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Review

Application of sample displacement techniques to the purification of synthetic oligonucleotides and nucleic acids: a mini-review with experimental results

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Abstract

Clinical development of antisense drugs is rapidly increasing requirements for large quantities of oligonucleotides. As part of the manufacturing process appropriately large scale separation techniques must be developed. This article reviews applications of sample self displacement techniques to the purification of oligonucleotides and nucleic acids, wherein sample components act as displacers and enhance purities. Applications for other solutes are also reviewed where similar methods may be of use in oligonucleotide purification. Experimental data is presented for sample self displacement anion-exchange chromatography of the clinically important phosphorothioate oligonucleotides. The technique can enhance full-length purity of the product and offers maximal utilization of the chromatographic media. Full-length purities of 95% and higher are achieved by this approach and recovery yields are up to 70% depending upon starting feed purity. Recycling and real time process monitoring can further improve recovery. Techniques for column saturation with multiple linked columns are presented and their applications to oligonucleotide purification are discussed. © 1998 Elsevier Science B.V.

Keywords: Reviews; Sample displacement techniques; Displacement techniques; Preparative chromatography; Oligonucleotides; Nucleic acids

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1. Introduction

The increasingly rapid development of antisense oligonucleotides as drugs and advances in large scale solid-phase synthesis of these drugs present a challenge to purification scientists to match this scale increase, provide very high purity levels, and do so in a cost effective manner. Demand for antisense drugs could reach the level of metric tons per year, if these drugs continue to be successful in systemic administration [1].

The most significant process-related impurities found in synthetic oligonucleotide drugs are the ($n-1$) deletion sequences, which differ from the full length oligonucleotide by omission of a single nucleotide. For phosphorothioate drugs 18–21 nucleotides in length, no commercial ion-exchange column is reported to separate the ($n-1$) from full-length oligonucleotide. However, since total charge on these molecules increases with length, displacement ion-exchange chromatography offers a potential way to enhance purity.

Since true displacement chromatography requires careful selection and subsequent removal of the displacer, use of sample components as displacers, is more attractive. This article will review and illustrate the application of this technique to purification of synthetic oligonucleotides, including separations under overload conditions where sample self displacement chromatography is expected to be operative. Since there are presently very few published applications of displacement chromatography purification of oligonucleotides, other applications are reviewed that might lead to enhanced oligonucleotide purification if similarly applied. Finally, efforts currently under way to deal with alternative paths for the scale up of oligonucleotide will be described.

1.1. Antisense oligonucleotides

Currently there are at least ten antisense oligonucleotide drugs undergoing phase I to phase III human clinical trials and many more in pre-clinical trials [2–4]. The first generation oligonucleotide in clinical development and uniform deoxy phosphorothioates of length between 8 and 30 nucleotides. In the case of phosphorothioates, a non-bridging oxygen atom in the diester backbone is replaced by a sulfur atom to enhance nuclease resistance and provide RNase H substrate activity in drug:mRNA duplexes.

1.2. The purification problem

The crude product of solid-phase synthesis comprises the full length phosphorothioate oligonucleotide, (n-mer), and nucleotide deletion sequences ($n-1$), ($n-2$) etc. Deletions arise at multiple positions along the chain. Hence there are multiple deletion sequences present of a given length. Other impurities present are the partial phosphodiesters [$(P{=}O)_1$'s], generated as a result of incomplete sulfurization of the non-bridging oxygen. Small amounts of $(P{=}O)_2$, $(P{=}O)_3$, etc. may also be present, though these levels depend on whether or not the oligonucleotide was synthesized in a convergent manner and the efficiency of the sulfurization reagent. In addition, impurities of length ($n+1$) may be present in amounts less than 1.5%. There is strong evidence that these arise due to double coupling, promoted by

organic acids during the solid-phase synthesis of oligonucleotides [5].

$(P{=}O)_n$ impurities can be separated from full-length phosphorothioate product by anion-exchange chromatography, as may $(n-2)$ and higher order deletion impurities. The $(n-1)$ deletion sequences are much more difficult to separate from full length all thioate product. For 20-mer phosphorothioate oligonucleotides, normal gradient elution anion-exchange chromatography does not fully separate $(n-1)$ impurities from the full-length product, but sample displacement can be used to enhance product purity. In this article we will review strategies used to take advantage of this effect, and we will present results demonstrating their use. Since there is limited data available for the application of these techniques to oligonucleotide purification, key applications using other solutes will be reviewed as well.

1.3. Terminology of sample self-displacement chromatography

To avoid ambiguities in terminology, the following definitions will apply. 'Displacement chromatography' will describe processes in which a stronger binding external component, a 'displacer', is added to elute samples in an isotachic train. This technique has been reviewed extensively [6–8]. The term 'sample displacement effect' describes the situation in which a stronger binding component within a sample displaces a weaker binding component. Sample self-displacement describes all techniques utilizing this displacement effect. Variants of this terminology have been employed by some authors, for instance, 'sample displacement chromatography' (SDC) [9–11] and 'solute-displacement chromatography, SD-HPLC' [12].

2. Theory

2.1. Displacement chromatography

Displacement chromatography can provide higher resolution, purity and, potentially a higher concentration of final product. The main disadvantage of the technique is that an external component must be added to the sample mixture to effect displacement. Identification of a suitable displacer, together with the subsequent verification of its removal from isolated final product, present major obstacles to use in drug purification applications. Displacement chromatography has been extensively reviewed for proteins and other biotechnology applications [6,7], therefore only application to purification of oligonucleotides is reviewed here.

The benefits of displacement can be realized without addition of a displacer through use of sample self-displacement chromatography. In this technique, sample components themselves are used in a displacer role. Experience gained from published work on separation of binary mixtures using a sample self-displacement effect, taken together with observations of other workers for oligonucleotides [13], provided the rationale for the experimental approach used here. This article will review the available literature on sample self displacement pertaining to DNA and oligonucleotide purification, and present experimental data from our on-going research in this field.

2.2. Sample self-displacement separations

There is considerable literature on this subject [14–27], and an excellent review of early results in the field was provided by Guiochon et al. [28]. This article will not revisit those theoretical points, except to consider some that bear strongly on the purification of oligonucleotides.

