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Rigid polymerics: the future of oligonucleotide analysis and purification

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Abstract

A family of rigid macroporous HPLC materials, reversed phase and anion exchange, has been evaluated for the analysis and purification of a range of de-protected, dimethoxytrityl-off, oligonucleotides. A 25-base pair (bp) double-stranded DNA ladder was used to determine the resolving range for the four pore sizes of reversed-phase media. The 100 Å pore size resolves up to 50–75 bp, the 300 Å up to 250–300 bp, the 1000 Å up to 400–450 bp and the 4000 Å pore size is capable of resolving in excess of 500 bp. The dynamic capacity of these four pore sizes was also determined using a synthetic oligonucleotide with two ion-pairing agents at ambient and 60 °C. The dynamic capacity was shown to decrease with increasing pore size and that with the triethylammonium acetate ion-pairing agent there was negligible temperature dependency. The dynamic capacity was higher when tetrabutylammonium bromide was used at elevated temperature. A strong anion-exchange functionality on a pH-stable polymeric particle was used to investigate the selectivity and resolution of the technique. Using a poly-T-oligonucleotide size standard, resolution of full length oligonucleotide (n) from the truncated species due to coupling failure ($n-1$, $n-2$, etc.) was demonstrated up to at least the 30mer. Resolution of a phospho diester contaminant from a phospho thioate oligonucleotide and a truncated sequence was demonstrated using anion-exchange HPLC at high pH.
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1. Introduction

Oligonucleotide synthesis is now a fast, extremely reliable and highly efficient technique, but the multi-step process does result in a target molecule and failure sequences, where coupling of a nucleotide onto a linear oligonucleotide chain fails and results in truncated oligonucleotides. As the length of the target oligonucleotide increases, so the product yield decreases and hence the quantity of impurities to be resolved increases; product yields of 50–80% are not uncommon. There are some oligonucleotide applications that do not require high purity, but there are many others, for example in genotyping and anti-sense therapies, where purity is essential.

To achieve the high purity required, polyacrylamide gel electrophoresis (PAGE) is conventionally used and oligonucleotide purities in excess of 98% can be obtained [1]. There are two major disadvantages with PAGE; the first is its labour-intensive/time-consuming nature and the second is the limited amount of oligonucleotide that can be purified. High-performance liquid chromatography (HPLC) can also be used [2]. For the separation of the protected, dimethoxytrityl-on (DMT-on) product from de-protected DMT-off failure sequences produced during the solid-phase synthesis, reversed-

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phase chromatography can be used [3]. Two HPLC methods have been used for the analysis and purification of de-protected DMT-off oligonucleotides, anion-exchange and ion-pair reversed-phase HPLC. Anion exchange separates the product from the earlier eluting failure sequences using a salt gradient. However, the relatively small pore silica-based weak anion exchangers do not have the required resolution or sample load for preparative separations [4]. It has been shown that quaternising the polyethyleneimine weak anion exchanger improves both the resolution and loading [5]. Ion-pair reversed-phase chromatography has been proposed as an alternative to the anion-exchange methods, but resolution decreases with increasing oligonucleotide length. A method has been proposed that improves resolution for oligomers in the range 20 to 30 chain lengths that utilises a triethylamine (TEA) and hexafluoroisopropanol (HFIP) eluent system [6]. However, there are difficulties associated with the use of HFIP for larger-scale purifications, including cost and toxicity.

