GRACE VYDAC **APPLICATION NOTE**

#0105

Purification of Synthetic Oligonucleotides

Phosphodiesters, Phosphorothioates, Reversed Phase and Anion Exchange

Synthetic oligonucleotides are often purified by reversed-phase HPLC. However, reversed phase alone may not be the best choice.

Oligonucleotide Purification Problems

The most common difficulty in purifying oligonucleotides is their tendency to form hydrophobic hydrogen-bonded complexes. Hydrophobic effects, base stacking for example, and hydrogen bonding act in concert: exclusion of water strengthens hydrogen bonds because it eliminates the competition of hydrogen bonding with water molecules. Complexes formed by synthetic oligonucleotides can be intrachain (hairpins), interchain, complexes with synthetic byproducts, or all three. Hydrophobic complex formation makes chromatography, especially reversed phase, more complicated.

Reversed-phase chromatography is solvent-eluted hydrophobic interaction. Conditions that favor hydrophobic complex formation also favor retention on reversed-phase columns. The organic solvents commonly used for reversed-phase elution, acetonitrile and isopropanol for example, also have chaotropic (complex breaking) effects on hydrophobic aggregates. Although hydrophobic substances in the sample may adsorb to the reversed-phase column as complexes, the increasing concentration of solvent in the mobile phase can either elute them intact or cause them to undergo rearrangements and elute as a variety of entities. This is a generally unpredictable situation that, at a minimum, leads to broader peaks. Chaotrope concentrations high enough to prevent complex formation in the sample will often also prevent retention on reversed-phase columns.

Rules to remember:

- Longer phosphodiester chains, and especially phosphorothioates, are likely to form complexes and cause problems.
- Short phosphodiester oligonucleotides are less prone to difficulties.

In many cases, anion-exchange chromatography or a combination of anion exchange with reversed phase offers a better solution.

Anion Exchange

In anion exchange, separation is driven by the negative charge of the oligonucleotide molecule. Each nucleotide residue adds one negative charge, but some bases also add positive charges. All oligos of the same length will not have the same charge.

An anion-exchange column generally has higher loading capacity than a reversed-phase column. However, this needs to be balanced against the fact that high sample concentrations can increase hydrophobic complex formation as shown by the chromatograms of Figure 1.

It is possible to do anion-exchange separations under conditions that break up hydrophobic hydrogen-bonded complexes. Inclusion of a chaotropic organic solvent in the mobile phase – acetonitrile, ethanol, or isopropanol for example – not only breaks up hydrophobic complexes, but also strengthens ionic retention. Some salts are also chaotropic – for example, $NaClO_4$ and NaBr. They are recommended for mobile phases used for separating oligonucleotides. Other salts – $(NH_4)_2SO_4$, NH_4OAc, NaCl – are cosmotropic (increase structure). They should not be used.

Purification of Phosphodiester 50-Mer on VYDAC® 301VHP Anion-Exchange Column

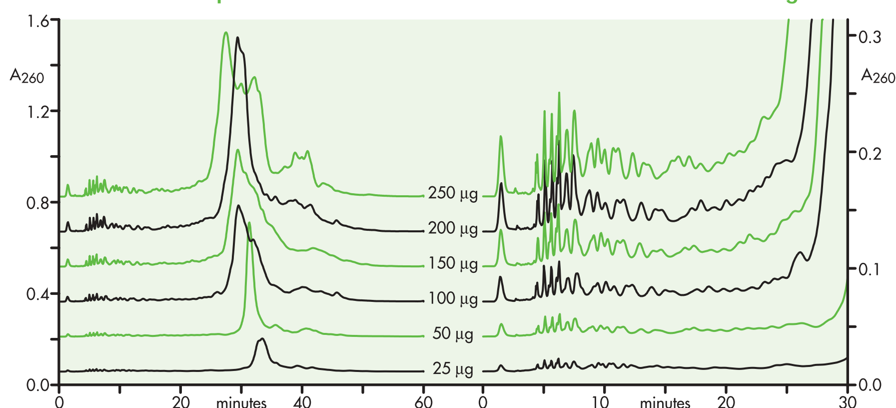

Figure 1. *Complete chromatograms and detail for separations of a crude 50-mer phosphodiester oligonucleotide at six different sample concentrations. There is a characteristic shift of retention to shorter times and distinct change in profile of the main product peak indicating complex formation with increased sample loading. Column: VYDAC 301VHP5410 900 Å 5 µm DEAE-type polymer-based anion exchange, 4.6 mm ID x 100 mm. Flow: 1.0 mL/min. Mobile phase: NaBr in 25% aqueous acetonitrile. Gradient: 0 to 75 mM NaBr in 2 minutes, then 75 to 150 mM NaBr in 50 minutes.*

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The chromatograms of Figure 2 actually violate this rule, combining NH_4OAc, a cosmotropic salt, with solvent to break up complexes. However, this provides the advantage of a completely volatile mobile phase and succeeds because the phosphodiester oligonucleotides being separated in this example have only a moderate tendency to aggregate.

Phophodiester oligonucleotides can usually be separated in aqueous systems using chaotropic salts only, and no organic modifier (Fig. 3). Phosphorothioates and some difficult phosphodiester oligos may require both chaotropic salts and solvent (Fig. 4).