In sample self-displacement chromatography of binary mixtures, the stronger binding component (component 2) displaces the less retained component (component 1) as a narrow concentrated band with a characteristic L-shaped profile. The sharp component 1 front is followed by a high concentration front of component 2 contaminated by a dilute tail of component 1. This generally favorable displacement effect is accompanied by a related adverse effect for overloaded injections, the 'tag-along effect', which causes component 2 to elute earlier than it would if injected alone. A theoretical explanation for these two effects is given by Golshan-Shirazi and Guiochon [29]. The displacement effect is intensified with the increased ratio of loading factor for the second component to loading factor of the first. Loading factor is defined as the ratio of the amount injected to

saturation capacity for the component. The opposite conditions favor the 'tag-along effect' which is more obvious when the relative amount of component 2 to component 1 is low, and a long tail of component 2 is observed.

The work of Newburger and Guiochon gives practical insight into use of the displacement phenomenon for isocratic purification of binary mixtures [30,31]. Yield of the first component was always much higher than the yield of the second component for a given purity target. As sample load increased, yields of the two components decreased, while the production rate (amount of pure product recovered per unit time) increased. This decrease in yield was due to tailing of the first component into the zone of the second. Tailing decreases as the displacement effect increases. Tailing was dependent on the sample size, relative concentration of the two components, their isotherm coefficients, and the saturation capacities of the two components [23]. These authors also showed that the displacement effect is greater on a more efficient column, by allowing lowering of the k' and by optimizing the time for competitive interaction. This technique has also been used for separating binary mixtures [32] and routine purification of chiral mixtures [33]. Guiochon and co-workers [34,35] have also simulated multi-component separations. While the presence of more impurities complicates the picture relative to a binary separation, the effect of most experimental parameters is similar to a trend for binary separations.

Gradient elution profiles under sample overload conditions have been simulated by Snyder and co-workers [14,26], and by Antia and Horvath [36], and reviewed for reversed-phase separations by Cretier and Rocca [15]. Their results reinforce observations that solute behavior under overloaded conditions is similar for isocratic and gradient elutions. Cox and Snyder [14] provide a guideline for the selection of gradient conditions to maximize sample self-displacement effects and increase the production rate. They suggest increasing the production rate by using the highest gradient slope possible, since the slope of the gradient does not have a significant effect on the yield of components. In most separations where the saturation capacity of the later eluting component is higher, a stronger starting eluent strength would enhance the production rate. Cox [37] has experimentally applied some of these conditions to the sample self displacement separation of two insulins by reversed-phase gradient elution.

2.3. Multi-component sample self displacement with column saturation

There are literature examples of separations carried out under the conditions utilizing the full capacity of the column. These techniques make use of sample self displacement and frontal chromatography, utilizing the differential breakthroughs of different components.

Tice et al. [38] used an overloaded column method, 'overfeeding', to purify soybean trypsin inhibitor. The sample was fed continuously until it began to break through. Weakly bound material eluted during feeding and the material left on the column was purer than the starting feed. Similar results were obtained by Graber and Condoret [39] when they compared the anion-exchange separation of soybean trypsin inhibitor using non-overloaded and overloaded sample displacement conditions. While yield for the overloaded case was only 26% compared to 70% in the non-overloaded case for similar product specific activity, throughput was 2.2 times higher in the overloaded separation.

Hodges and co-workers [9–11,40–43] described a novel reversed-phase method that used the sample displacement effect produced during sample loading. Due to competition of solutes for the same sites, the major separation of hydrophilic or hydrophobic impurities from the product takes place in water, and an organic eluent is used primarily to clean the column. This strategy was used to separate a product peak from hydrophilic and hydrophobic impurities (Fig. 1), and was then extended to a two-column approach, where the feed was passed through a short pre-column trap into the main column until sample breakthrough occurred [10]. At this stage the pre-column was by-passed, and a gradient was used to elute the sample from the main column. In the multi-component approach, the sample was injected isocratically into a series of small interconnected columns [11] at a controlled flow-rate and duration. There was a distribution of impurities due to sample self displacement. Following this displacement, each segment was eluted separately. Column sizes were

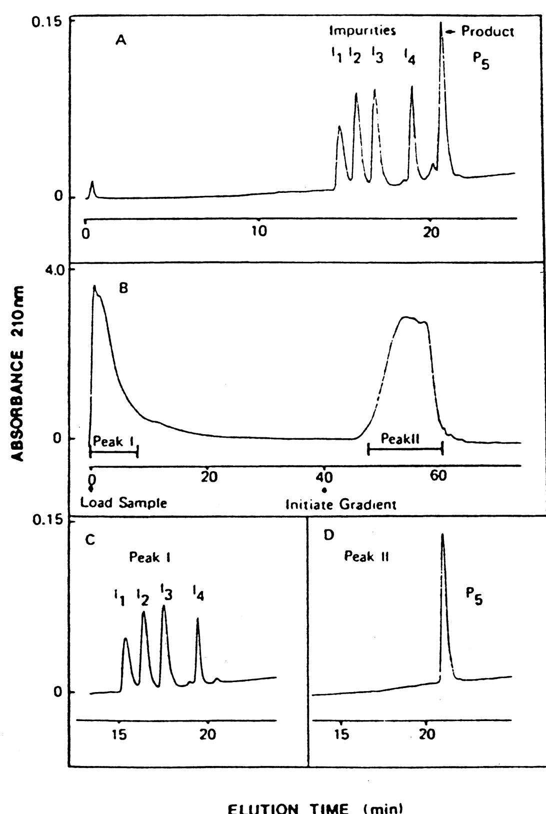

Fig. 1. Application of sample self-displacement chromatography for purification of peptides. Product (P_5) is separated from hydrophilic peptide impurities (I_1, I_2, I_3, I_4) using sample displacement chromatography on a reversed-phase column. Isocratic elution was used in the first 40 min, followed by a linear gradient. Panel A indicates the analytical separation profile of the peptide mixture, Panel B shows the preparative separation, and Panel C and D show the analysis of peaks I and II obtained in the preparative separation. (From Ref. [9], courtesy of Marcel Dekker.)

adjusted to ensure that there was just sufficient binding capacity to accommodate the target compound. Thus, regulating capacity of the column to affect purification became a way of optimizing the purification. The multi-segmented strategy was duplicated on a single column by using an isocratic hold during loading or immediately after the loading step, to provide a prolonged period of competitive binding. Using sample self displacement in combination with column saturation kinetics resulted in much higher recoveries, 95% as compared to 75% when simply overloading the column for sample self-displacement. Additionally, approximately 7-fold more material could be processed using this strategy [10].

