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Purification of an oligonucleotide at high column loading by high affinity, low-molecular-mass displacers

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Abstract

The development of efficient techniques for large-scale oligonucleotide purification is of great interest due to the increased demand for antisense oligonucleotides as therapeutics as well as their use for target validation and gene functionalization. This paper describes the use of anion-exchange displacement chromatography for the purification of 20-mer phosphorothioate oligonucleotide from its closely related impurities using low-molecular-mass amaranth as the displacer. Experiments were carried out to examine the effect of the feed load on the performance of the displacement chromatography. In contrast to prior work, displacement chromatography was successfully scaled-up to high column loadings while maintaining high purity and yields. Experiments carried out on a Source 15Q column indicated that crude oligonucleotide loading as high as 39.2 mg/ml of column were readily processed, resulting in product recovery of 86% and purity of 92%. These results demonstrate that anion-exchange displacement chromatography can indeed be employed for large-scale oligonucleotide separations at high column loading.

Keywords: Displacement chromatography; Oligonucleotides; Amaranth

1. Introduction

Significant advances in antisense therapeutics over the last decade have generated substantial interest in the use of antisense oligonucleotides as drug candidates. There are currently over a dozen antisense oligonucleotide drug candidates in clinical trials and Vitravene has achieved Food and Drug Administration (FDA) approval [1]. The completion of the Human Genome Project has also brought attention to the use of antisense oligonucleotides for gene functionalization and target validation. The rapid development of oligonucleotide therapeutics and the projection that demands for oligonucleotides could reach the level of metric tons per year, poses a significant challenge to separations scientists to develop cost effective processes that can deliver drugs with high purity [2–4].

The most significant process-related impurities found in synthetic oligonucleotide drugs are the ($n-$

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1) deletion sequences, which differ from the full-length oligonucleotide by omission of a single nucleotide. Depending on which base is deleted in the $(n-1)$ failure sequence, that species could exhibit very similar retention to the full-length product (n). Other impurities that may exist include $(n-2)$, $(n-3)$ etc., $(n-x)$ failure sequences and some $(n+x)$ longmers. The impurities of length $(n+1)$ have been shown to be present at levels of less than 1.5%. It has been suggested that these impurities arise due to double coupling, promoted by organic acids during the solid-phase synthesis [3]. Partial phosphodiesters $(P{=}O)_x$ are other process-related components generated in phosphorothioate oligonucleotides as a result of the sulfur exchange. These are mainly $(P{=}O)_1$ and some $(P{=}O)_2$ and $(P{=}O)_3$ may be present. The presence of 2^{n-1} (where n = number of bases) centers of stereoisomerism about the phosphorus–sulfur bond causes considerable conformational heterogeneity to the oligonucleotides. Thus, the purification of oligonucleotides requires a chromatographic process capable of very high resolutions at preparative scale. In addition, phosphorothioate oligonucleotides exhibit an extremely high binding affinity for anion-exchange resins as compared to molecules typically encountered in biopharmaceutical processing. All these factors combined make the process scale purification of oligonucleotides extremely challenging [4–7].

Ion-pair reversed-phase HPLC (IP-RPLC) has been extensively used for the purification of oligonucleotides [8–12] and for oligonucleotides containing strong intra- or inter-molecular interactions (up to 12 bases) [13]. This method has been mostly applied for oligonucleotides with the hydrophobic 5′-*O*-dimethoxytrityl (DMT) protecting group (DMT-on) and requires a subsequent chemical step to remove the protecting group from the purified product. It has been shown that DMT-on oligonucleotides, up to 143 nucleotides in length, can be separated with high resolution using the IP-RPLC technique [8]. Germann et al. [14] have shown the use of polystyrene–divinylbenzene stationary phases to purify DMT-on oligonucleotides up to 50 bases. In addition, it has also been shown that IP-RPLC was applicable for the purification of detritylated oligonucleotides (DMT-off) where a volatile buffer system was employed. Huber et al. [12] has employed IP-RPLC on highly cross-linked polystyrene–divinylbenzene particles and Hill et al. [9] has shown the use of silica based materials for the separation of DMT-off oligonucleotides (25- to 100-mer). Reversed-phase chromatography using polytstyrene–divinylbenzene resins have also been shown to be effective for the separation of heptenated oligonucleotide from the non-heptenated form [15].

