HPLC Purification of mRNA with Reverse Phase and Size Exclusion Chromatography

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Abstract: RNA based therapeutics represent a powerful new tool for gene therapy. In RNA production, impurities such as nucleotide triphosphates, abortive transcripts, and DNA template can affect down-stream applications by triggering immune responses. Gel-based methods of purification, such as preparative denaturing polyacrylamide gel electrophoresis (PAGE) and agarose gels, are limited by poor resolution of RNA size and potential chemical modifications from reagents such as formaldehyde. To date, high performance liquid chromatography (HPLC) remains a staple method for purification of messenger RNA (mRNA) and RNA oligonucleotides. Presented here are two HPLC methods for the purification of RNA, comparing reverse-phase and size exclusion chromatography.

Reverse Phase Methods

COSMOSIL Application Data

Column:	3.5NP-Cholester
Column size:	4.6 x 100 mm
Mobile phase:	A- 100mM TEAA in water
	B- Acetonitrile
Gradient:	0-20% B over 15 min
Flow rate:	0.3 mL/min
Temperature:	75 Celsius
Detection:	uv 260nm
Sample:	mRNA (1450 nt)
Inj. Vol.:	12 uL (100µg/mL)

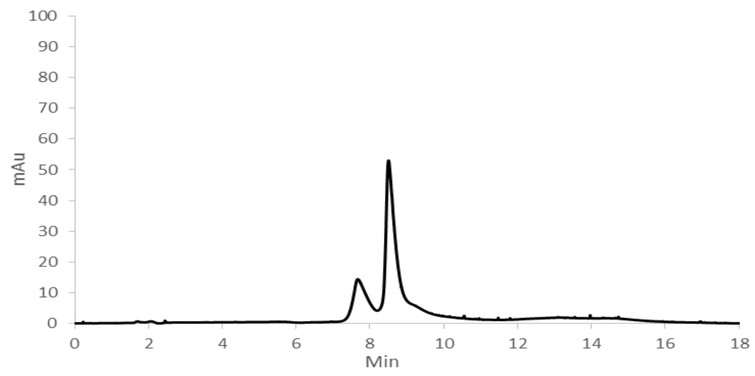


Figure 1: Analysis of 1450 nucleotide (nt) RNA by reverse phase chromatography. The main RNA peak at 8.5 minutes was preceded by an impurity at 7.8 minutes. This impurity results from the incomplete modification of the RNA with a poly-A end-cap. The use of a column with non-porous (NP) particles and Cholesterol as the stationary phase resulted in good separation of the known impurity.

COSMOCORE Application Data

Column:	***
Column size:	2.1x100mm
Mobile phase:	A- 100mM TEAA
	B- 100mM TEAA 75%Acetonitrile
	Grad: 8-12% ACN, 0-20min
Flow rate:	0.5mL/min
Temperature:	60°C
Detection:	UV260nm
Sample:	ds siRNA (20 nt each)
Inj. Vol.:	2.0µL, 25µM

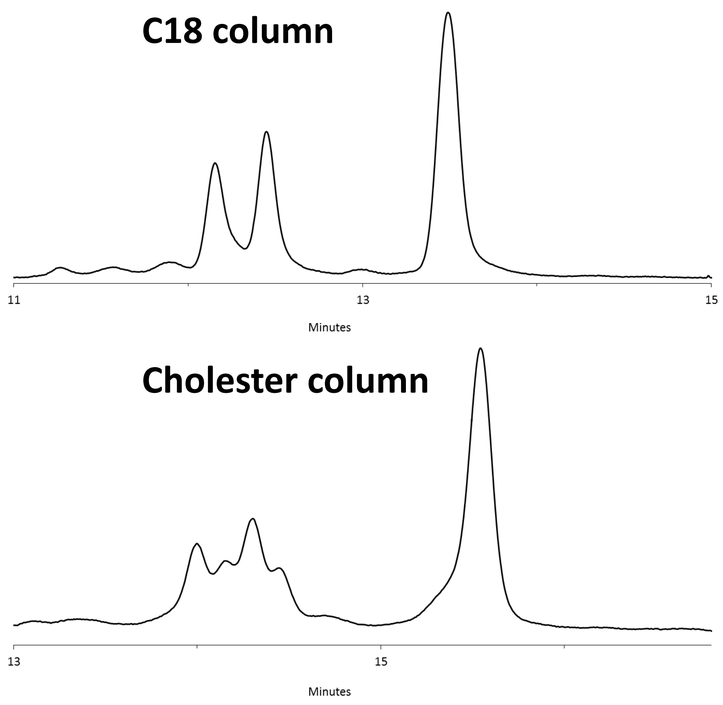


Figure 2: Denaturing HPLC analysis of siRNA with octadecyl (C18) and Cholesterol-based HPLC columns. The siRNA was modified to contain unlocked nucleic acids (UNA), 2'-methoxy, and phosphorothioate bonds. On both columns, the individual strands appear around 12.25 and 12.5 minutes. Interestingly, the C18 column showed only 2 peaks, one for each strand, while the Cholester column exhibited 4 distinct peaks. It may be that the incomplete modification of the siRNA is able to be better detected on a Cholesterol-based stationary phase.

