Oligonucleotide Purification Strategies using a New High-Capacity Anion Exchange Resin

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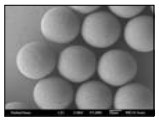
Introduction

With the advent of nucleic acid silencing technologies and the need for high purity diagnostic and therapeutic oligonucleotides, there is a need for high-capacity chromatographic supports that can deliver economic purification processes. A new, 30 micron, mono-sized, polymeric resin has been recently developed that provides high resolution and high capacity for synthetic oligonucleotides. Physical properties of this new resin are described, including particle size uniformity, capacity and pressure stability. The purification of four synthetic DNA oligonucleotides are demonstrated under a variety of conditions.

Introduction – Monodisperse Technology



Resin	Uniformity Coefficient
Toyopearl™ 650 (Tosoh)	1.34
Source™ 30 (GE)	1.65
Sepharose™ HP (GE)	1.68
Rohm and Haas Prototype	1.06

Advantages of New Rohm and Haas Anion Exchange Resin: Higher throughput from…

- **Uniform Particle Size**
  - better resolution and lower pressure drop
- **High Capacity**
  - increased column loading
- **Rigidity**
  - lower compressibility → faster flow rates

Figure 1
Physical Stability

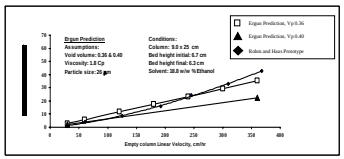


Table 1
Anion Exchange Resin Capacity

Resin	Batch Capacity
Rohm and Haas Prototype	60 g/L
TSKgel Super Q-5PW	42 g/L
Source 30Q	41 g/L

Conditions:
Sample: GGT GGT GGT GGT phosphodiester
Binding Buffer: 10mM NaOH
Detection: UV @ 254nm

Experimental

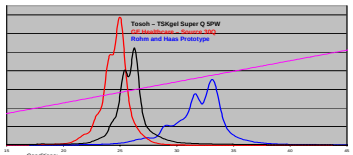
Oligonucleotides
The crude phosphorothioate oligonucleotide was provided by Monomer Sciences Inc. (Huntsville, AL). Two phosphodiester (AAA CCT GAT GTG and GGT GGT GGT GGT) , deprotected oligonucleotides were synthesized at the 10µmol level by the Biotechnology Center at the University of Illinois at Urbana-Champaign. The fourth oligonucleotide is a confidential sequence that was supplied for methods development purposes.

Purification Conditions
All purifications were performed on stainless steel Alltech columns, 4.6mm ID x 10cm L, on an GE Healthcare AKTA Explorer system. The specific purification conditions are listed in the results section.

Analytical Conditions
All analyses were performed on an Agilent 1100 HPLC system under the following conditions:

Column: Dionex NucleoPac PA-100, 4.0mm ID x 250mm L
Injection: 50uL
Temperature: 80°C
Flow Rate: 0.9mL/minute
Mobile Phase: A: 75% 25mM sodium phosphate, pH 7.0/25% acetonitrile
B: 75% 25mM sodium phosphate, pH 7.0 w/ 400mM sodium perchlorate/25% acetonitrile
Gradient: 10 – 65% B in 16.5 minutes
Detection: UV @ 260nm

Figure 2
Purification of Phosphorothioate Oligonucleotide
(G*CTGGTGTCCCGCATGG*A*G*G)

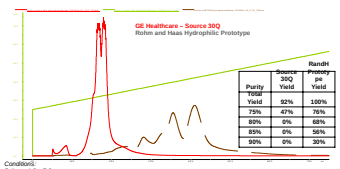


Conditions:
Column 1.0 x 7.0 cm
Linear Gradient: 0 to 100% B in 60 min
Sample: 1mg/mL of 20mer, 0.5mL injection
A: 50mM Tris, pH 8.0
B: 50mM Tris, pH 8.0 + 2M NaCl
Flow rate: 1 mL/min

* Indicates Phosphorothiolate Linkage

Crude Phosphorothioate Provided by Monomer Sciences, Inc.