Another technique that has recently been applied for the purification of crude DMT-on oligonucleotides is solid-phase extraction using a novel hydrophilic–lipophilic balanced sorbent in the presence of an ion-pairing agent containing buffer [16]. Membrane chromatography has also been employed for large-scale purification of DMT-off oligonucleotides producing high purity and high yields [17]. Mixed-mode chromatography with reversed-phase resins coated with tetraalkyammonium salts has also been shown to be effective for the resolution of oligonucleotide and tRNA [18].

Anion-exchange chromatography is another technique that is frequently employed for separating detritylated (DMT-off) oligonucleotides and eliminates the need for a chemical step after purification. In addition, the availability of base stable stationary phase materials now enables high-resolution oligonucleotide separations. Separation of oligonucleotides on non-porous anion-exchange resins was shown to be very effective for the rapid separation of oligonucleotides with high resolutions and recovery [19]. Anion-exchange chromatography has been successfully employed for the purification of 30 to 50 base oligonucleotides using polyethyleneimine (PEI) functionalized silica materials [20,21]. In addition, anion-exchange resins with zirconium treated silica particles has also shown to result in high recoveries and purities of oligonucleotides up to 44 bases [22].

Among different modes of chromatography, displacement offers several advantages for preparative scale processes. These advantages are: high purity and high product concentration which can be obtained in a single step process, successful purification of bioproducts from their closely related impurities, and very high process yields at high column loadings. Low-molecular-mass displacers ($M_r < 2000$) have several operational advantages [23] as compared to large polyelectrolyte displacers and have

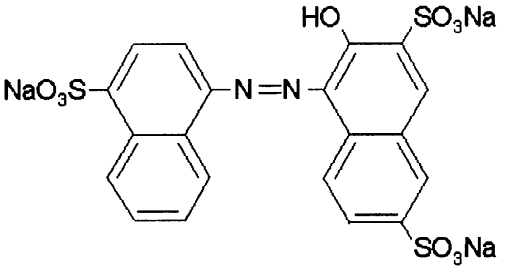

Fig. 2. Chemical structure of amaranth.

generated significant interest from the biotechnology industry. A variety of low-molecular-mass displacers have been employed to purify both model protein mixtures and more complicated industrial feeds [24–27].

The displacement mode of chromatography on anion-exchange resins has been successfully employed for the semi-preparative scale purification of phosphorothioate oligonucleotides using high molecular mass dextran sulfate as the displacer [7]. In addition, large-scale purification of oligonucleotides using sample self-displacement mode has been used resulting in 70–85% yields after a few cycles [3].

Recently, it has been shown that low-molecular-mass displacers can be employed for purification of oligonucleotides [28]. Despite the high purities and yields achieved, the amount of oligonucleotides that could be purified on the Poros HQ/M resins was low. Herein, we demonstrate that the displacement mode of chromatography can successfully be employed at very high column loadings on Source Q resins using amaranth (Fig. 2) as the displacer. These results indicate that the displacement chromatography with low-molecular-mass displacers is a promising technology for providing large amounts of oligonucleotides at high purity and yield.

2. Theory

The theoretical framework employed in this work is the steric mass action (SMA) isotherm of protein ion-exchange. Using this framework, one can evaluate the efficacy of a displacer by determining its dynamic affinity [29]:

$$\lambda = \left(\frac{K}{\Delta}\right)^{\frac{1}{\nu}} \quad (1)$$

where ν is the characteristic charge (average number of sites that a molecule interacts with on the chromatographic surface) and K is the equilibrium constant of the exchange reaction between the solute and the salt counter-ions on the surface. The $\Delta = Q_d/C_d$, where Q_d and C_d are the displacer concentrations in the stationary phase and mobile phase, respectively. The dynamic affinity is a measure of the ability of a solute to displace another solute at a specific displacement condition (given by the value of Δ).

