TOSOH

APPLICATION NOTE

SIMPLE AND EFFECTIVE METHOD FOR PURIFICATION OF DMT-ON OLIGONUCLEOTIDES USING HIC RESINS

INTRODUCTION

The use of synthetic oligonucleotide therapeutics continues to grow because of their effectiveness in the treatment of devastating diseases. Within the biopharmaceutical industry, oligonucleotide drug pipelines have increased significantly, along with the need for purification techniques of these highly valuable materials.

Dimethoxytrityl (DMT), a 5′ protecting group, is used in the synthesis of oligonucleotides to temporarily mask the characteristic chemistry of a 5′-hydroxy functional group. In many preparations of delicate oligonucleotides, DMT may be left on an oligonucleotide following synthesis to give stability to the molecule during subsequent processing.

In this note, a novel, effective and high recovery method for purification of a DMT-on oligonucleotide and the effective removal of a DMT-group from an oligonucleotide in a single purification step are described. This purification can be achieved by using hydrophobic interaction chromatography (HIC) since the DMT-on group is strongly hydrophobic.

MATERIALS AND METHODS

Oligonucleotide: 5′-GAA TTC ATC GGT TCA GAG AC-3′, a single stranded DNA oligonucleotide, 20-mer in length with a molecular weight of 6.141 kDa. It was supplied (AEX-HPLC) at ~55% purity from Trilink.

Salts: 3 different salts purchased from Millipore Sigma were used in the study: sodium chloride (NaCl), sodium sulfate (Na_2SO_4) and ammonium sulfate $(NH_4)_2SO_4$.

HIC resins: Four TOYOPEARL® HIC resins were selected for the study: PPG-600M, Phenyl-650M, Butyl-650M, and Hexyl-650C.

Conditions: See chromatograms

RESULTS AND DISCUSSIONS

Resin selection

Hydrophobic interaction chromatography is a powerful tool for the process purification of biomolecules. The technique utilizes the accessible hydrophobic regions located on the surfaces of the molecules and their interactions with a hydrophobic stationary phase.

Proteins and other molecules with hydrophobic surfaces are attracted to the hydrophobic ligands of HIC resins by employing an aqueous high salt mobile phase. The salt conditions contribute to a lyotropic effect, which allows the proteins to bind to a hydrophobic ligand. Bound molecules are eluted by decreasing the salt concentration. Most therapeutic targets are eluted in a low salt or a no salt buffer. Since HIC separations are done under mild eluting conditions, biological activity is typically retained.

In order to determine the ligand able to provide the best purity, recovery and yield for the purification of an oligonucleotide, four TOYOPEARL HIC resins were selected: PPG-600M, Phenyl-650M, Butyl-650M, and Hexyl-650C. These stationary phases are ranked from the least to the most hydrophobic, as shown in Figure 1. These HIC resins are polymethacrylic polymer beads featuring ligands with different degrees of hydrophobicity and selectivity.

DEGREES OF HYDROPHOBICITY OF TOYOPEARL HIC RESINS

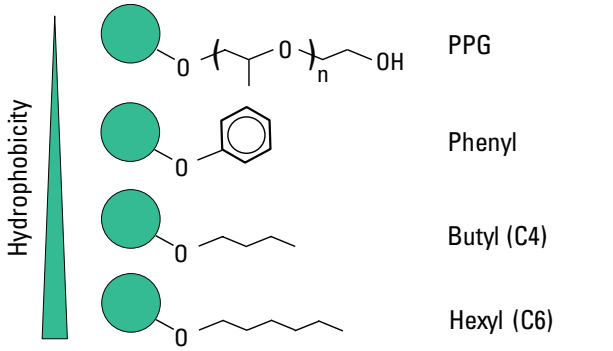

Figure 1

Impact of varying salt conditions on purification

Prior to the resin screening process, different salts were used to determine the salt tolerance limit for the oligonucleotide that will be purified. Different concentrations of salts added to the sample were tested in order to verify the concentration at which precipitation occurs: 0.5 mol/L, 1.0 mol/L and 1.5 mol/L. Experimental results (data not shown) indicated that at 1.5 mol/L salt concentration, the oligonucleotide was bound strongly to all of the HIC resins without sample precipitation. Therefore, 1.5 mol/L salt concentration was selected for use in subsequent studies.