The best strategy for anion exchange is to ramp rapidly to an ionic strength slightly below the elution point of the desired product. Then run a shallow linear gradient of salt concentration to elute the product in a short time with high resolution.

Anion Exchange of Phosphodiester Oligonucleotide 70-Mer and 80-Mer

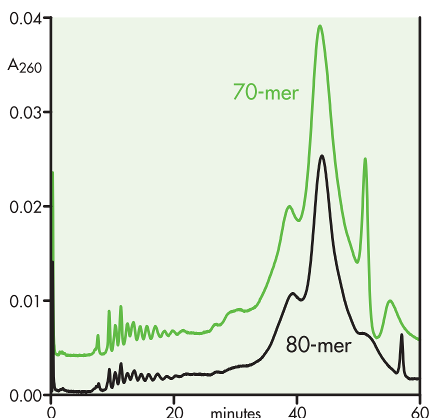

Figure 2. *Purification of crude 70-mer and 80-mer phosphodiesters by anion exchange. Column: VYDAC 301VHP552 900 Å 5 µm polymer-based DEAE-type anion exchange, 5 mm ID x 25 mm. Flow: 1.0 mL/min. Mobile Phase: A = 25 mM NH_4OAc, pH 7, in 50% IPA. B = 500 mM NH_4OAc, pH 7, in 50% IPA. C = 1.0 M NH_4OAc, pH 7, in 25% ACN. Gradient: 100% A to 79% A/21% B in 5 minutes, then ramp to 41% B in 45 minutes, then to 100% B in 5 minutes, and 100% C in 0.1 minute.*

Buffer Effects on Anion Exchange of Phosphodiester 40-Mer

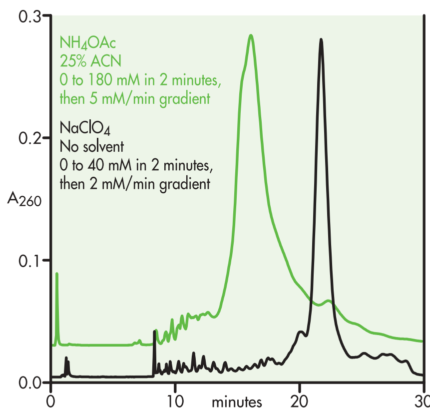

Figure 3. *Purification of 40-mer phosphodiester by anion exchange. $NaClO_4$ is a better chaotrope and provides a stronger anion than NH_4OAc. Column: VYDAC 301VHP552 900 Å 5 µm polymer-based DEAE-type anion exchange, 5 mm ID x 25 mm. Flow: 1.0 mL/min. Mobile Phase: As shown.*

Uncharged reaction byproducts that might form complexes with oligonucleotides will normally fall through without retention on anion exchange. It is therefore an excellent first purification step and can be followed by reversed phase for final cleanup. A C18 column and TEAA-containing mobile phase will also easily accomplish removal of salts from the anion-exchange eluate.

Reversed Phase

Whether or not it is used as a first or second purification step, reversed phase does have useful features.

Trityl groups are much more hydrophobic than acetylated failure sequences. Therefore, reversed phase is the best step to accomplish their separation. With a chemical-resistant polymer reversed-phase column (VYDAC® 259VHP), you can elute acetylated failure sequences, base-cleave the trityl group on the column, and elute the desired oligo ahead of the trityl residue. Both purification

Anion Exchange of Phosphorothioate Oligonucleotide 38-Mer

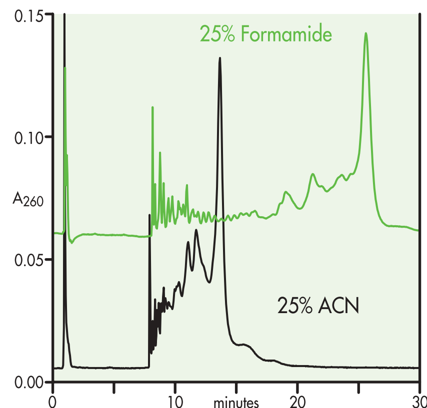

Figure 4. *Purification of phosphorothioate 38-mer by anion exchange. For some S-oligos, formamide is needed to break up complexes. Column: VYDAC 301VHP5410 900 Å 5 µm polymer-based DEAE-type ion exchange, 4.6 mm ID x 100 mm. Flow: 1.0 mL/min. Mobile Phase: A = 25 mM NH_4OAc in 25% solvent. B = 500 mM $NaClO_4$ in A. Gradient: 0 to 10% B in 2 minutes, then 10% to 30% B in 75 minutes.*

and detritylation can thereby be achieved in one chromatographic step.

Triethylamine acetate (TEAA), a volatile ion-pairing agent for anions, is often used in mobile phases for oligonucleotide reversed phase chromatography. It binds to the reversed-phase surface but also interacts ionically with phosphodiester linkages. For unprotected oligos, it can be convenient to think of reversed phase with TEAA as a sort of solvent-eluted anion exchange. A C4 column usually works well and will not bind hydrophobic reaction products as tightly as C18. (See Vydac Application Note #9801.)

Reversed-phase chromatography can provide a good single-step purification for non-problematic oligonucleotides as well as initial research purification. In a process environment, and with phosphorothioates or difficult-to-purify phosphodiesters, it is best used as a followup to ion exchange.

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