Veeraragavan et al. [44] have duplicated the two-column strategy of Hodges et al. [10] by using two Mono-Q ion-exchange columns connected in series. Since overlap between adjacent peaks was not eliminated entirely, product recovery was low. Pessi et al. [45] used an approach similar to Hodges et al. [10] by using an extended isocratic step with a low organic to initiate separation of hydrophilic impurities from histidine containing peptides. This was followed by short duration increasing steps of organic solvent to elute the remaining adsorbed material. While 98% pure material was obtained at 70% yield, the elution profile required substantial fractionation to allow the proper product pooling. These authors point out that overlap needs to be minimized for the optimization of these purifications methods and that random selection of column sizes would not guarantee an optimized purification [44,45].

3. The application of displacement techniques to oligonucleotides and nucleic acids

3.1. Displacement chromatography

Displacement chromatography is one way to separate deletion sequence impurities from the full length product because the impurities differ in charge from product as a function of length. Gerstner [46,47] successfully applied this method to the purification of synthetic phosphorothioate oligonucleotides. Dextran sulfate was used as a displacer and full length purities as high as 96% were achieved, with typical yields in the range of 60–70%. A typical displacement chromatogram is shown in Fig. 2 for a 20-mer phosphorothioate, with a sample loading of 6.5 mg ml^{-1} column volume (C.V.) [46]. These results were very encouraging, and Gerstner also showed that a similar protocol could be applied to a wide variety of other oligonucleotides. This technique is linearly scaleable. The main limitation is that it may be difficult to ensure removal of dextran sulfate from the isolated product. This disadvantage is avoided in sample self-displacement where no additional displacer components are added. Published reports of

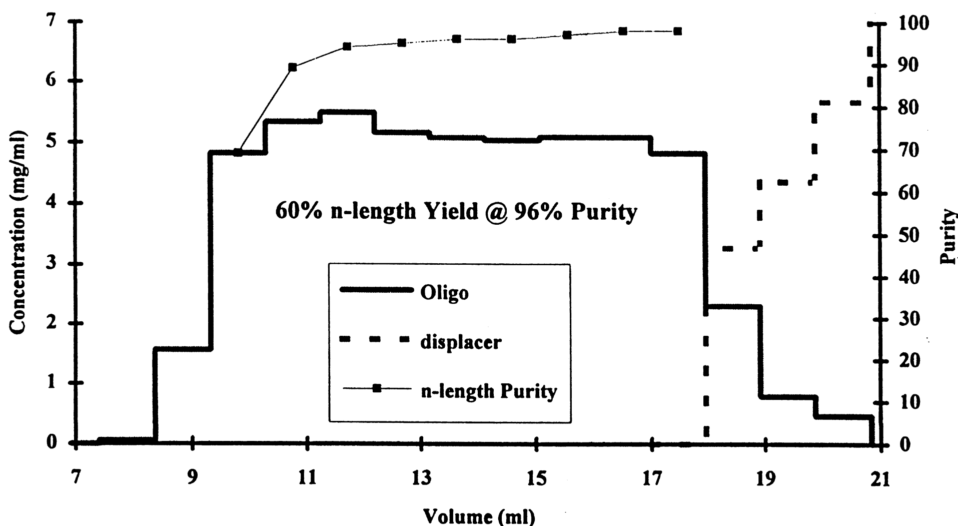

Fig. 2. Displacement chromatography of a 20-mer phosphorothioate on Poros HQ/M column with dextran sulfate as the displacer. Column loading was 6.5 mg ml^{-1} C.V., and the linear velocity was 180 cm h^{-1}. (From Ref. [46], by permission of Oxford University Press.)

such applications for oligonucleotides and nucleic acids are discussed below.

3.2. The purification of oligonucleotides and nucleic acids using sample self displacement effects

An overload phenomenon has long been known to take place in gradient chromatography when the loaded mass is high and the gradient is very shallow. Thayer et al. [13] used the term 'quasi-displacement' mode to describe this situation for a preparative purification of 25-mer oligonucleotide (Fig. 3). In this purification a 250 mm×4.0 mm I.D. analytical column was overloaded with 1 mg of 25-mer phos-

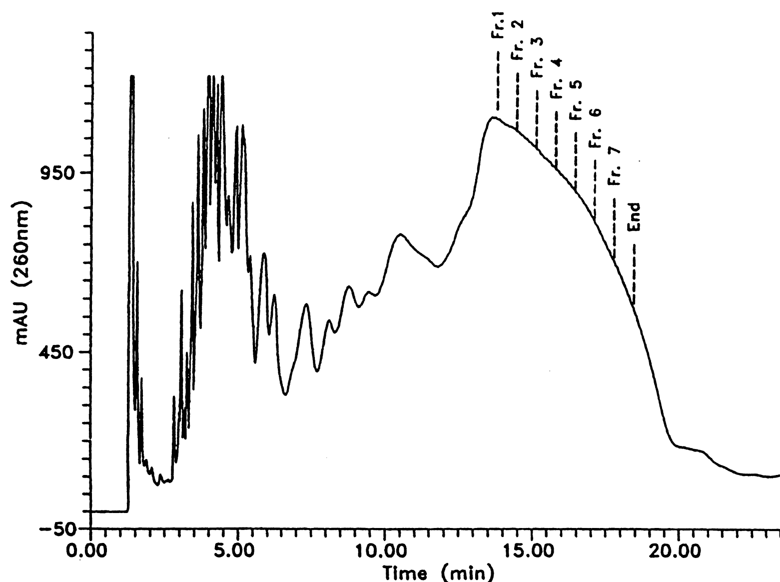

Fig. 3. Sample self-displacement of detritylated 25-mer phosphodiester oligonucleotide (Cetus PCR-01 Primer) on NucleoPac (Dionex, Sunnyvale, CA) column. The marked fractions contain mostly full-length material. (From Ref. [13], by permission of Academic Press, Inc.)

