[APPLICATION NOTE]

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OLIGONUCLEOTIDE SEPARATION TECHNOLOGY: SYNTHESIS CHALLENGES AND HPLC ISOLATION OPTIONS

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INTRODUCTION

Origins of synthetic oligonucleotides impurities

Use of synthetic oligonucleotides is increasing in areas ranging from clinical diagnostics to novel biopharmaceutical therapeutics. While the automated synthesis of oligonucleotides is a highly efficient process, small amounts of impurities are created at each step throughout the synthesis cycle. Consequently, manufacturing organizations as well as individuals who depend on the quality of delivered products have a vested interest in cost effective and efficient ways to purify and analyze these important biological tools. Failure to achieve these goals can seriously impede the ability of an organization or individual to achieve desired results. An example might involve a delay in obtaining FDA approval for a new diagnostic reagent or drug.

A closer inspection of how synthesis coupling efficiency impacts the amount of manufactured full length product is shown in Figure 1. Regardless of average coupling efficiency, longer oligonucleotide sequences contain a greater concentration of shorter length contaminants. The failure products, typically labeled N-1, N-2..., N-x, are prematurely halted shorter oligonucleotides. Some are missing a nucleotide(s) in the middle of sequence, rather than at the end. These products are called mismatch failure sequences.

Some by-products of synthesis may have greater molecular weight (often labeled N+x) than the target oligonucleotide. This is a result of incomplete post-synthesis deprotection, or due to the branching of an oligo backbone during the synthesis. For labeled oligonucleotides, the failure products are also generated by failure to conjugate the label with the target sequence.

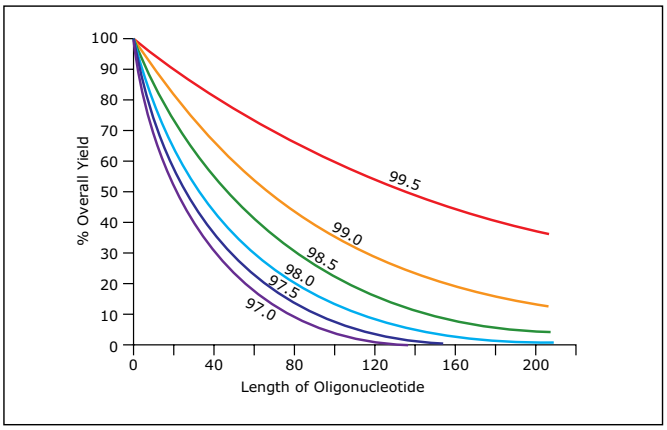

Figure 1. Synthetic oligonucleotide length compared to theoretical yield at various coupling efficiencies.

This application note addresses how Waters® Oligonucleotide Separation Technology Columns are the most viable option for handling the challenges of purification and isolation of synthetic oligonucleotides.

DISCUSSION

Lab-scale isolation options

Once a synthesis is complete, the synthetic oligonucleotide must be cleaved from the solid-phase support (e.g. controlled pore glass). The base and phosphate groups must then be fully deprotected prior to use of any subsequent purification technique. Table 1 highlights commonly used methods for the lab-scale purification (25 to 500 nmole) of synthetic oligonucleotides. The advantages as well as disadvantages of each technique are presented.

Technique	Advantages	Disadvantages
Polyacrylamide gel electrophoresis (PAGE)	Well-established and efficient method. It separates long oligonucleotides (>50 to 60 mer).	Low mass loading capacity. Gels are typically overloaded for purification and the resolution is compromised. PAGE does not separate N+x sequences. Manual band cutting. Excision is based on markers without detailed knowledge of target oligo retention. Samples need to be extracted from the gel and desalted; recovery of target oligonucleotides is low. Method is laborious; it is typically used only when no other technique is suitable for the task.
Ion exchange liquid chromatography (IEX-LC)	Trityl-off method. Separation of failure sequences is due to the backbone charge.	IEX-LC is efficient only for relatively short oligos (<20 to 25 mers); longer oligos are poorly resolved. Sample is contaminated with high concentration of salts; further desalting is required. IEX columns packed with non-porous sorbent offer improved resolution, but suffer with low mass load capacity. When loading exceeds 10 to 20 nmoles (for 4.6 mm I.D. columns), the resolution is compromised. IEX-LC does not separate N+x sequences.
Trityl-on liquid chromatography (Trytil-on LC; DMT-on LC)	Elegant, fast, and universal method for oligos of various length and sequence. RP columns used with this method have sufficient mass load capacity.	Does not adequately remove mismatch failure sequences (similarly as the target oligo, they carry DMT group). DMT group is labile; part of the product may be lost due to the spontaneous detritylation. DMT residue and remaining acid have to be removed after the detritylation.
Trityl-off liquid chromatography (Trityl-off LC; DMT-off LC)	Effectively removes practically all types of failure products. Uses volatile solvents; samples do not have to be further desalted. Collected fractions are simply lyophilized and ready for use. RP columns used with this method have sufficient mass load capacity. Labeled and dually-labeled oligonucleotide probes can be also purified. Method is suitable for LC/MS analysis (with MS compatible ion-pairing buffers).	Method requires efficient columns packed with small particle size sorbent. Oligo retention and resolution partially depends on the sequence. Method development for different oligo sequence and length probes is necessary.