Figure 3
Purification of Phosphorothioate Oligonucleotide
(G*CTGGTGTCCCGCATGG*A*G*G)



Purity	Source 30Q Yield	RandH Prototype Yield
Yield	92%	100%
75%	47%	76%
80%	0%	68%
85%	0%	56%
90%	0%	30%

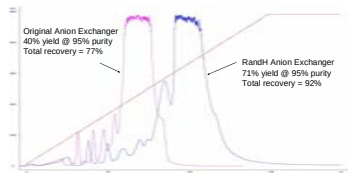
Conditions:
Column 1.0 x 7.0 cm
Linear Gradient: 25 to 75% B in 120 min
Sample: 1mg/mL of 20mer, 4mL injection, 32% purity as measured by AIEC HPLC chromatography
A: 50mM Tris, pH 8.0
B: 50mM Tris, pH 8.0 + 2M NaCl
Flow rate: 1 mL/min

* Indicates Phosphorothiolate Linkage

Purification of Phosphorothioate Oligonucleotide
(G*CTGGTGTCCCGCATGG*A*G*G)

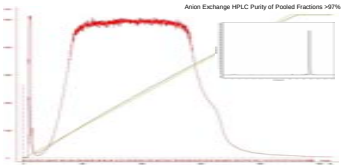
Discussion
A phosphorothioate oligonucleotide with an initial purity of 32% was purified using anion exchange chromatography, as shown in Figures 2 and 3. The Rohm and Haas strong anion exchange prototype demonstrated a much different elution pattern than either of the commercial anion exchange resins, Source 30Q or TSKgel Super Q-5PW (30 micron). Additionally when the yields and purities of the Source 30Q and the anion exchange prototype were compared, as shown in Figure 3, the Rohm and Haas resin demonstrated superior separation capabilities.

Figure 4
Oligonucleotide Purification Comparison



Conditions:
Load: 10g/L (269 OD/mL) on 1.0cm ID x 7.0cm L column, 4mL/min
Buffer A: Tris-HCl
Buffer B: A + 1M NaCl
Gradient: 0 to 100% B in 60 minutes
Flow Rate: 2.5mL/minute

Figure 5
Oligonucleotide Purification - 45g/L Load



Conditions:
Load: 45g/L (1209 OD/mL) on 1.0cm ID x 11.8cm L column, 8mL/min
Buffer A: in Tris-HCL
Buffer B: Tris-HCl
Gradient: A + 1M NaCl
Flow Rate: 0 to 100% B in 180 minutes
2.5mL/minute

Purification of Oligonucleotide

Discussion
Purification of a synthetic oligonucleotide with a confidential sequence and length was developed on the new anion exchange prototype and that purification was compared to the current competitive anion exchange process. A comparison of purifications using the two resins is shown in Figure 4. Under identical conditions, the Rohm and Haas prototype was able to provide improved yields and purities. Additionally, the loading was increased to 45g/L on the anion exchange prototype, as shown in Figure 5. The highest achieved purity was 97% with an influent purity of 75%. The process using the new Rohm and Haas resin had the following benefits over the existing process:

- Simplified process conditions:
  - Ambient vs. elevated temperature
  - No organic used in the process
- Increased throughput - 50% increase in loading level
- Lower pressure drop – 30 micron versus 20 micron particle size.

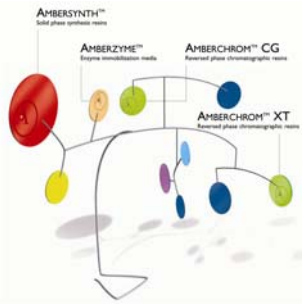
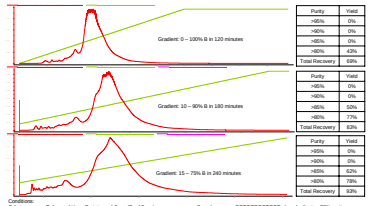


Figure 6
GGT GGT GGT GGT - Purification Development



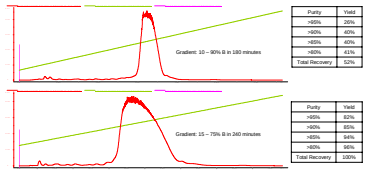
Purity	Yield
>95%	0%
>90%	0%
>85%	0%
>80%	43%
Total Recovery	69%

Purity	Yield
>95%	0%
>90%	0%
>85%	50%
>80%	77%
Total Recovery	83%

Purity	Yield
>95%	0%
>90%	0%
>85%	62%
>80%	78%
Total Recovery	93%

Conditions:
Column: Rohm and Haas Prototype, 4.6mm ID x 10cm L
Buffer A: 50mM Tris-HCl, pH 8.0
Flow Rate: 0.5mL/min (load and elution)
Fractions: 0.5mL
Buffer B: A + 2M NaCl
Gradient: listed above
Detection: UV @ 254nm
Sample: GGTGGTGGTGGT phosphodiester, 78% purity
0.2g/L in 50mM Tris-HCl, pH 8.0