Taking the logarithm of both sides of Eq. (1) and rearranging the following relation can be written [30]:

$$\log K = \log \Delta + \nu \log \lambda \quad (2)$$

Thus, on a plot of $\log K$ vs. ν (dynamic affinity plot), Eq. (2) defines two regions demarcated by a line of slope $\log \lambda$ and intercept $\log \Delta$. The dynamic affinity line of a solute originates at the point Δ on the ordinate axis and passes through the point defined by the linear equilibrium properties K and ν of the solute. The region above the affinity line includes all solutes which will displace the solute of interest when traveling at a velocity characterized by Δ. Conversely, solutes in the region below the affinity line will be displaced by the solute of interest under these conditions.

While the dynamic affinity plot can graphically represent the order of affinities of various solutes, the order of their relative affinities is restricted to the specific operating conditions of the experiment (reflected in the value of Δ). To enable a comparison of displacer efficacies over a range of operating conditions, Eq. (2) can be rearranged to yield an equation for the displacer ranking plot:

$$\log \lambda = \frac{1}{\nu} \log K - \frac{1}{\nu} \log \Delta$$

A displacer ranking plot provides a useful means of comparing the dynamic affinities of various displacer molecules over a range of operating conditions. It has been employed [31] to rank homologous series of displacers in cation-exchange systems. In this paper, the displacer ranking plot is employed to

study the relative affinities of a variety of high affinity and low molecular mass anionic displacers.

3. Experimental

3.1. Materials

Strong anion-exchange columns (quaternary ammonium), Source 15Q (15 μm), 100×4.6 mm I.D. was donated by Amersham Pharmacia Biotech (Uppsala, Sweden) and Poros HQ/H (10 μm), 100×46 mm I.D. was obtained from Perseptive Biosystems (Framingham, MA, USA). The 50-cm J&W μPAGE-10 gel filled capillaries and μPAGE (Tris–borate and urea) buffer to use for capillary gel electrophoresis (CGE) were purchased from J&W Scientific (Folsom, CA, USA). The VWSP, 0.025-μm membranes used for desalting were purchased from Millipore (Bedford, MA, USA). Sodium hydroxide and sodium chloride were purchased from Fischer Scientific (Pittsburgh, PA, USA), and amaranth was purchased from Aldrich (Milwaukee, WI, USA). The crude phosphorothioate oligonucleotide ISIS 2302 (sequence: 5′-GCC CAA GTC GGC ATC CGT CA-3′) was synthesized at 80 m*M* scale on OligoProcess (APB, Piscataway, NJ, USA) at Isis Pharmaceuticals (Carlsbad, CA, USA).

3.2. Apparatus

All displacement experiments were carried out using a Waters 590 HPLC pump (Waters, Milford, MA, USA) connected to a chromatography column via a model C10W 10-port valve (Valco, Houston, TX, USA). Linear gradient experiments for parameter estimation were carried out using a fast liquid chromatographic (FPLC) system, consisting of two Pump-500 pumps and LCC-500 controller donated by Amersham Pharmacia (Uppsala, Sweden). The column effluent during both displacement experiments and parameter estimation was monitored using a Waters 484 UV–Vis absorbance detector. Fractions of the column effluent were collected using a LKB 2212 Helirac fraction collector (LKB Bromma, Sweden). Oligonucleotide and displacer analysis of the collected fractions was carried out using a Waters 600 multisolvent delivery system, a Waters 712 WISP autoinjector and a Waters 484 UV–Vis absorbance detector controlled by a Millenium chromatography manager (Waters). Capillary gel electrophoresis experiments were carried out using a WatersQuanta4000 system (Waters) at New York State Health Department, Wadsworth Labs., Albany, NY, USA.

3.3. Procedures

3.3.1. Determination of steric mass action (SMA) parameters for oligonucleotide and amaranth

The linear SMA parameters for oligonucleotide and amaranth were obtained using linear gradient experiments [31]. Linear gradient experiments were carried out with different slopes between 20 m*M* NaOH and 20 m*M* NaOH+2.5 *M* NaCl solutions. The retention times were measured from the UV absorbance profiles at 254 nm for both oligonucleotides and amaranth.