A limitation reported with both anion-exchange and reversed-phase HPLC methods is that they both suffer from oligonucleotide mass transfer restrictions within the porous particles that reduces the efficiency of the separation [7]. Working at elevated temperatures, 60 °C, does improve the mass transfer, but limitations of pore diffusion or exclusion of large oligonucleotides still exists. In an attempt to overcome the mass transfer limitations, work has been carried out using reversed-phase non-porous 1.5 μm silica-based particles [8] and non-porous ion exchangers [9], but the capacity, due to the loss of the internal pore volume, is low and the operating pressures high. An alternative approach is to look at the pore size and structure of the HPLC material. Wide-pore polymeric materials have traditionally been used for the separation of biomolecules [10], but the original materials were semi-rigid and had only limited mechanical stability, making them inappropriate for HPLC. With the availability of rigid macroporous polymers which could be packed in HPLC columns and run with high flow-rates, biomolecule separations can be achieved in much shorter times; reversed-phase separations of proteins [11] and anion-exchange separation of oligonucleotides [12] have been reported. With advances in the production of rigid polymeric particles it is now possible to produce reversed-phase and ion-exchange particles that are mechanically rigid and stable to temperature and pH in a range of defined pore sizes, 100 to 4000 Å. It would therefore be expected that the wide-pore HPLC packings would exhibit improved mass transfer characteristics for the larger oligonucleotides when compared with small-pore silica-based materials.

The work presented here aims to demonstrate the feasibility of using a family of small particle size, high-performance poly(styrene–divinylbenzene)-based reversed-phase, PLRP-S, and a strong anion-exchange material, PL-SAX, for the analysis and purification of oligonucleotides and to define the resolving ranges and selectivity for the various materials.

2. Experimental

2.1. Dynamic loading capacity

The dynamic binding capacity was determined for each poly(styrene–divinylbenzene) reversed-phase material, PLRP-S, pore size and with two ion-pairing agents, triethylammonium acetate (TEAA) and tetrabutylammonium bromide (TBuABr), by frontal loading. A 4 mg/ml solution of the 20mer oligonucleotide prepared in either 0.1 *M* TEAA, pH 7.0, or 0.025 *M* TBuABr was pumped through a 150×4.6 mm I.D. column packed with the PLRP-S material at a flow-rate of 1.0 ml/min. The oligonucleotide breakthrough curve was obtained by monitoring the column eluent at 260 nm. The volume of oligonucleotide solution required to saturate the column was determined at 20% of the adsorption of the oligonucleotide solution with no column in place. The column packing was washed using a strong eluent, acetonitrile–0.1 *M* TEAA pH 7.0 (50:50, v/v) or acetonitrile–0.025% TBuABr (99:1, v/v), and conditioned using the binding eluent, acetonitrile–0.1 *M* TEAA pH 7.0 (1:99, v/v) or acetonitrile–0.025 *M* TBuABr (1:99, v/v), before repeating the frontal loading experiment.

2.2. HPLC instrumentation and columns

A quaternary low-pressure mixing gradient system

consisting of a Model LC 1150 pump, a PL-DG 804 degasser and a PL-LC 1200 UV–Vis detector set at 260 nm (Polymer Labs., Church Stretton, UK) was used. A manual Rheodyne 7725 injector fitted with a 20 μl loop (Polymer Labs.) was used to inject the oligonucleotide samples. The PLRP-S 100 Å, 300 Å 1000 Å and 4000 Å and PL-SAX 1000 Å media were packed into stainless-steel column hardware with the following dimensions: 50×4.6 mm I.D., 150×4.6 mm I.D., 150×2.1 mm I.D. (Polymer Labs.). The HPLC conditions are given in the captions to the figures.

2.3. Eluents and chemicals

The water used for eluent preparation was purified using an Elgastat Prima RO system coupled to an Elgastat UHP system (Elga, High Wycombe, UK) and the eluent additives were of analytical or HPLC grade (Fisher, Loughborough, UK).

3. Results and discussion

3.1. Reversed-phase analysis

Molecular biology applications require the analysis and purification of molecules with widely differing molecular sizes. The choice of media pore size will depend on the size of the oligonucleotide to be separated/purified. The smaller oligonucleotides require small pore sizes and as the chain length increases larger pore size materials will be required to minimise diffusion issues and maximise available surface area and hence loading. Rigid polymeric materials are therefore ideal as they can be produced with controlled pore sizes in the range of $<$60 Å to $>$4000 Å. For high-performance resolution the PLRP-S reversed-phase material is available in a range of pore sizes, 100 Å, 300 Å, 1000 Å and 4000 Å. In all cases the particles have an open pore structure that minimises mass transfer restrictions, but all are sufficiently rigid to be able to operate under HPLC conditions of pressure and flow-rate.