Table 1. Advantages vs. disadvantages of synthetic oligonucleotide lab isolation techniques.

Oligonucleotide Separation Technology

Waters Oligonucleotide Separations Technology (OST) Columns are specifically designed for the HPLC purification and HPLC or UltraPerformance LC® (UPLC®) analysis of synthetic oligonucleotides. Its separation mechanism is based on highly efficient ion-pairing reversed-phase (IR-RP) chromatography of the "trityl-off" synthetic oligonucleotide species, where the oligonucleotide is detritylated at the last step of synthesis. IP-RP LC separates the trityl-off full length product from failure sequences.

Waters OST columns were developed following a series of comprehensive investigations that helped Waters scientists and engineers better understand limitations of existing technologies for this application area. Our flexible separation chemistry technology is designed to assist manufacturers deliver quality products that can help researchers make profound discoveries (e.g. via siRNA research) that lead to novel drug therapies or diagnostic reagents.

As shown in Figure 2, separation of N from N-1 species on OST Columns rivals separations obtained using capillary gel eletrophoresis techniques. OST Columns are useful for the purification and analysis of DNA or RNA-based oligonucleotide products. This method has significant advantages over current technologies used to purify oligonucleotides. For example, compared to purification with cartridges, gel electrophoresis, desalting, or ion-exchange chromatography, OST Columns offer the highest level of product purity without sacrificing product recovery (Table 2). As such, OST Columns represent a new standard in synthetic oligonucleotide purification.

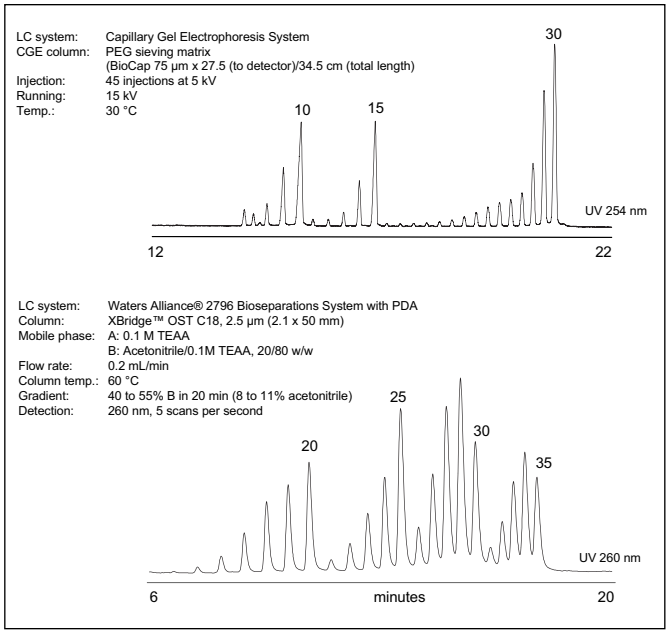

Figure 2. Separation of detritylated oligodeoxythymidine ladders by capillary gel electrophoresis (CGE) vs. ion-pair reversed-phase (IR-RP) chromatography.

Technique	Published expected purity	Actual purity* estimated by HPLC/CGE	Target recovery*
Desalted (gel filtration)	60 to 70%	70%	~80%
Anion exchange	85 to 95%	90%	~37%
PAGE	85 to 95%	91%	~8%
Waters OST Column	>95%	>95%	>90%

*At standard mass loads.

Table 2. Comparison of purity between available methods. Comparison of methods was performed with 100 nmole of 25 mer oligonucleotide. The IP-RP HPLC purification was accomplished in a single injection using a Waters OST C_{18} 2.5 µm, 4.6 x 50 mm column.

The XBridge™ OST C_{18} Column chemistry consists of Waters' patented Bridged Ethyl Hybrid (BEH) base particles (Figure 3) functionalized with C_{18} ligands. The small particles (e.g. XBridge OST 2.5 µm particles and ACQUITY UPLC® OST 1.7 µm particles) and large surface area of the BEH sorbent material yields high separation efficiency and large sample capacity. In particular, the small particle size of sorbent improves the mass transfer of the oligo macromolecules in the stationary phase and is key for successful separation efficiency.