3.3.2. Displacement chromatography

A feed mixture of the crude ISIS 2302 phosphorothioate oligonucleotide was purified by displacement chromatography using amaranth as the displacer in the Source 15Q column at room temperature. The column was initially equilibrated with the carrier solution (20 m*M* NaOH+500 m*M* NaCl) and then sequentially perfused with feed, displacer and regenerant solutions. Several experiments were carried out with different amounts of oligonucleotides (49–69 mg). Amaranth (5 m*M*) was employed as the displacer. Appropriate fractions (200 μl) of column effluent were collected during the displacement experiments for subsequent analysis of oligonucleotide and amaranth as described below. The column was regenerated by sequentially perfusing with 20 column volumes each of 1 *M* NaOH+25% (v/v) Acetonitrile, and 20 m*M* NaOH+2.5 *M* NaCl solution.

3.3.3. Oligonucleotide and amaranth analysis by HPLC

Fractions from the displacement experiment were evaluated by high temperature (70°C) anion-exchange chromatography using a Poros HQ/H (100×46 mm I.D.) column for the analysis of oligonucleotides and amaranth. HPLC analysis provides high

resolution and enables the determination of the *n*-mer oligonucleotide, $(P{=}O)_x$ and the displacer concentrations in the fractions. For fractions containing only the oligonucleotides, a linear gradient was run from 20 m*M* NaOH to 20 m*M* NaOH+2.5 *M* NaCl in 30 min. For fractions containing the amaranth displacer, a 60-min linear gradient was employed to enable base line resolution of amaranth from any oligonucleotides in the fractions. The column effluent was monitored at 254 nm and 5-μl samples were injected at a flow-rate of 1 ml/min.

3.3.4. CGE analysis of oligonucleotide

CGE analysis [32] was carried out to determine the length-based purity of the oligonucleotide contained in the fractions collected during the displacement experiments. Prior to CGE analysis, samples were diluted and desalted using Millipore membranes. Capillaries were cut into 47 cm lengths and the effective length before the detector was 40 cm. After electrokinetic injection of samples for 5 s at 7.5 kV, separation was carried out at 14.1 kV using tris–borate and urea buffer as the electrolyte at room temperature.

4. Results and discussion

As described in Section 2, displacer ranking plots [31] enable a comparison of the efficacy of different displacers for a given purification problem over a range of operating conditions. The linear SMA parameters of amaranth and the 20-mer (ISIS 2302) oligonucleotide were obtained using linear gradient experiments on the Source 15Q stationary phase material. The parameters were, for oligonucleotide: $\nu=11.58$, $K=5.71\cdot10^5$ and for amaranth: $\nu=1.58$, $K=1.79\cdot10^3$. These parameters were then used to generate the displacer ranking plot shown in Fig. 1.

As seen in Fig. 1, according to the theory, these results indicate that amaranth possesses sufficient dynamic affinity over a wide range of conditions to enable displacement of the ISIS 2302 oligonucleotide on the Source 15Q material. Displacement experiments were carried out at various feed loads to examine whether the purification of this oligonucleotide could be successfully carried out at high loadings while maintaining high purity and yield. The

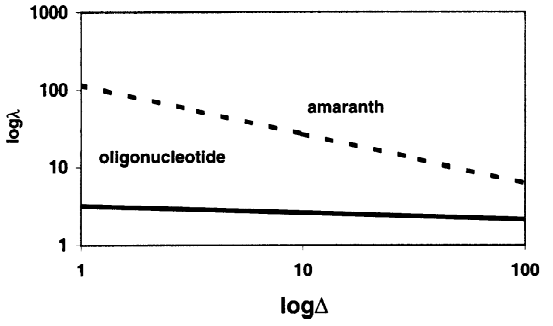

Fig. 1. Displacer ranking plot for oligonucleotide and amaranth.

first displacement experiment was conducted at a feed load of 49 mg (for the 1.66-ml column) and the resulting separation is shown in Fig. 3. For the simplicity of this presentation, ''impurities'' denotes all the $(n-x)$ deletions and partial phosphodiester $(P{=}O)_x$ impurities observed during the displacement experiments. As seen in the figure, the low-molecular-mass displacer amaranth was able to produce a good separation at this loading using the Source 15Q material.