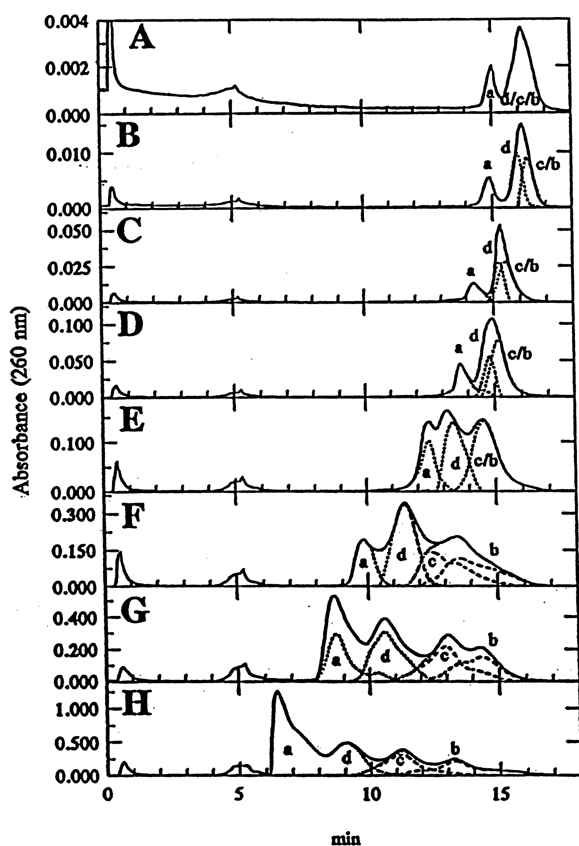

Fig. 4. Enhanced visual effects of sample self-displacement by increasing the mass of the sample from 1 μg to 750 μg. The sample comprised four plasmid DNA digests of sizes 873 bp (a), 1337 bp (b), 1388 bp (c) and 2.7 kbp (d). Dotted lines indicate the elution pattern analyzed by agarose gel electrophoresis. The two close fragments 1337 and 1388 show visual resolution at high loads. (From Ref. [12], by permission of Oxford University Press.)

phodiester. A short step-gradient was used to remove a major portion of the deletion sequences, followed by a very shallow gradient. Analysis of the fractions indicated that a large portion of the major visible peak is composed of full-length product. The recovery of full-length 25-mer was 60% with purity greater than 97%. There was a large zone of the elution profile containing the full length oligonucleotide. Similar procedures developed independently at Isis Pharmaceuticals for antisense oligonucleotides are discussed in the Experimental section.

In a remarkable demonstration of sample self-displacement effect, Waterborg and Robertson [12] showed that by varying column load, resolution of two DNA restriction fragments significantly improved over an analytical injection (Fig. 4). These restriction fragments were very close in size, 1337 and 1388 base pairs (bp). This method permits a much higher column loading than previously demonstrated. The authors point to significant overlap of the adjacent peaks as reason for caution in the use of this technology. It was noted that the loading in this case was high, but still lower than the maximum binding capacity of the column. This study suggests that chromatographic supports with lower binding avidity are better targets for this type of separation because they increase the solute–solute competitive binding. This work shows that sample load can be a powerful variable in optimizing a particular purification.

The following section presents some original experimental data for sample self displacement, and in some cases using techniques similar to those discussed in this section.

4. Experimental

4.1. Reagents

Reagent grade NaCl and HCl were obtained from Sigma (St. Louis, MO, USA), and NaOH from Mallinkrodt (Paris, KY, USA). Deionized water used in the experiments was obtained on site through the Milli-Q system (Millipore, Newton, MA, USA). Electrophoresis grade Tris-borate was obtained from Beckman (Fullerton, CA, USA).

All oligonucleotides used in this work are 20-mer phosphorothioates, manufactured in-house via automated solid-phase synthesis. The sequences used in this study are Isis 3521/CGP 64128A a drug under development for targeting PKC-α, Isis 5132/CGP 69846A is another antisense compound being developed for cancer treatment, and Isis 2302 which is an antisense inhibitor of cell adhesion molecule ICAM-1, being developed to treat inflammatory diseases. All crude materials were DMT-off, i.e. the acid labile hydrophobic group 4,4′-dimethoxytrityl (DMT) was removed. The DMT group on Isis 2302 was removed on the synthesizer itself, whereas for Isis 3521/CGP 64128A and Isis 5132/CGP 69846A the DMT group was removed after pre-purification by reversed-phase chromatography.

4.2. Chromatographic supports and columns

Two chromatographic supports were used in the preparative experiments, Q HyperD F (BioSepra, Marlborough, MA, USA) and Poros HQ/M (PerSeptive BioSystems, Framingham, MA, USA). HyperD media is a 35 μm ceramic coated silica particle, and the Poros HQ/M is a 20 μm particle with a polystyrene base matrix. Most experiments in this study were conducted on the 100 mm×4.6 mm I.D. polyether ether ketone (PEEK) column. The large scale run was carried out on a 200 ml, AP-5 (Waters, Milford, MA, USA) 100 mm×50 mm I.D. glass column.

4.3. Apparatus

Most preparative experiments were performed on a BioCad 60 workstation (PerSeptive BioSystems) connected to a model SF-2120 fraction collector (Advantec Toyo Kaisha, Japan). Some experiments were conducted on a Vision workstation (PerSeptive BioSystems) with an integrated fraction collection system, and a robotic autoinjector system, for automated analysis of the collected fractions after preparative runs. Analytical HPLC runs were carried out on a Waters chromatographic system, with 717 auto sampler, 600E system controller, 991 photo diode array detector and Millennium 2.10 operating software. Capillary gel electrophoresis (CGE) was performed on a P/ACE 5000 system (Beckman).

4.4. Preparative conditions

The buffer system used was Buffer A: 50 m*M* NaOH, pH 12.6 and Buffer B: 50 m*M* NaOH +2.5 *M* NaCl. Elution profile varied in each experiment, but the general scheme included injection under no-salt condition, followed by a step change, and a very shallow gradient. Fractions were collected manually or through an automated fraction collector. Signals were monitored at two wavelengths, 266 nm and 290 nm.

4.5. Analytical conditions

The quantity of oligonucleotide in each fraction was determined by measuring absorbance at 266 nm, converting AU to milligrams at of 20 AU mg^{-1}. For this paper, the same factor was used for all compounds. Unless stated otherwise, phosphodiester content of the fractions was measured by analytical anion-exchange chromatography, using a 100 mm× 4.6 mm I.D. Poros HQ/H (10 μm) column. The analytical separation was performed at 70°C with the same buffer system used in the preparative run. Length based purity was determined by CGE. Fractions were desalted multiple times using Centricon desalting cartridges (Amicon, Beverly, MA, USA), then analyzed on a 47 cm (40 cm to detector) Beckman eCAP ssDNA 100 gel filled capillary at a temperature of 40°C, with Tris-borate as carrier electrolytes [48].

4.6. Calculations

Using the total mass calculation based on UV spectroscopy and concentration data from anion-exchange and CGE, individual component concentrations in mg ml^{-1} were calculated and used to plot the trends. The pooling was determined on a spreadsheet and the estimated pool purity and recovery yields were calculated. The product recovery yield was calculated as the percentage of full-length oligonucleotide in the product pool versus the total amount of full-length collected after the experiment.