Size Exclusion Methods

COSMOSIL Application Data

Column:	5Diol-1000-II
Column size:	7.5 x 300 mm
Mobile phase:	A- 100mM TEAA in water
	B- Acetonitrile
Flow rate:	0.3 mL/min
Temperature:	60 Celsius
Detection:	uv 260nm

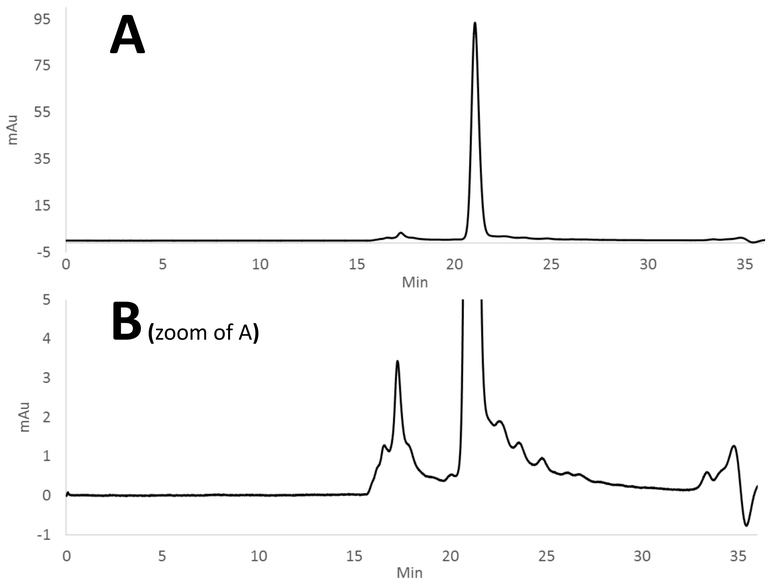
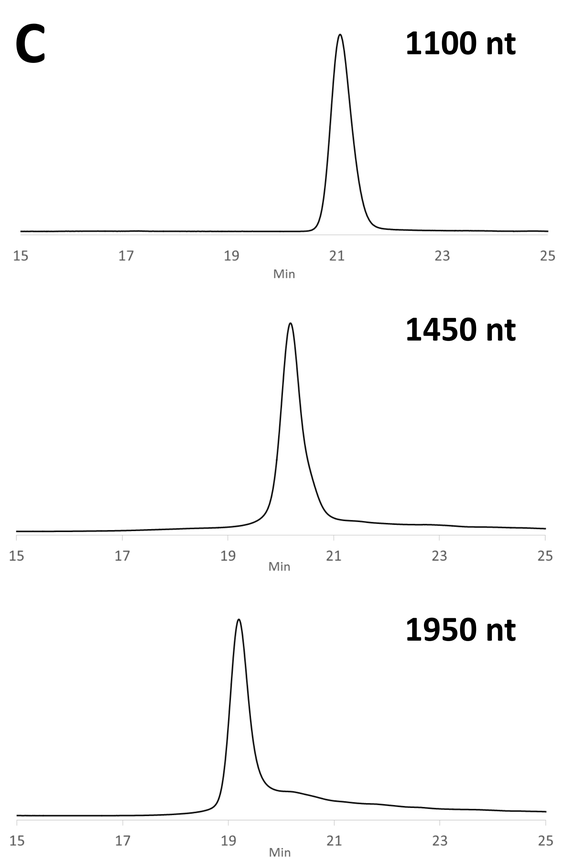
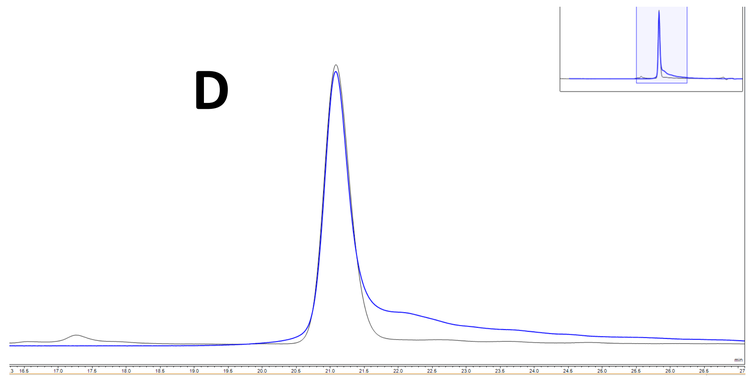


Figure 3: Analysis of mRNA with size exclusion chromatography (SEC). mRNA ranging in size from 1100-1950 nt were purified with a column containing particles with 1000Å pore size. Good peak shape was obtained using a mobile phase of 100mM triethylammonium acetate (TEAA) and acetonitrile (**A**). Higher molecular weight impurities, potentially template DNA were seen before the main peak at 21 minutes, and several smaller mRNA species were seen between 22-25 minutes (**B**). The 3 different sized mRNA tested showed good resolution of 2.03 and 2.36. (**C**). A simulated chromatogram of purified mRNA by HPLC compared to un-purified samples (**D**).





Conclusions: HPLC remains a powerful technique in the purification of RNA impurities. Reverse-phase chromatography was useful in the purification of species with different chemistries, such as incomplete chemical modification. Size exclusion methods were adept at separating degraded or aborted RNA. When comparing size, resolutions greater than 2.0 were seen for RNA species with differences of 300-400 nt.

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