A 25-base pair (bp) double-stranded DNA ladder was used to determine the separation range for the four pore sizes of PLRP-S media. The sample was analysed using the same chromatographic conditions and the individual peaks collected for PAGE analysis to show the size and purity of each fraction. Fig. 1 shows the HPLC chromatograms and PAGE analysis for each pore size. Lane 1 in the PAGE shows the 25-bp ladder and the lanes from left to right the collected peak fractions with increasing elution time. It is clear from these chromatograms that the PLRP-S 100 Å material has a very restricted resolving range with three distinct peaks and the remaining ladder components all co-elute, excluded from the pores. Two fractions were collected across peak two which from the PAGE analysis can be seen to contain the same oligonucleotide size, 50 bp. As the pore size of the HPLC media increases so the resolving range increases, and the larger oligonucleotides can permeate the porous structure. The PLRP-S 100 Å media resolves up to 50–75 bp, the 300 Å up to 250–300 bp, the 1000 Å up to 400–450 bp and the 4000 Å pore size is capable of resolving in excess of 500 bp.

Oligonucleotides designed as polymerase chain reaction (PCR) primers are typically 15–35 nucleotides long and are synthesised with a production yield in the range 60–80%. For purification it is essential to achieve resolution and also to have high capacity so enabling rapid, economic purifications to be achieved. It is therefore necessary to know not only which of the PLRP-S pore sizes has the optimum resolving range, but also which has the highest available surface area and, hence, capacity. A typical 20mer oligonucleotide was therefore chosen to look at capacity, frontal loading analysis, as a function of media pore size. Fig. 2 shows the frontal loading curves obtained with the range of PLRP-S pore sizes, 100 Å, 300 Å, 1000 Å and 4000 Å, using the ion-pairing agent TEAA at ambient and 60 °C with the dynamic capacities summarised in Table 1. The first curve shows the zero to full-scale deflection, no column in place, and the subsequent curves, left to right, the effect of decreasing pore size. In all cases, including the smallest 100 Å pore size, the frontal loading curves are sharp, indicating good mass transfer characteristics and the ability to use virtually the entire column volume for preparative/process work. Increasing the column temperature has little effect on the shape of the frontal loading curves or oligonucleotide capacity.