This result is significant because it shows that it is possible to purify four times as much oligonucleotide than previously achieved using Poros HQ/M [28]. In the foregoing experiment, the yield was 91% and purity was ~99% for the product pool as determined by the high-temperature anion-exchange (HPLC) assay. When the displacement was carried out at a total column loading of 49 mg, the separation took place with very little tailing of impurities [$(P{=}O)_x$

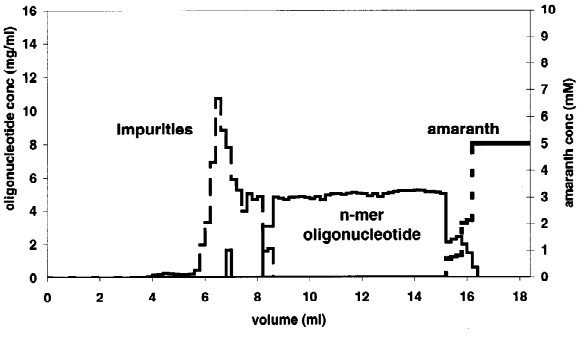

Fig. 3. Displacement separation of 49 mg of oligonucleotides using amaranth as a displacer. Column: 100×4.6 mm I.D. Source 15Q; carrier: 20 m*M* NaOH+500 m*M* NaCl; displacer: 5 m*M* amaranth; flow-rate: 0.2 ml/min.

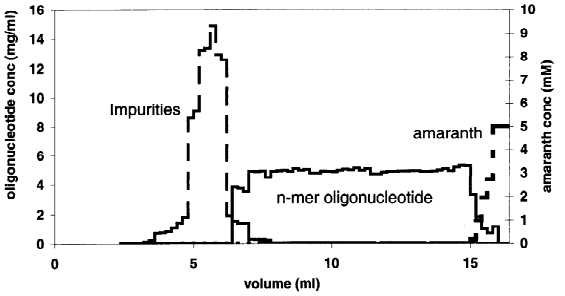

Fig. 4. Displacement separation of 59 mg of oligonucleotides using amaranth as a displacer. Column: 100×4.6 mm I.D. Source 15Q; carrier: 20 m*M* NaOH+500 m*M* NaCl; displacer: 5 m*M* amaranth; flow-rate: 0.2 ml/min.

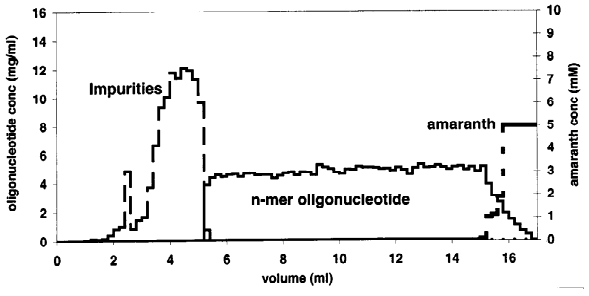

Fig. 5. Displacement separation of 65 mg of oligonucleotides using amaranth as a displacer. Column: 100×4.6 mm I.D. Source 15Q; carrier: 20 m*M* NaOH+500 m*M* NaCl; displacer: 5 m*M* amaranth; flow-rate: 0.2 ml/min.

and $(n-x)$] into the main *n*-mer oligonucleotide zone (Fig. 3). The displacement zone was broad due to the high loading of oligonucleotides, further improving the performance. A series of experiments were then carried out to examine the effect of the feed load on the performance of this displacement system.