IMPACT OF VARYING SALT CONDITIONS ON HIC OLIGONUCLEOTIDE PURIFICATION

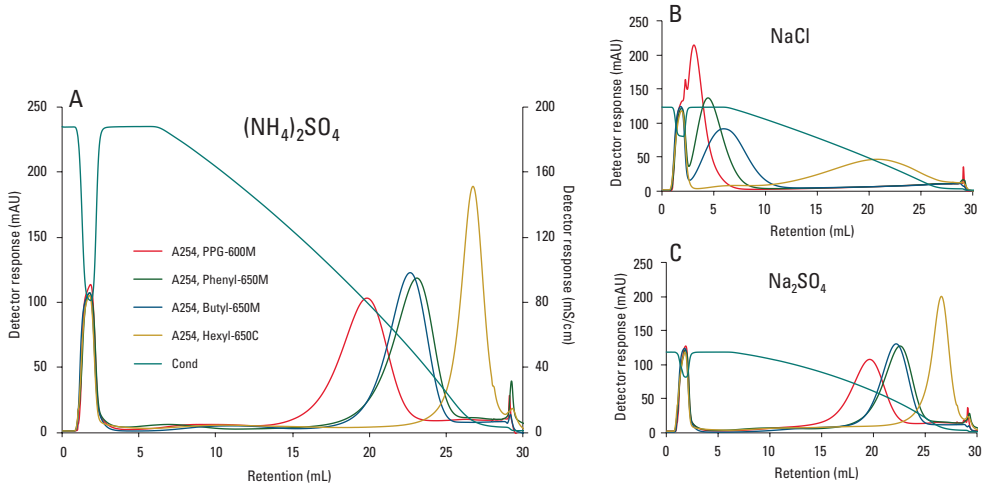

Figure 2

Media:	TOYOPEARL PPG-600M, Phenyl-650M, Butyl-650M, or Hexyl-650C
Column:	5 mm ID × 5 cm (1 mL)
Mobile phase:	A: 20 mmol/L Tris, 1 mmol/L EDTA, 1.5 mol/L salt as indicated, pH 7.5, B: 20 mmol/L Tris, 1 mmol/L EDTA, pH 7.5
Gradient:	0 - 100% B, 20 mL, 100% B, 4 mL, columns were washed with 5 CV of 30% isopropyl alcohol between runs
Flow rate:	1.0 mL/min (1 min residence time)
Detection:	UV @ 254 nm (mAU), conductivity (mS/cm)
Temperature:	ambient
Injection vol.:	1 mL (0.1 mg/mL load ratio)
Sample:	unpurified DMT-oligo, 0.1 mg/mL

In this study, 3 different salts were screened: sodium chloride (NaCl), sodium sulfate (Na_2SO_4) and ammonium sulfate ($(NH_4)_2SO_4$). Figure 2, panels A and C, show that $(NH_4)_2SO_4$ and Na_2SO_4 generated very similar oligonucleotide peaks. In fact, all HIC resins gave similar elution profiles and all selected resins effectively separated a DMT-off (flow-through peak) from a DMT-on oligonucleotide. In addition, the data also showed that TOYOPEARL Phenyl-650M and Butyl 650M bound the oligonucleotide stronger compared to the TOYOPEARL PPG-600M resin. TOYOPEARL Hexyl-650C showed the strongest binding, therefore, the oligonucleotide peak was eluted at the latest retention time. It was also noticed that NaCl (Figure 2, panel B) did not provide strong binding for the oligonucleotide on the resin, thus the reason why the oligonucleotide was eluted very early at the beginning of the decrease of the salt gradient.

It is known that sulfates of sodium and ammonium are most effective at promoting ligand-oligonucleotide interactions and are known to have little destructive effect on sample structure. Ammonium sulfate demonstrated a more linear conductivity response when used in a gradient (compare Fig. 2, panel A to panel C) and thus was chosen for these studies.