Furthermore, compared to the use of traditional silica-based small particle C_{18} offerings, Waters BEH-based OST Columns demonstrate outstanding packed bed stability over repeated conditions of elevated temperature and pH conditions.[1]

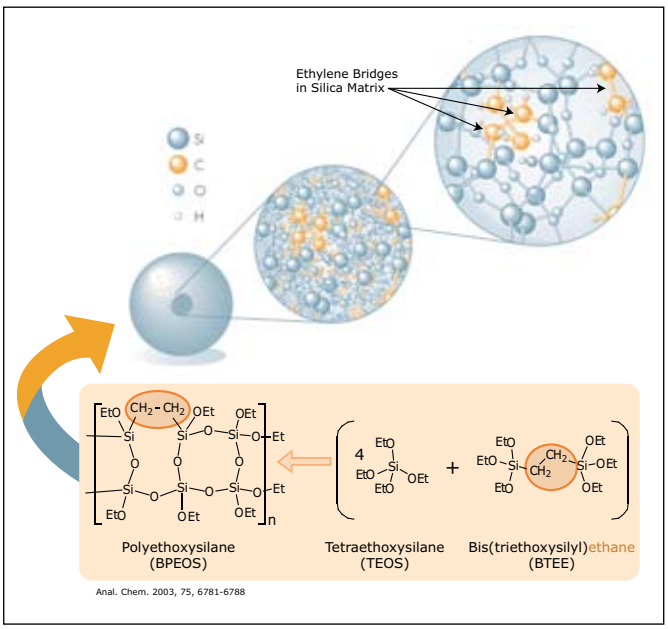

Figure 3. BEH Technology™ particles are prepared from two high purity monomers: tetraethoxysilane (TEOS) and bis (triethoxysilyl) ethzane (BTEE, which incorporates the pre-formed ethylene bridge). This structure results in greater temperature and pH stability compared to that seen with traditional silica-based, reversed-phase materials.

CONCLUSION

Scalable separations with OST Columns

XBridge OST C_{18} Columns are the preferred offering for detritylated oligonucleotide purifications due to their resolving ability (Figure 4) and availability of column sizes designed to meet laboratory-scale isolation requirements in a cost effective yet efficient manner.

LC conditions

LC System:	Waters Alliance® HPLC 2695 System
Column:	Waters XBridge OST C_{18}, 2.5 µm 4.6 x 50 mm
Column temp.:	80 °C
Flow rate:	1.26 mL/min.
Mobile phase A:	0.1M TEAA, pH 7.5
Mobile phase B:	Acetonitrile
Gradient:	5 to 50% B in 9.6 min
Detection:	UV 260 nm

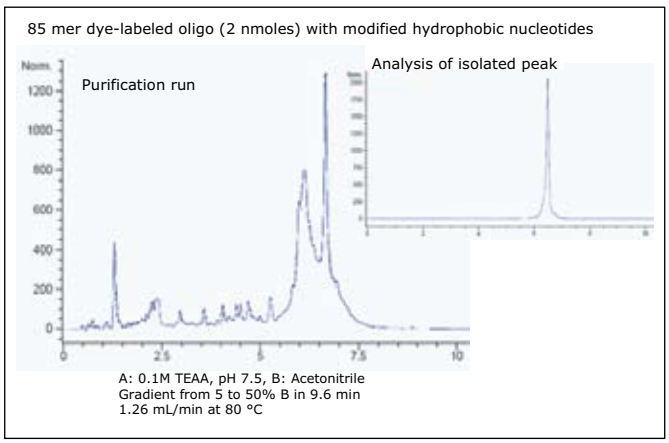

Figure 4. XBridge OST C_{18} isolation (2 nmoles injected) and analysis of isolated 85 mer dye-labeled oligo with modified hydrophobic nucleotides.

As indicated in Table 3, the choice of XBridge OST C_{18} column dimension and operating flow rate depends primarily on the scale of the synthesis reaction mixture. Typically, 2.5 µm particle sorbents are used for HPLC analytical- or lab-scale purification applications using 4.6 x 50 mm columns. Additional column dimensions are offered for larger-scale applications. Up to 0.5 µmole of synthetic oligonucleotide material can be successfully purified on a 10 x 50 mm column without compromising isolation product purity or recovery.

Higher mass loads, up to 2.5 µmole, can be purified with the same high purity and only moderate reduction in recovery. Selection of the appropriate column size for the amount of oligonucleotide sample loaded is recommended to maximize component resolution and recovery of the target product from non-desired failure sequences.

Column (mm)	Approx. mass load (µmoles)**	Flow rate (mL/min)
2.1 x 50	0.04	0.2
4.6 x 50	0.20	1.0
10.0 x 50	1.00	4.5
19.0 x 50*	4.00	16.0
30.0 x 50*	9.00	40.0
50.0 x 50*	25.00	110.0

**Custom OST Column*
***Values are only approximates and vary depending on oligonucleotide length, base composition, and "heart-cutting" fraction collection method used.*

Table 3. XBridge OST C_{18} Column selection guide for detritylated oligonucleotide purification.

Figure 5. Oligonucleotide Separation Technology (OST) Columns.

For the latest listing of Waters XBridge OST and ACQUITY UPLC Column offerings for the high-resolution HPLC isolation and UPLC or HPLC analysis of synthetic oligonucleotides, go to www.waters.com/ost.

References

1. Wyndham K, et al. Characterization and evaluation of C18 HPLC stationary phases based on ethyl-bridged hybrid organic/inorganic particles. Anal. Chem. 2003; 75: 6781. Waters Application Note WA32741.

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