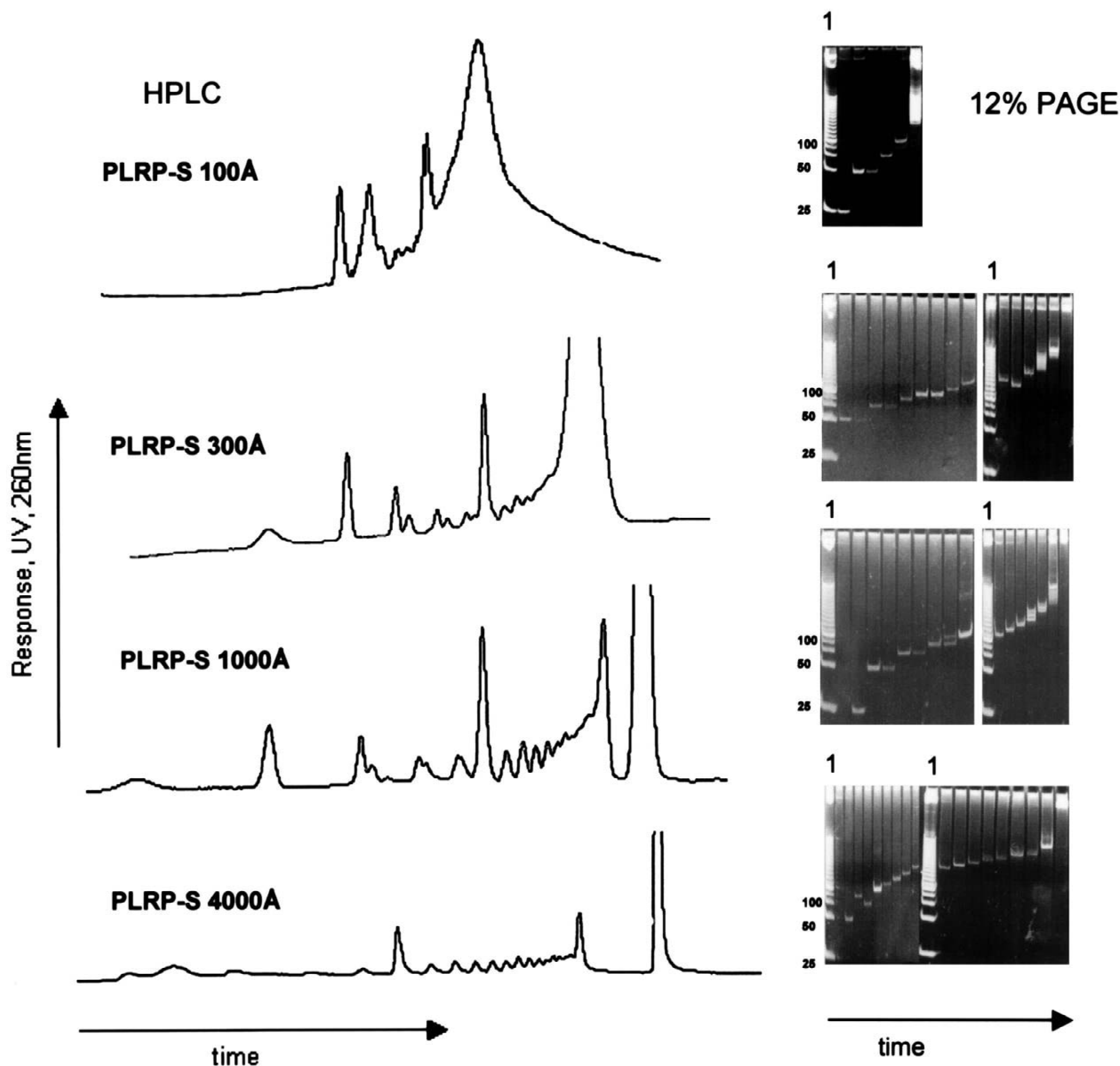

Fig. 1. Reversed-phase chromatograms of a 25-bp ladder and PAGE analysis of each peak fraction. Column, PLRP-S 150×2.1 mm I.D.; eluent A, 0.1 *M* TEAA; eluent B, 0.1 *M* TEAA in 50% acetonitrile; gradient, 12.5–50% B in 150 min at a flow-rate of 200 μl/min.

Triethylammonium acetate is a volatile buffer, which is advantageous for preparative separations, but it has been reported that some oligonucleotide sequence dependency is observed with this eluent system. Tetraalkylammonium ions are more strongly adsorbed onto the surface of the reversed-phase particles than trialkylammonium ions and there is less oligonucleotide sequence dependency. The frontal loading curves at both ambient and 60 °C were repeated using the TBuABr eluent system (Fig. 3) and the dynamic capacities summarised in Table 1. As with the TEAA eluent the frontal loading curves are sharp and there is no obvious temperature dependency, however the dynamic capacity is higher and increases with increasing temperature. It should be noted that, although the loading is higher, there is