5. Results

5.1. Purification of phosphorothioate oligonucleotides using sample self-displacement chromatography

Sample self-displacement chromatography was used to purify a sample of Isis 3521/CGP 64128A with a low starting 89.6% full-length purity. An oligonucleotide load of 2.6 g was applied to a 200 ml column packed with Q HyperD F media. Saturation capacity of the media was estimated as 40 mg ml^{-1}, therefore, the load for this experiment was 32% of saturation load. The sample was injected over 13 min at 10 ml min^{-1}, and all other chromatographic steps were at 35 ml min^{-1}. The elution gradient was a sharp step to 45% B, followed a very shallow gradient to 48% over 25 C.V.s. The total run duration

was 120 min, but run time could be reduced to less than 60 min. Samples were collected manually. The second and third fractions were 100 ml, and the remaining fractions were 50 ml each. The re-constructed chromatogram is shown in Fig. 5, showing only the 60 min of elution. The cumulative profile is a classical overload profile. Profiles of the major impurities ($n-1$) and (P=O), show that there is a grouping of the impurities towards the front of the peak and a gradual tailing into the main peak. While sampling in the initial portion of the chromatogram was coarse, the available data shows a general trend similar to other sample displacement chromatography examples discussed in the earlier sections. The displacement effect is less dramatic in this experiment than noted for binary mixtures. However, interaction between the n-mer peak and impurities, can be visualized in a plot comparing the profiles of the n-mer and the cumulative concentration of deletion sequence components, as shown in Fig. 6. This plot shows that there is an advantage in increasing this interaction to increase purity of n-mer in the tailing region. The starting full-length purity of 89.6% could be increased to 93.5% purity at 72% yield of the full-length oligonucleotide. The fractions used for pooling are marked in Fig. 6, and the corresponding impurity concentrations are given in Table 1. The maximum purity of any fraction in this experiment was 95.5%.

A similar method can be applied to other oligonucleotides with different base composition, with only minor modifications in starting eluent strength. A reconstructed chromatogram from sample self displacement purification of Isis 5132/CGP 69846A on Q HyperD media is shown in Fig. 7. Operating conditions were similar to those described above. While only anion-exchange analysis was performed on the fractions, the general trend is similar to that

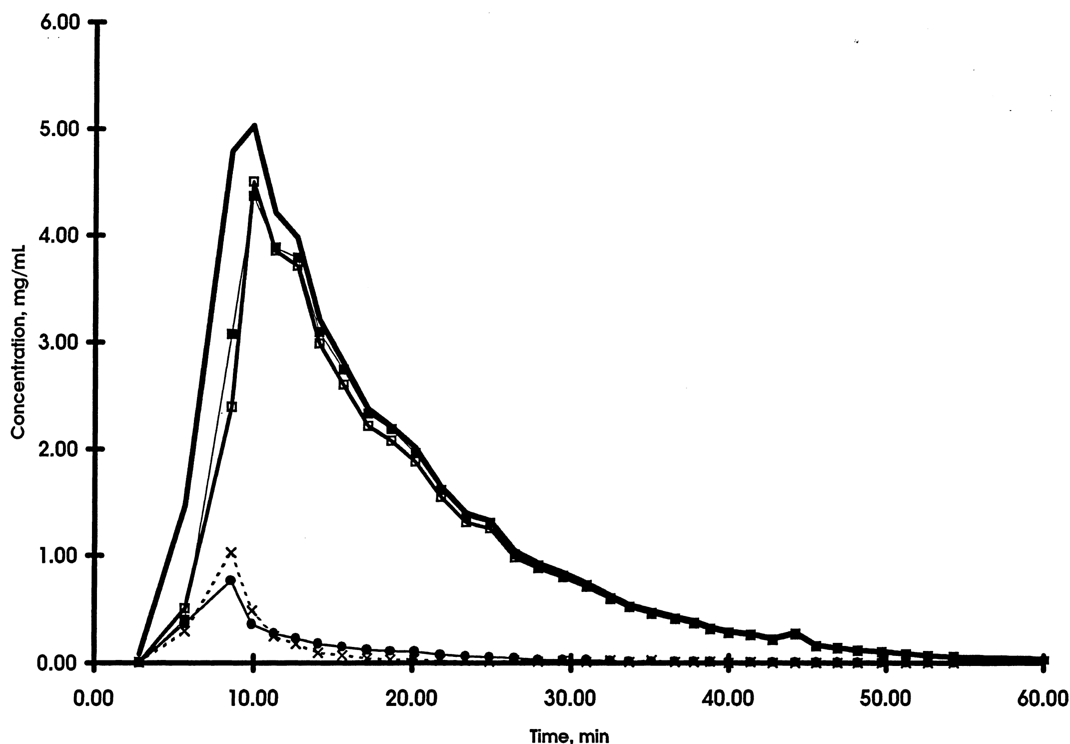

Fig. 5. Reconstructed chromatogram for the purification of Isis 3521 (CGP 64128 A) on Q HyperD F 35 μm anion-exchange column. The oligonucleotide profile is indicated by ———, n-mer –□–, ($n-1$) –●–, (P=O) –×–, and (P=S) by –■–. Buffer A: 50 mM NaOH, Buffer B: 50 mM NaOH +2.5 M NaCl, pH 12.6. Elution: Step 0% B to 45%B, 45%B to 48% B in 25 C.V.. flow-rate: 35 ml min^{-1}, loading at 10 ml min^{-1}. Column: 200 ml (100 mm×50 mm I.D.). Equipment: BioCad 60 (PerSeptive Biosystems, Framingham, MA, USA).