Subsequent experiments were then carried out at 59 and 65 mg total column loadings and the results are shown in Figs. 4 and 5, respectively. Again, these high loading experiments resulted in no premature breakthrough of the product and the product recovery for these experiments were 87 and 86%, respectively. The purities by the chromatographic assay were maintained at ~99% (Figs. 6 and 7). In order to further examine the length-based purity obtained in these experiments, analysis by CGE was carried out as described in the experimental section. The CGE technique was shown to be very selective in discriminating between $(n-x)$ impurities and the full-length oligonucleotide due to its high number of theoretical plates [32]. The results of fraction analysis by both HPLC and CGE methods are shown in Figs. 7 and 9, respectively, for the 65 mg experiment. For this paper all impurities with higher affinity than the full-length oligonucleotide were lumped together and denoted as $(n+x)$. As seen from the HPLC analysis of the crude feed (Fig. 6), the partial phosphodiester $(P{=}O)_x$ impurities that

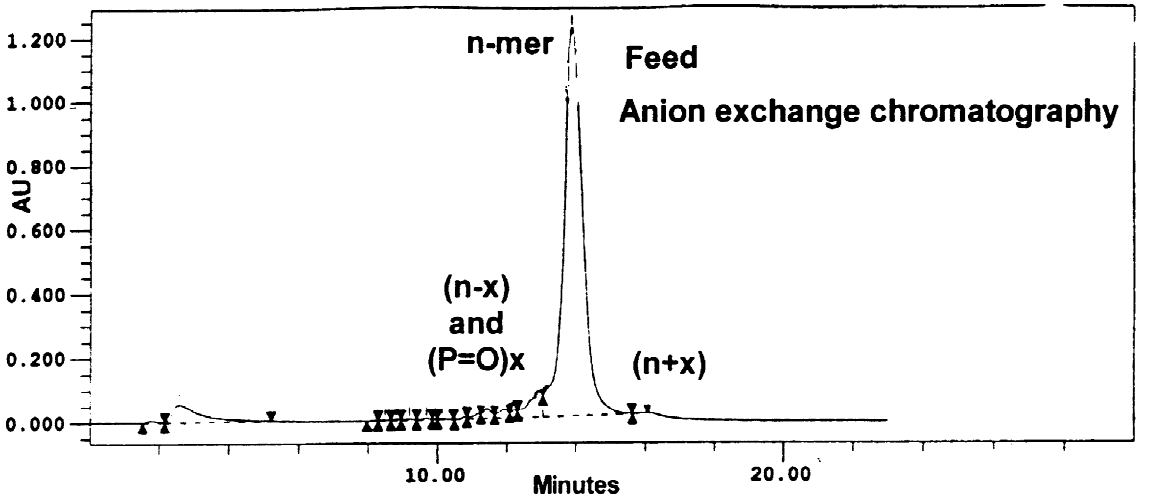

Fig. 6. High-temperature anion-exchange analysis of ISIS 2302 feed.

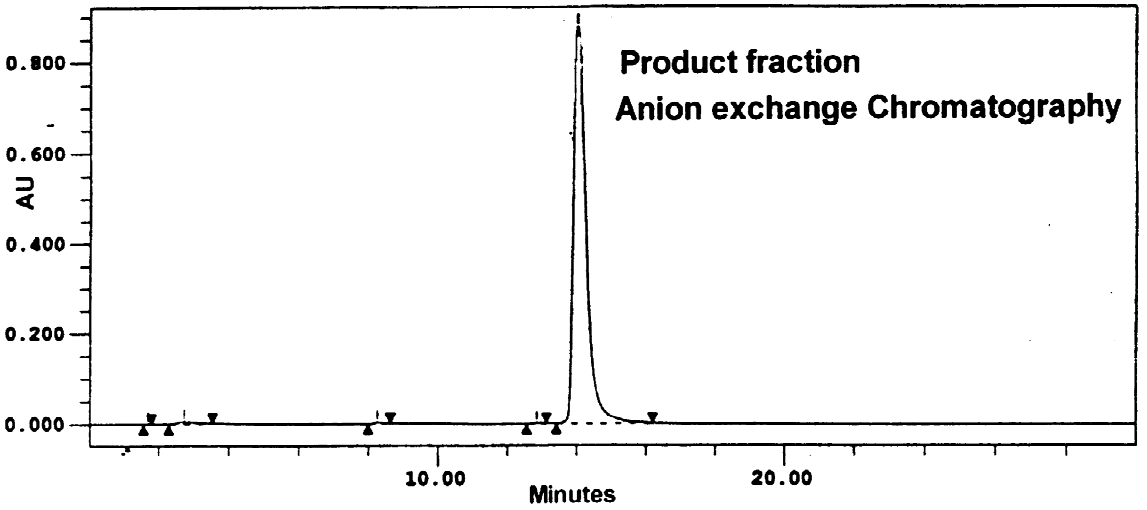

Fig. 7. High-temperature anion-exchange analysis of product fraction from the displacement experiment with 65-mg loading.