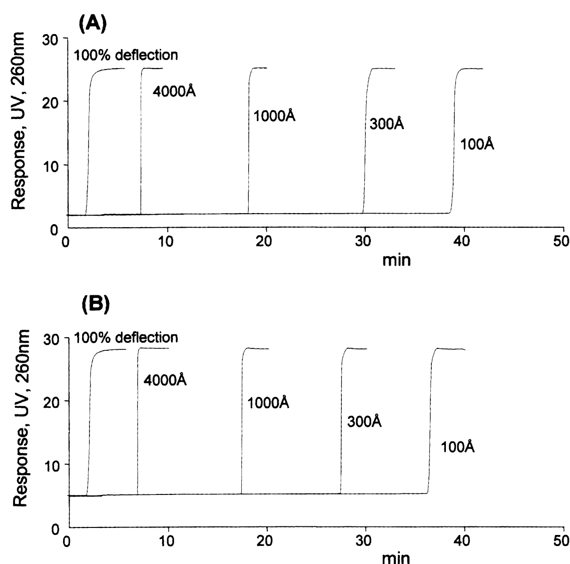

Fig. 2. Frontal loading curves for a de-protected 20mer oligonucleotide using TEAA as the ion-pairing agent. Column, PLRP-S 150×4.6 mm I.D.; conditioning buffer, acetonitrile–0.1 *M* TEAA pH 7.0 (1:99, v/v); washing buffer, acetonitrile–0.1 *M* TEAA pH 7.0 (50:50, v/v); loading solution, 4 mg/ml oligonucleotide prepared in 0.1 *M* TEAA pH 7.0; flow-rate, 1.0 ml/min. (A) Curves obtained at ambient temperature; (B) curves obtained at 60 °C.

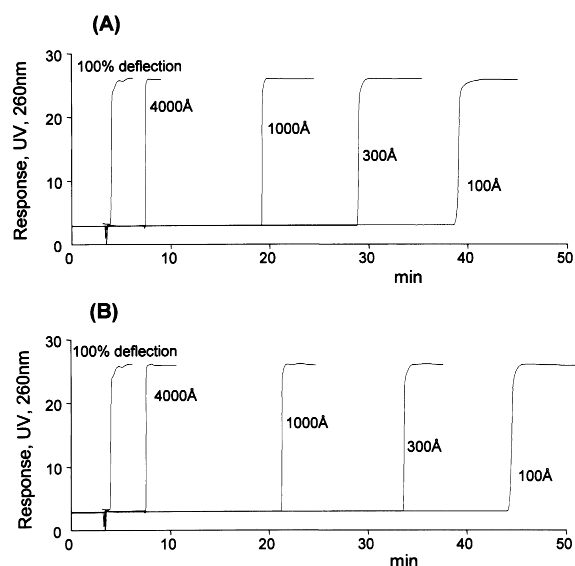

Fig. 3. Frontal loading curves for a de-protected 20mer oligonucleotide using TBuABr as the ion-pairing agent. Column, PLRP-S 150×4.6 mm I.D.; conditioning buffer, acetonitrile–0.025 *M* TBuABr (1:99, v/v); washing buffer, acetonitrile–0.025 *M* TBuABr (99:1, v/v); loading solution, 4 mg/ml oligonucleotide prepared in 0.025 *M* TBuABr; flow-rate, 1.0 ml/min. (A) Curves obtained at ambient temperature; (B) curves obtained at 60 °C.

a disadvantage of using the TBuABr eluent for preparative separations in that it is not volatile and therefore unlike the TEAA mobile phase additive cannot be removed by lyophilisation [13].

There is also much interest in the use of oligonucleotides as therapeutic agents, for example antisense therapy against viral infections and for enhanced cancer therapies. To improve the in vivo resistance to nuclease degradation, phospho thioate analogues of DNA are utilised. There is a requirement to produce large quantities of well-defined oligonucleotides in an economic and timely fashion for clinical trials, etc. Reversed-phase ion-pair chromatography is an obvious choice, as excellent selectivity and high capacity, as has been demonstrated above, is achieved. However, with the phospho thioate analogues, impurities from incomplete thiolation must be separated in addition to the synthesis failure sequences. In order to assess the feasibility of reversed-phase ion-pair chromatography for the analysis and purification of these therapeutic agents a separation of two phospho thioate (PS) oligonucleotides, an 18mer and 20mer, together with a phospho diester (PO) oligonucleotide contaminant was attempted using the two ion-pairing agents, TEAA and TBuABr. The two chromatograms are shown in Fig. 4. Using both ion-pairing agents it is possible to resolve the two PS oligonucleotides, with the 18mer eluting before the 20mer, but resolution of the PO oligonucleotide contaminant could only be achieved with the TEAA. The TEAA is the least strongly adsorbed ion-pairing agent and the one reported to