Figure 7
GGT GGT GGT GGT - Purification Development



Purity	Yield
>95%	20%
>90%	40%
>85%	40%
>80%	41%
Total Recovery	52%

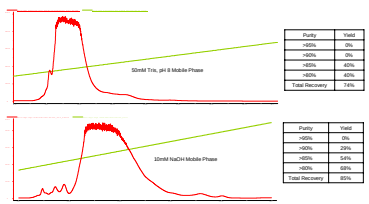
Purity	Yield
>95%	82%
>90%	85%
>85%	94%
>80%	96%
Total Recovery	100%

Conditions:
Column: Rohm and Haas Prototype, 4.6mm ID x 10cm L
Buffer A: 10mM NaOH
Flow Rate: 0.5mL/min (load and elution)
Fractions: 1mL
Sample: GGTGGTGGTGGT phosphodiester, 0.2g/L in 10mM NaOH
Buffer B: A + 2M NaCl
Gradient: listed above
Detection: UV @ 254nm

GGT GGT GGT GGT Purification Development

Discussion
Synthetic oligonucleotides containing a high number of guanidines are typically difficult to purify. Initial purification development was performed using a 50mM Tris mobile phase at pH 8. A linear salt gradient was varied during the course of three purifications, and by increasing the shallowness of the gradient, we did observe some improvements in the yield and purity as demonstrated in Figure 6. However, there was still room for improvement as there were no fractions with purities greater than 90%. Therefore, the mobile phase was modified to 10mM sodium hydroxide, pH 11 and similar salt gradients were employed. As shown in Figure 7, a dramatic improvement in yield and purity was achieved with sodium hydroxide mobile phase.

Figure 8
AAA CCT GAT GTG Purification Development

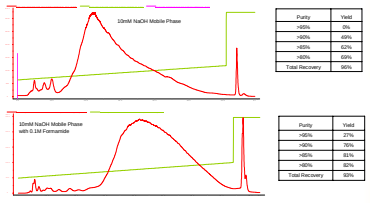


Purity	Yield
>95%	0%
>90%	0%
>85%	40%
>80%	40%
Total Recovery	74%

Purity	Yield
>95%	0%
>90%	29%
>85%	54%
>80%	69%
Total Recovery	85%

Conditions:
Column: Rohm and Haas Prototype, 4.6mm ID x 10cm L
Buffer A: see above
Flow Rate: 0.5mL/min (load and elution)
Fractions: 1mL
Sample: AAA CCT GAT GTG phosphodiester, 0.2g/L in 10mM NaOH
Influent Purity: 58%
Buffer B: A + 2M NaCl
Gradient: 15% to 75% B in 240 minutes
Detection: UV @ 254nm

Figure 9
AAA CCT GAT GTG Purification Development



Purity	Yield
>95%	0%
>90%	49%
>85%	62%
>80%	69%
Total Recovery	96%

Purity	Yield
>95%	27%
>90%	76%
>85%	81%
>80%	82%
Total Recovery	93%

Conditions:
Column: Rohm and Haas Prototype, 4.6mm ID x 10cm L
Buffer A: 10mM NaOH
Flow Rate: 0.5mL/min (load and elution)
Fractions: 1mL
Sample: AAA CCT GAT GTG phosphodiester, 0.2g/L in 10mM NaOH
Influent Purity: 58%
Buffer B: A + 2M NaCl
Gradient: 15% to 45% B in 240 minutes with stop to 65% B
Detection: UV @ 254nm

AAA CCT GAT GTG Purification Development

Discussion
The initial gradient conditions of 15% to 75% B in 240 minutes with a 50mM Tris mobile phase provided unsatisfactory yields and purities. By switching to a sodium hydroxide mobile phase, there were improvements in the yields and purities, as shown in Figure 8. With a 58% influent purity we were not able to achieve purities above 95%. As shown in Figure 9, by using a shallower gradient of 15% to 40% in 240 minutes, the overall recovery and yields at 80%, 85%, and 90% purity improved. However, the yield at >95% purity did not improve. Chaotropic agents such as urea and formamide have been used previously for the reversed phase purification of oligonucleotides. Therefore, 0.1M formamide was added to the mobile phase while keeping the gradient identical to the previous purification. The addition of this chaotrope improved yields at all purity levels, and dramatically increased the yield at the >95% purity.

Conclusions

- A new strong anion exchange resin has been developed for the purification of synthetic oligonucleotides. This resin provides unique selectivity and high capacity for the purification of phosphodiester and phosphorothioate backbone oligonucleotides.
- This new anion exchange resin provided excellent purification results for a high "G" content oligonucleotide.
- Mobile phase conditions have a strong impact on the yields and purities of oligonucleotides. A sodium hydroxide mobile phase provided optimal results.
- The addition of chaotropic agents such as formamide can also improve yields and purities of synthetic oligonucleotides.

References

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