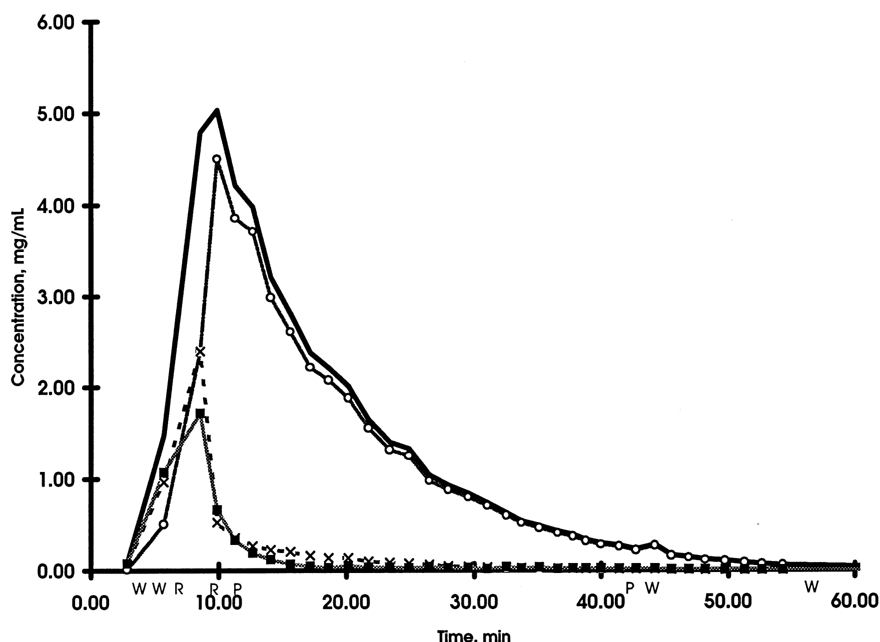

Fig. 6. Cumulative impurity distribution in purification of Isis 3521 (CGP 64128A). Concentration profiles of n-mer (– ○ –), and cumulative concentrations of all other CGE impurities (– × –) and AX impurities (– ■ –) are shown. The chromatogram trace is shown by the total oligonucleotide concentration (———). The cumulative impurity concentration is obtained by subtracting the n-mer concentration from the total oligonucleotide concentration. Suggested fraction pooling is indicated below the X-axis. Fractions marked between P are pooled for product, similarly fractions flanked by W are waste fractions and fractions indicated between R could be recycled. Experimental details are given in Fig. 5.

seen in Fig. 5. Pooling fractions at the end of the (P=O) impurity tail resulted in a purified pool of 99.4% pure (P=S), with 0.6% (P=O), at 72% yield of phosphorothioate; starting purity of the feed to the column was 86.4% (P=S) with 8.15% (P=O).

This method was used to purify a batch of crude Isis 5132/CGP 69846A. Starting DMT-on crude was pre-purified on a reversed-phase column, detritylated and then purified using the sample self-displacement method. In each run, three pools were collected

Table 1
Starting and final compositions for sample self-displacement of Isis 3521/CGP 64128 A in area percent purity (Figs. 5 and 6).

	CGE			AX	
	$(n-1)$	n-mer	$n+$x	(P=O)	(P=S)
Starting	5.6	89.6	1.2	7.3	89.0
Final (yield=72%)	5.0	93.5	0.8	2.8	96.3
(yield=63%)	4.8	93.7	0.9	2.3	96.9

The final pooling composition is calculated by considering different combinations of fractions in the product pool.

waste, recycle and product, based on the analysis of fractions. The recycle pool was diluted with water to reduce conductivity for loading and reprocessed using the same preparative method. Recycle pools from multiple runs were combined for reprocessing. Six anion-exchange runs, inclusive of reprocessing recycle runs, were used to process 10.2 g pre-purified crude, giving a cumulative yield of 70%. The purity of the feed was 86.4% (P=S) with 8.15% (P=O). The final product purity achieved was 97.3% full-length purity by CGE, with 2.65% $(n-1)$, and 2.8% (P=O) impurities.

This method was easily extensible to other oligonucleotides of various chain lengths and base sequences, with very minor changes in the gradient conditions. The method appears broadly useful for purification of this class of molecules. Recovery was increased by selective recycling of the mixed zones. While extensive fractionation is shown here, the number of fractions can be greatly reduced after optimizing operating factors. We are exploring on-

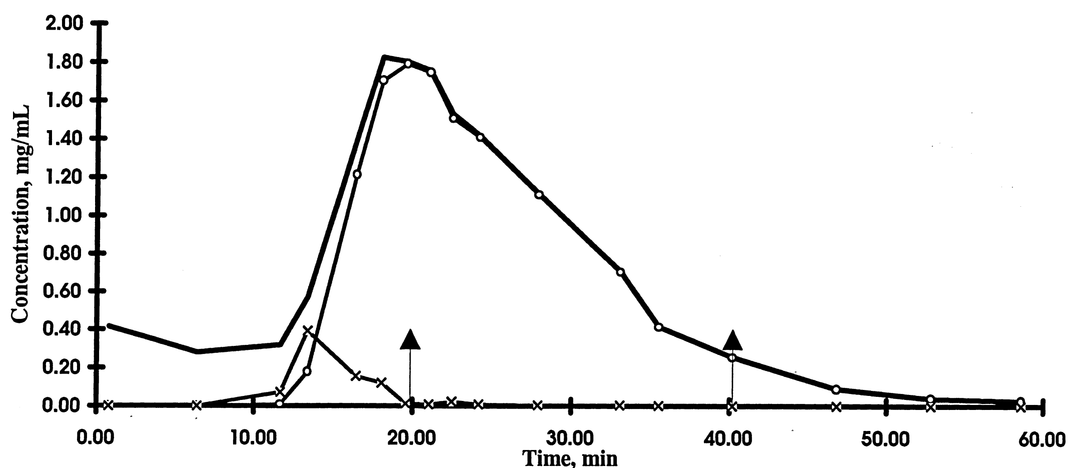

Fig. 7. Reconstructed chromatogram for the purification of Isis 5132 (CGP 69846 A) on Q HyperD F 35 μm anion-exchange column. Only the anion-exchange analysis is shown. The oligonucleotide profile is indicated by ———, (P=S) by – ○ –, and (P=O) by – × –. The product cut is shown by arrows. The 200 ml column was used (100 mm×50 mm I.D.), and 1.5 g of sample was loaded on it. The flow-rate was 40 ml min^{-1}. Buffers and equipment same as in Fig. 5.

line analysis as a way to make automatic fractionation decisions. Automated recycling will also adapt this process to large scale production. The loading afforded by this method and the resulting purity of oligonucleotides are very attractive.