Table 1
Oligonucleotide capacity (mg/ml packed bed) when TEAA and TBuABr are used as the ion-pairing agent

	PLRP-S 100 Å	PLRP-S 300 Å	PLRP-S 1000 Å	PLRP-S 4000 Å
TEAA				
Ambient	58	45	26	11
60 °C	59	43	27	10
TBuABr				
Ambient	63	46	29	11
60 °C	72	54	34	11

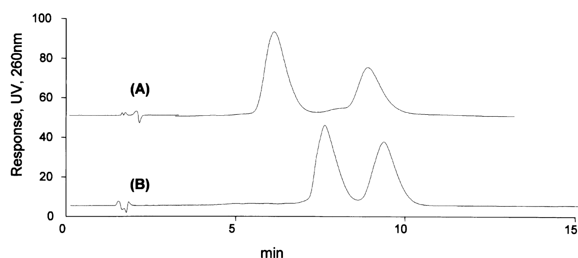

Fig. 4. Separation of de-protected 18mer and 20mer PS oligonucleotides with a small PO contaminant. Column, PLRP-S 100 Å 5 μm 150×4.6 mm I.D.; detector, UV 260 nm; flow-rate, 1.0 ml/min; temperature, 60 °C. (A) Using TEAA ion-pairing agent, gradient 15 to 25% acetonitrile in 0.1 *M* TEAA pH 7.0. (B) Using TBuABr ion-pairing agent, eluent A acetonitrile–0.025 *M* TBuABr (1:99); eluent B acetonitrile–0.1 *M* TBuABr (99:1); gradient, 60–70% B in 20 min.

give some sequence-dependent selectivity and would therefore be most likely to discriminate between the PO and PS oligonucleotides.

3.2. Anion-exchange analysis

From the previous data it is clear that ion-pair reversed-phase HPLC can be used for the analysis and purification of oligonucleotides. By judicious choice of the ion-pairing agent selectivity and capacity can be achieved for synthesis failure sequences $n-1$, $n-2$ separations, and for contaminants due to incomplete chemical modification of the DNA analogues. However, these methodologies do require the use of organic solvents, such as acetonitrile, which are both expensive to purchase and dispose of and require specific safe-handling facilities. Whilst this is often acceptable for analytical and small-scale preparative work, as the quantity requirement increases so the production costs increase and a process may become uneconomical. An alternative purification strategy is required. One such alternative would be the use of anion-exchange materials for de-protected, DMT-off oligonucleotide analysis and purification. The PL-SAX strong anion exchanger uses the poly(styrene–divinylbenzene) base particle with a quaternised polyethyleneimine layer and so has a strong anion-exchange functionality coupled with chemical stability and would therefore be expected to show good resolution [5].

It is still essential that failure sequence, $n-1$, $n-2$, etc., resolution is obtained. To assess the feasibility of achieving this level of selectivity for a range of oligonucleotides the separation of a poly-T-oligonucleotide size standard was performed (Fig. 5). The four main peaks correspond to the 10mer, 15mer, 30mer and 50mer. It is clear that using the PL-SAX 1000 Å 8 μm material good resolution of the failure sequences is achieved with $n-1$ resolution up to at least the 30mer.

