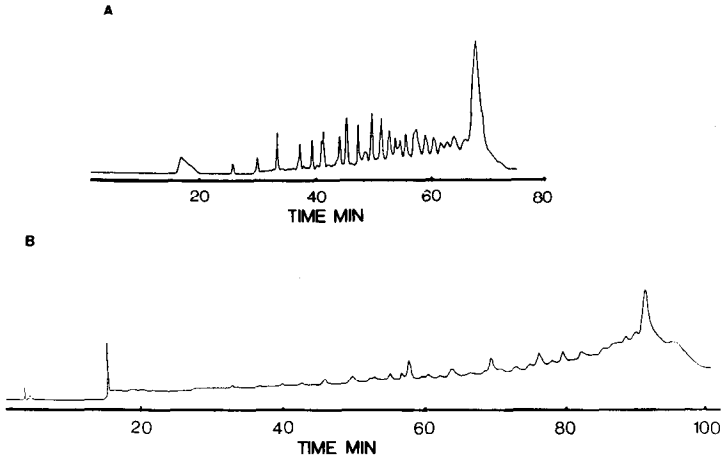

Fig. 7. Chromatography of longer oligonucleotides. (A) 27-mer; (B) 44-mer. The column was eluted using gradient D; a.u.f.s. = 0.2.

oligonucleotides, leading to a change in the apparent molecular size of the chromatographing species, which results in an increased retention time and peak distortion.

In order to investigate the effect of increasing chain length, a number of longer oligonucleotides were purified. Fig. 7 shows the elution profiles of 27-mer and 44-mer hetero-oligonucleotides. To improve the separation of these longer molecules the gradient time was increased to 120 min and the flow-rate reduced to 0.5 ml/min. Gel electrophoresis of the eluted oligonucleotides showed single bands and clearly demonstrated the removal of shorter-failure sequences. Larger oligonucleotides of this type could be isolated in greater than 90% purity, with gel electrophoresis showing only traces of the (n − 1)-mer (data not shown).

CONCLUSIONS

A novel bonded-phase packing is reported which allows the rapid purification of both homo- and hetero-oligonucleotides. The matrix is believed to interact with the oligonucleotides by a mixed-mode mechanism involving both polar and hydrophobic interactions. Rapid separations of oligonucleotides were obtained with high recoveries and purities in excess of 90% over a size range up to 44 base units in length. Baseline resolution between *n*- and (*n* − 1)-mer oligomers was achieved under normal preparative loading conditions. Loading studies indicated that sufficient oligonucleotide for normal hybridisation requirements could be isolated from a single chromatographic run.

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