Effect of HIC stationary phase on purification

After the initial resin screening, TOYOPEARL PPG-600M, Phenyl-650M and Butyl-650M were selected for the loading study consisting of both a DMT-off and DMT-on oligonucleotide. These resins were selected because they generated similar peak elution profiles under similar gradient conditions (Figure 3). TOYOPEARL PPG-600M eluted the DMT-on oligonucleotide earlier than the TOYOPEARL Phenyl-650M and Butyl-650M resins.

The expanded view chromatogram indicated that the DMT-off oligonucleotide was not bound to any of the three resins and was eluted in the void peak, whereas, the DMT-on oligonucleotide was bound to all three resins and eluted when the salt concentration was decreased. TOYOPEARL PPG-600M did not bind the DMT-on oligonucleotide as strongly as the other two resins. High resolution and baseline separation between DMT-off and DMT-on oligonucleotide peaks were provided by TOYOPEARL Phenyl-650M and Butyl-650M.

COMPARISON OF DIFFERENT TOYOPEARL HIC RESINS

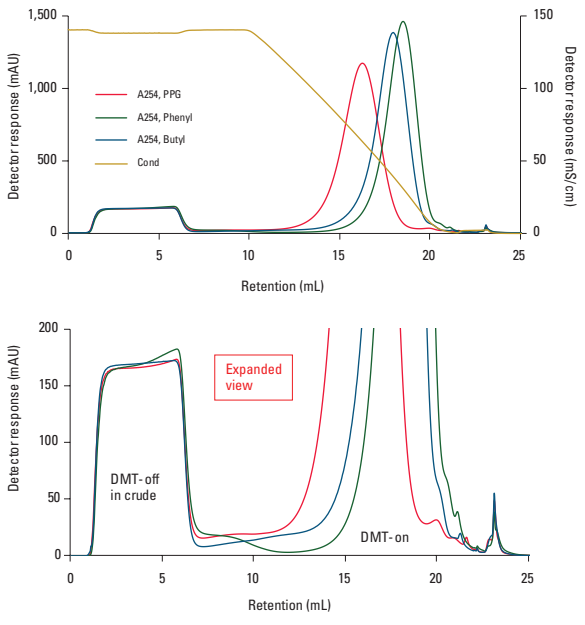

Figure 3

Table 1 demonstrates that TOYOPEARL PPG-600M, Phenyl-650M and Butyl-650M gave similar performance in the purity (99%) and recovery (89%) of a DMT-on oligonucleotide as determined by reversed phase HPLC (RP-HPLC).

SUMMARY OF PURITY AND RECOVERY

Fraction	Fraction Volume (mL)	Avg. conductivity (mS/cm)	Purity (% DMT-on)	Recovery (% DMT-on)
Load			77.9	
PPG elution	4.9	68.0	98.7	89.1
Butyl elution	4.5	45.9	99.0	89.0
Phenyl elution	4.2	36.8	99.0	88.9

Table 1

One-step removal of DMT protection group

For the on-column removal of the DMT-group from the oligonucleotide, TOYOPEARL Phenyl-650M was chosen for further study as a representative of the screened TOYOPEARL HIC resins. Figure 4 shows that removal (cleavage) of the DMT-group from the oligonucleotide was successfully accomplished directly on the column. DMT was cleaved by acidification at approximately pH 4 and the DMT-off oligonucleotide eluted. The DMT-group was removed from the stationary phase during cleaning-in-place (CIP) of the column.

Figure 5 shows the analysis of a crude oligonucleotide sample (panel A) and the on-column cleaved former DMT-on oligonucleotide fraction from Figure 4 (panel B) using a TSKgel® OligoDNA-RP HPLC column. Data confirms that the DMT-group was effectively removed from the oligonucleotide and that on-column DMT cleavage resulted in a > 99% pure DMT-off oligonucleotide at 99% recovery.