For the analysis and purification of large oligonucleotides it is essential that a large-pore particle is used and that there is an open pore structure so that any restriction to mass transfer is minimised. One significant advantage of the rigid macroporous HPLC materials is their mechanical stability. Even large-pore materials, 1000 Å and 4000 Å, can be packed into high-efficiency HPLC columns and operated with high eluent flows, in excess of 1440 cm/h, and at system pressures up to 3000 p.s.i. (1 p.s.i. = 6894,76 Pa). The PL-SAX 1000 Å material has an open pore structure and shows minimal band broadening even with large oligonucleotides at high eluent flow-rates. Fig. 6 shows a 20 min purification of a 91mer oligonucleotide from the shorter failure sequences.

Strong anion-exchange functionalities on chemically stable polymeric particles are extremely advantageous for the analysis and purification of de-protected oligonucleotides. It is possible to extend the normal operating pH range for anion-exchange chromatography to higher pH, due to the stability of

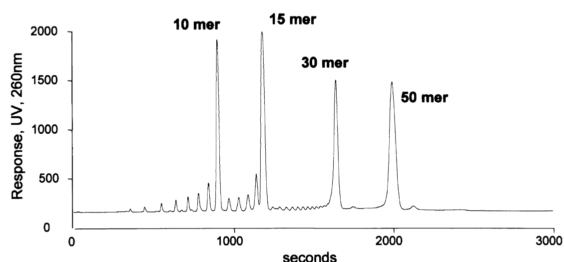

Fig. 5. Separation of a poly-T-oligonucleotide size standard spiked with a 10mer, 15mer, 30mer and 50mer (main peaks). Column, PL-SAX 1000 Å 8 μm 50×4.6 mm I.D.; eluent A, acetonitrile–0.1 *M* TEAA pH 8.5 (7:93); eluent B, acetonitrile–0.1 *M* TEAA 1 *M* ammonium chloride pH 8.5 (7:93); gradient, 0–40% B in 10 min followed by 40–70% B in 14 min and 70–100% B in 25 min; flow-rate, 1.5 ml/min.