appear as a shoulder of the main peak disappear in the analysis of the displacement product fractions shown in Fig. 7. The CGE analysis (Figs. 8 and 9) further confirms that the $(n-x)$ and $(n+x)$ impurities have been removed by displacement chromatography. The detailed purity information determined both by HLPC and CGE for different fractions collected during the experiments are summarized in Tables 1 and 2.

The column loading of these oligonucleotide displacement separations was pushed even further to 69 mg. Under these conditions, the separation resulted in some premature elution of the oligonucleotide product (Fig. 10) due to insufficient capacity of

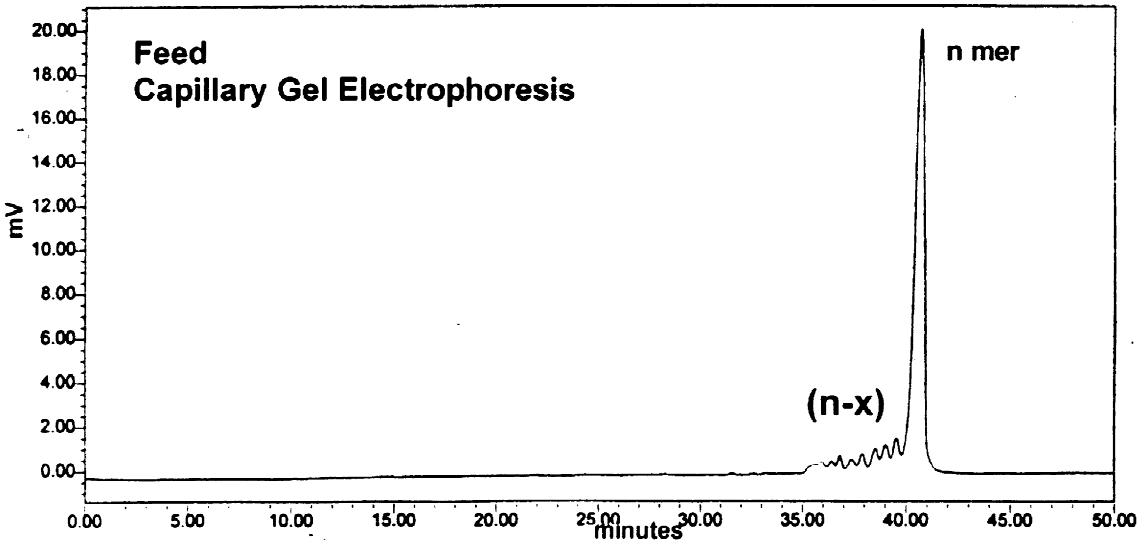

Fig. 8. Capillary gel electrophoresis analysis of ISIS 2302 feed.