5.2. Flow-through column saturation experiments

A tandem column experiment was performed to demonstrate sample self-displacement of impurities from one coupled column onto the other during loading. This approach is similar to that suggested by Hodges [10,11] and used by Veeraragavan et al. [44]. Column I was a small 50 mm×4.6 mm I.D. Poros HQ/H (10 pm) column with 0.83 ml C.V., and column II was a larger 100 mm×4.6 mm I.D. Poros HQ/M column. The columns were connected to separate multi-port valves on the Vision workstation. Using the automated control afforded by this workstation, the solvent flow path could be changed to go through column I to II in series or individually through each column under automatic control. A sample of 12.5 mg ml^{-1} Isis 2302 DMT-off crude product was injected into the two columns in series via sample loop at 500 μl, and washed with five times the cumulative column volumes of the two columns. The columns were then eluted separately. The complete chromatogram is shown in Fig. 8. The fraction numbers start with eluents from the precolumn. The analysis on an anion-exchange column having the same packing as the column I of the preparative run, shows that most of the impurities were displaced to column II and the main product were retained on column I. The estimated capacity of the columns is 15 mg ml^{-1} C.V. for the HQ/M column and 5 mg ml^{-1} for the HQ/H column. Fractions were analyzed as indicated earlier, and an estimated value for the two broad pools, the eluate from column I, and the eluate from column II are tabulated in Table 2. Fractionating the eluate from column I is expected to produce much higher purity product at the expense of some yield. These conditions were not optimal, but analysis clearly shows that there is a distinct advantage to using sample self-displacement in purifying oligonucleotides. The main limitation of such flow-through techniques is that tolerance on feed purity is tight, and maintaining a robust method under varying feed concentrations is thus difficult. At smaller scales selecting appropriate column sizes and number of segments is complicated but feasible.

6. Discussion

This work, which began as research to maximize

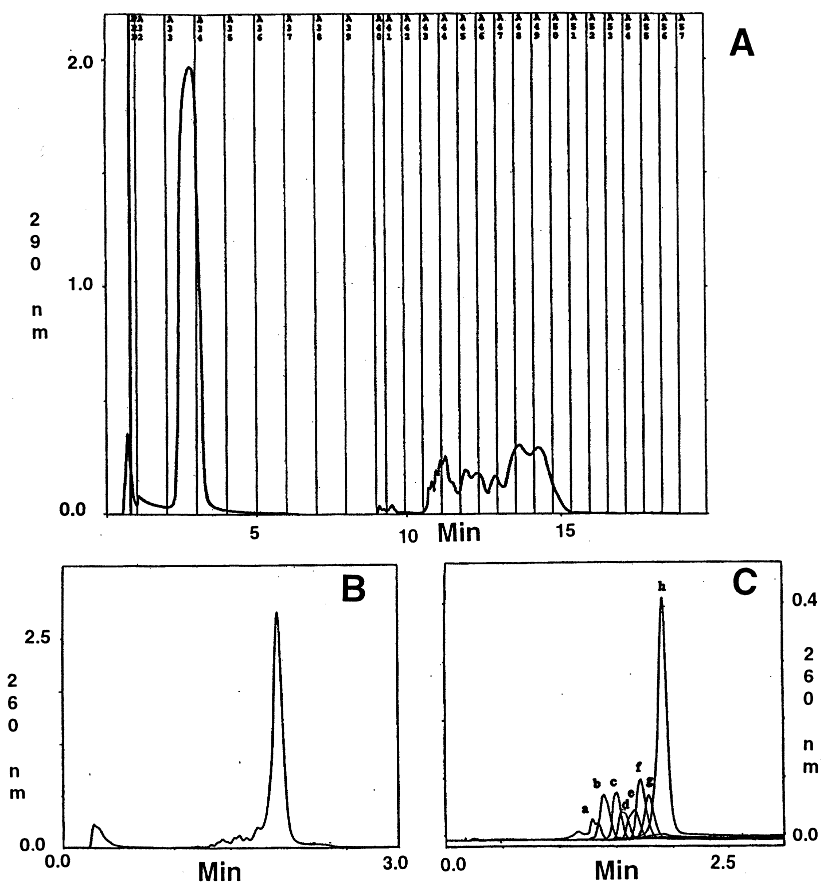

Fig. 8. Two-column purification of Isis 2302 DMT-off crude. Panel A shows the preparative chromatogram. Fractions marked A33–A37 are 5 ml fractions that elute off column I (Poros HQ/H 50 mm×4.6 mm I.D.), and fractions A40–A56 are 3 ml fractions that elute off column II (Poros HQ/M 100 mm×4.6 mm I.D.). The overflow and fraction A 32 were not analyzed. Panel B shows the analytical chromatogram for the crude. Panel C shows the superimposed analysis of fractions A43–A49 (a–g), and the main peak from column I, fraction A33 (h). Fraction 34 and 50 are not shown as they overlap significantly with fraction A34.

Table 2
Mass balances and purities in a tandem column purification of Isis 2302

	Column 1 eluate (Product pool)	Column 2 eluate (Waste pool)
Oligonucleotide amount, mg	4.21	1.77
(% of total)	(70.2%)	(29.5%)
% recovery yield of (n-mer)	98.2%	0.9%
Purity of n-mer in the fraction	81.9%	1.7%
Starting purity of n-mer	73.7%	
Enhancement in purity (purity of column 1 eluate-feed purity)	8.2%	

Experimental details are given in the text and Fig. 8

solute–solute interactions, has also shown promise for purification of phosphorothioates at multi-gram scale using sample self-displacement. The technique enhances purification of oligonucleotides, is reproducible and scaleable. While additional efforts will be needed to develop oligonucleotide sample self-displacement to full utility, the data presented here show the utility of the method in this application.

The purification of Isis 3521/CGP64128A by sample self-displacement shows that, while overall purity of the full-length oligonucleotide is enhanced by sample self displacement at acceptable yield, reduction in $(n-1)$ impurity is not significant, and must be explored further. Purification of crude Isis 5132/CGP69846A on the other hand, resulted in significantly lower $(n-1)$ content as more conservative pooling was made to allow mixed zone recycling. Since base sequence contributes to some degree to resolution of oligonucleotides on analytical anion-exchange chromatography, it is anticipated that the performance for two different sequences would also be slightly different under overloaded conditions. Thus, 95% and higher purities are easily attainable using this method. This performance is comparable to that obtained through displacement chromatography, but without the use of additive displacers. The use of high pH buffers in our experiments minimized any interferences due to possible secondary structure or self-complementary behavior. The higher loading afforded by this method enables processing of larger batches using smaller hardware.