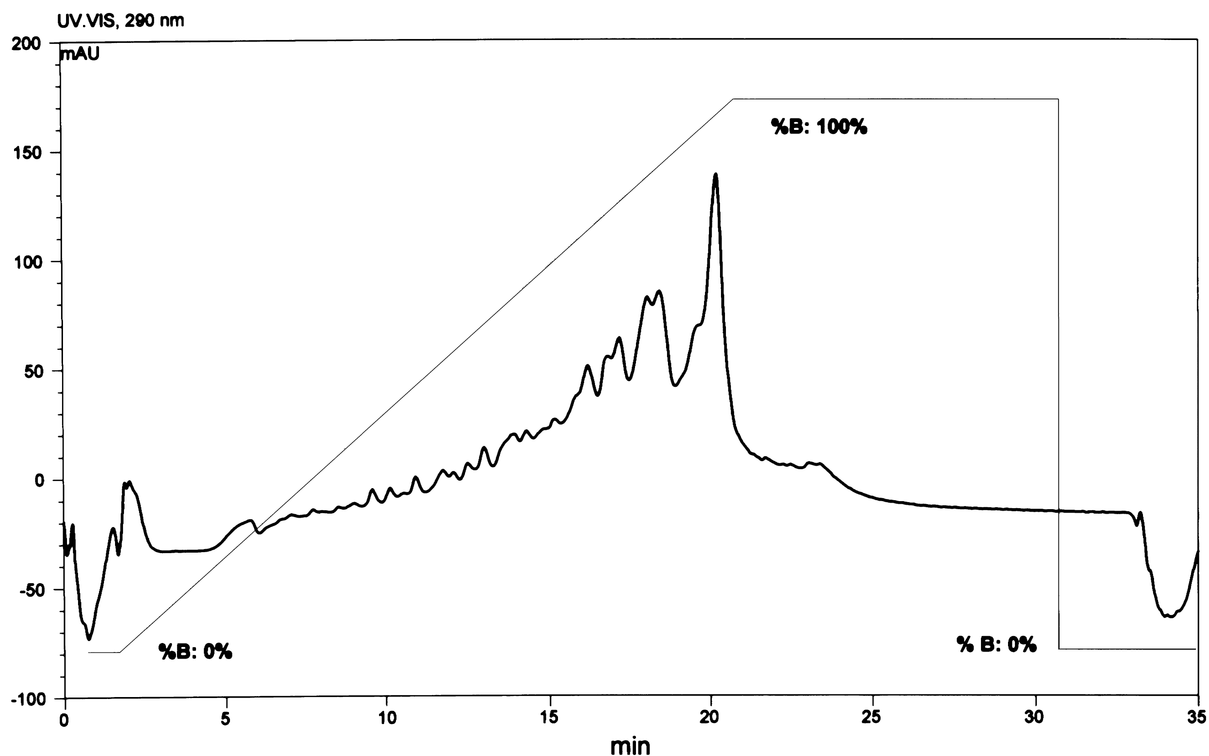

Fig. 6. Purification of a large, 91mer, synthetic oligonucleotide. Column, PL-SAX 1000 Å 8 μm 150×4.6 mm I.D.; eluent A, acetonitrile–0.1 *M* TEAA pH 7 (7:93); eluent B, acetonitrile–0.1 *M* TEAA 3.24 *M* ammonium acetate pH 7 (7:93); gradient, 0% B for 2 min followed by 0–100% B in 20 min; flow-rate, 1.5 ml/min; temperature, 60 °C.

the particle, with little change in the ionic capacity of the media, due to the strong anion-exchange functionality. The ability to operate over the extended pH range is particularly useful where an oligonucleotide is prone to self-association/aggregation as this is often reduced if not eliminated at high pH. The example in Fig. 7 shows a preparative separation of a PO oligonucleotide contaminant from a PS oligonucleotide using a 1 *M* NaOH eluent.

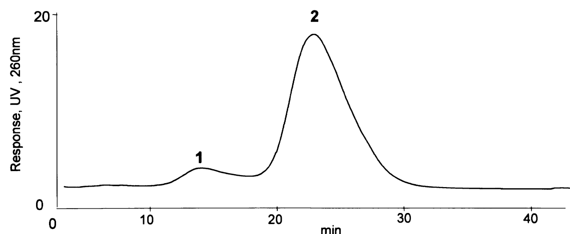

Fig. 7. Separation of a PO contaminant (1) from a PS oligonucleotide (2). Column, PL-SAX 1000 Å 10 μm 50×4.6 mm I.D.; eluent A, 1 *M* sodium hydroxide; eluent B, 1 *M* sodium hydroxide 2 *M* sodium chloride; gradient, 75–100% B in 25 min then held at 100% B for 15 min; flow-rate, 1.0 ml/min.

4. Conclusions

The relationship between pore size and resolving range has been established using a 25-bp double-stranded DNA ladder and ion-pair reversed-phase chromatography. With the small-pore 100 Å material resolution up to 50–75 bp is achieved increasing to over 500 bp with the 4000 Å pore size. Two ion-pairing agents have been compared, TEAA and TBuABr, and it has been shown that capacity is independent of temperature for the TEAA system, but that a higher capacity is obtained with the TBuABr at 60 °C. Improved resolution of a phospho thioate oligomer from the phospho diester contaminant and a phospho thioate failure sequence is

obtained with the TEAA ion-pairing agent. When using a chemically stable, wide-pore anion exchanger it is possible to achieve n from $n-1$ resolution to at least 30mer and purify a large 91mer oligonucleotide. High pH was used to improve the resolution of a phospho diester contaminant from a phospho thioate synthetic oligonucleotide.

5. Nomenclature

n	oligonucleotide with n nucleotide units
$n-1$	oligonucleotide truncated by one nucleotide unit
$n-2$	oligonucleotide truncated by two nucleotide units

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