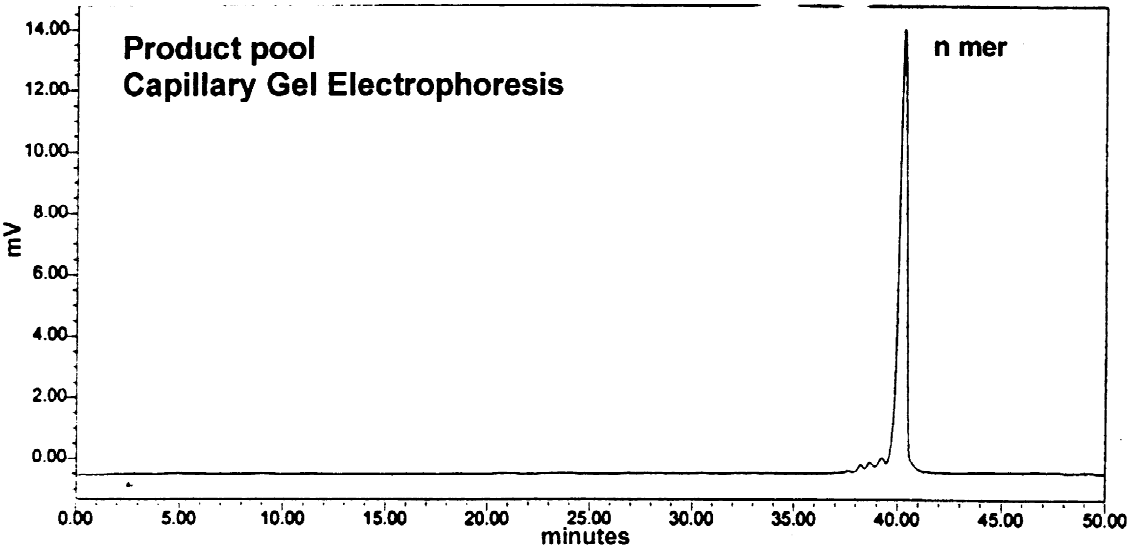

Fig. 9. Capillary gel electrophoresis analysis of product pooled from the displacement experiment with 65-mg loading.

Table 1
Purity analysis of representative fractions from the 65-mg displacement experiment (determined by anion-exchange chromatography)

Fraction (ml)	Anion-exchange	
	% Impurity	% *n*-mer
Feed	21.7	78.3
3.8–4.0	65.16	34.84
5.2–5.4	15.93	84.07
7.8–8.0	0	100.0
11.4–11.6	0	100.0
Product pool	0.05	99.95

the column at this loading. Nevertheless, high yields and purity were still maintained in this separation. In addition, all purified fractions were checked for the presence of amaranth by HPLC and found to be at the undetectable levels (data not shown).

Table 2
Purity analysis of representative fractions from the 65-mg displacement experiment (determined by CGE analysis)

Volume (ml)	CGE	
	% $(n-x)$	% *n*-mer
Feed	20.51	79.49
8.6–8.8	6.55	93.45
11.0–11.2	3.81	96.19
12.6–12.8	0.76	99.24
Product pool	5.44	94.56

5. Conclusions

In this paper, we have demonstrated that displacement chromatography with low molecular mass displacers can be successfully employed for the purification of phosphorothioate oligonucleotides (*n*-mer) from closely related impurities at high column loads. Column loads ranging from 49 to 65 mg resulted in isotachic displacement profiles with high yields and purities. In contrast to prior work, these

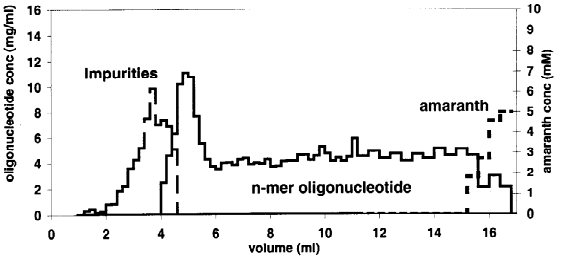

Fig. 10. Displacement separation of 69 mg of oligonucleotides using amaranth as a displacer. Column: 100×4.6 mm I.D. Source 15Q; carrier: 20 m*M* NaOH+500 m*M* NaCl; displacer: 5 m*M* amaranth; flow-rate: 0.2 ml/min.

results show that it is indeed possible to carry out oligonucleotide displacements at elevated column loads while maintaining the desired yield and purity. It is expected that displacement chromatography will be a useful tool for large-scale oligonucleotide purifications. Furthermore, since the Source 15Q media has good chemical stability for routine use between pH 2–12 and cleaning at pH 1–14 we believe that displacement chromatography with Source 15Q may provide a high throughput purification method for oligonucleotides in the process setting. The affinity of amaranth could be attributed to the three negatively charged sulfonic acid groups on the molecule as well as the aromatic moieties. Further investigation of the effects of various structural components on the efficacy of displacers for oligonucleotide separations is in progress in our lab.

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