Empirical and simulation studies for binary experiments discussed in the Theory section can be used as an effective starting point for determining suitable experimental parameters. The best approach is to optimize key experimental variables of load per run, flow-rate, and starting elution strength for maximizing the production rate [49]. Similar to Thayer et al. [13] a short step followed by a shallow linear gradient worked best for our conditions, as more sample could be loaded on the column. All our experiments were conducted under gradient elution conditions, and as theoretical work has predicted [14,15,26,36], there is no significant difference relative to the isocratic operation. Experiments to vary the gradient slope did not produce significant variation in product purity or yield, again as shown by prior published work [26]. In our experiments, the gradient slope was kept approximately the same for all oligonucleotide sequences, a suitable flow-rate was chosen, and only the starting eluent strength and sample load were varied for each product. The starting eluent strength, or $\%B$, was the main parameter optimized for each sequence, estimated by finding the $\%B$ required to elute the main peak on an analytical column with similar loading. Although a lower capacity media enhances competition for smaller binding sites, we used a higher capacity media at a lower loading factor. Amount loaded was optimized for each separation, however in most separations loading was about 30% of the maximum saturation capacity. The separations can be scaled-up by maintaining the same linear velocity and loading factor at higher scale.

The tailing observed in such methods reduces throughput, and is a significant drawback in contrast to the sharp, square profiles in displacement chromatography. A novel method developed by Newburger and co-workers [50] may offer another solution. This technique combines the beneficial aspects of sample self-displacement and displacement chromatography. In contrast to the continuous displacer injection in displacement chromatography, a finite amount of the displacing material is injected in this method, followed by the elution buffer. The displacing compound increases displacement effects between all components and reduces the tailing of strongest binding sample component. This method minimizes displacer usage and reduces the column regeneration time.

One way to increase isolated yield of a slower eluting component in overload experiments is to recycle the mixed zone between the two components [38,39,44]. Charton et al. [51] studied the effect of different recycling modes in enhancing the yield of a binary separation. Yield of the second component increases when the mixed zone is recycled. This is the method we used above, since a simple dilution was required prior to re-chromatography. However, injection time increases for the subsequent reprocessing runs and may reduce throughput. Automation strategies will be explored in the future to simplify this procedure.

There are several disadvantages to the sample

displacement approach. While the technique is easily carried out once developed, the development itself can be challenging. Parameters such as column length, concentration of the feed, sample size, and elution parameters are important and need to be optimized if the optimal interaction is to be obtained. Preparative profiles do not give a visual indication of separation and direct correlation with analytical data is not easy. The yield of product per cycle is relatively low; while this is easily remedied by recycling mixed zones, this reduces throughput. The main advantages are that the strategy provides higher purity product. Recycling gives acceptable cumulative yields in the range of 70 to 85%, depending on the number of cycles. Thus, the same performance as displacement chromatography is obtained, with a slightly higher loading, and no product contamination by the displacer. The initial work on this method began on compounds with strong tendency to form secondary structures. As the work progressed, it was discovered that the high pH buffers necessary to eliminate the formation of secondary structures, also provided sufficient robustness to purify six other nucleotide sequences currently under study with very minor modifications.

Although large-scale production is the main use for this technique, it is well suited for producing small quantities of oligonucleotides (up to 10 g) scale directly from DMT-off crude. It is relatively simple to recycle the mixed pool at this scale and the development of an acceptable purification protocol is also faster. The strategy employed would be as follows. The first series of runs can be used to increase the purity of the DMT-off crude from $<$70% to $>$85% purity. The fractions can be split into three pools; waste, recycle and product pools. The product pools are diluted and reprocessed, using a gradient that enhances the sample self-displacement effect. The number of recycles depends on the acceptable product purity and yield required. Since the amount processed decreases after each round of purification, the total number of runs required is quite manageable. In addition to saving the detritylation step, this general method assures a high purity for different sequences.

Methods that use saturation kinetics coupled with sample self-displacement chromatography can add one more tool for optimizing purifications. The approaches described by Hodges and co-workers [9–11,40] offer the best applications to date of such techniques. In oligonucleotide purifications, such methods can be used in a two step purification, where the crude is first pre-purified from 75% purity to a high 85% purity, and then again subjected to sample self-displacement with finer fractionation. The two-column experiment described in the previous section shows feasibility of enhancing crude purity with negligible product loss. Much higher product purity can be obtained by sample self-displacement at the expense of some product loss and additional fractionation. The use of saturation kinetics along with sample self-displacement has the advantages of minimal fractionation, reduced analysis, high capacity and higher resolution, resulting in higher yields. A major disadvantage is low robustness. The multiple column approach allows some flexibility in the system, as some pure material is always obtained due to the segmented approach. However, at larger scales it is difficult to manipulate more than two columns. Given the low separation factors for closely related oligonucleotides impurities, overlapping is difficult to eliminated entirely. Overlap needs to be minimized and columns sized according to impurity levels to optimize sample displacement purification methods.

In this article we have assumed that the isotherm profiles of oligonucleotides favor displacement effects and that the saturation capacity of the full-length product is higher than its deletions sequences. We have not made any attempt to quantify the pure component saturation capacities or other isotherm parameters. Also, demonstration of sample self-displacement as presented elsewhere [30] for binary mixtures has not been carried out for pure components of these solutes.

7. Conclusions

Sample self-displacement techniques can increase the purity of oligonucleotide product obtained by anion-exchange chromatography. Purity and production rate can be increased by reducing the overlap between peaks. Results presented here show that a sample self-displacement approach is effective for a number of oligonucleotide sequences. The high

loading in these separations makes efficient use of hardware. The recovery yield using such techniques may be low in some cases, however, recycling and on-column analysis of product purity and two avenues that can alleviate these problems. Superimposing frontal chromatography on overload chromatography yielded better results in the literature [10]. Our preliminary data using such strategies shows that while the purity obtained could be increased, the optimization of these strategies can be challenging. Band overlapping can reduce the yield, and predicting in-situ transient column saturation can be difficult. Displacement chromatography with dextran sulfate as displacer works well with oligonucleotide purification [46]. However, our results indicate that similar results are possible if the solute–solute interactions are enhanced in the sample self-displacement chromatography, with the added benefit that no displacer is added. Thus, sample self-displacement effects can be extremely favorable for oligonucleotide and nucleic acid separations and proper optimization of operating parameters can yield a process with increased throughput and purity.

8. Abbreviations

AX:	Anion-exchange
CGE, CE:	Capillary (gel) electrophoresis
C.V.:	column volumes (ml)
n-mer:	full length oligonucleotide
$n+$x:	impurities observed on CGE that migrate slower than n-mer
$(n-1)$:	single nucleotide deletion
(P=O):	monophosphodiester impurity, where all except one backbone linkages are phosphorothioates
(P=S):	fully thiated compound, where all non-bridging oxygen atoms have been replaced by S
SDC:	sample displacement chromatography.

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