CLEAVAGE OF DMT-GROUP DIRECTLY ON COLUMN

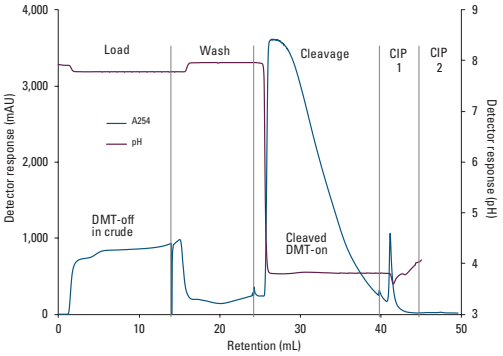

Figure 4

Media: TOYOPEARL Phenyl-650M
Column: 6.6 mm ID × 3.0 cm L (1 mL)
Flow rate: 0.25 mL/min (4 min residence time)
Detection: UV @ 254 nm (mAU), pH
Temperature: ambient

Phase	Volume (mL)	Buffer
Equilibrium	10	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$
Load	14	DMT-oligo, 0.5 mg/mL (7 mg)
Wash	10	10 mmol/L NaOH, 1.0 mol/L $(NH_4)_2SO_4$
Cleavage	15	50 mmol/L acetic acid, 1.0 mol/L $(NH_4)_2SO_4$
CIP 1	5	water
CIP 2	5	30% (v/v) 2-propanol

CLEAVAGE OF DMT-GROUP DIRECTLY ON COLUMN

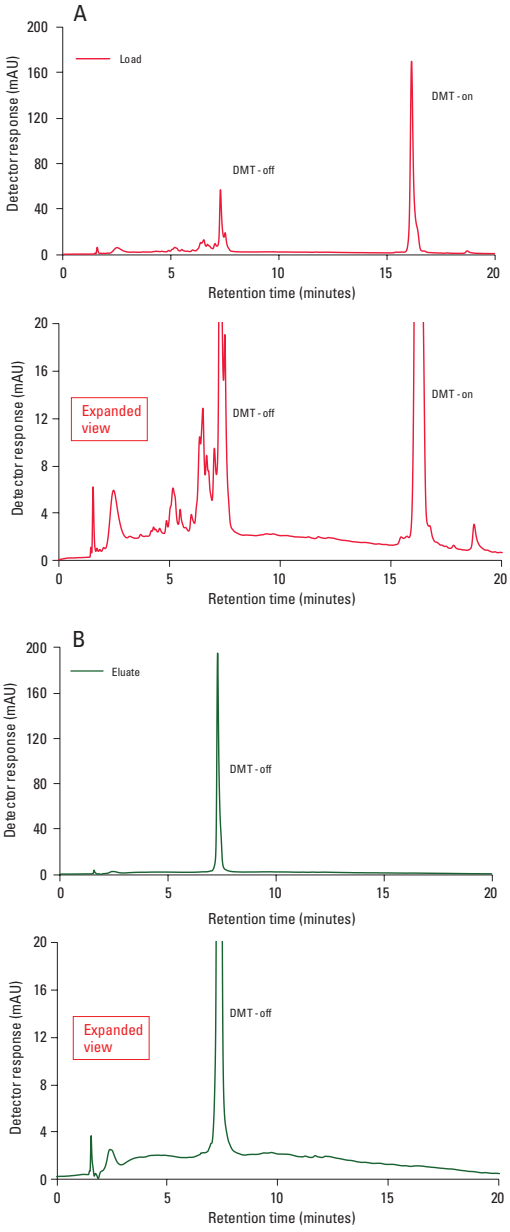

Figure 5

Column: TSKgel OligoDNA-RP, 4.6 mm ID × 15 cm L
Mobile phase: A: 100 mmol/L TEAA, pH 7.0
B: acetonitrile
Gradient: 5 - 35% B, 20 min
Temperature: 45 °C
Flow rate: 1.25 mL/min
Detection: UV @ 254 nm (mAU)
Injection Vol.: 10 µL

CONCLUSION

TOYOPEARL PPG-600M, Phenyl-650M and Butyl-650M hydrophobic interaction chromatography resins are effective in separating DMT-on and DMT-off oligonucleotides in a crude preparation. On-column cleavage at low pH was used effectively to remove a DMT-group and elute a DMT-off oligonucleotide. High purity and recovery were achieved for an on-column cleavage procedure.