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Note d'application

Coupled IEX Chromatography for Stoichiometric Analysis of CRISPR Ribonucleoprotein Complexes

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Abstract

Recently, the first CRISPR edited cell therapy-based therapeutic was approved for treatment of sickle cell anemia, with other CRISPR-based therapies in the pipeline.[1–5] However, despite the growing market for CRISPR-based therapeutics, technologies for comprehensive quality control analysis of CRISPR components and ribonucleoprotein (RNP) complexes are not well-established. To ensure safe and effective CRISPR drug substances, thorough quality control assessment of CRISPR reagents and characterization of RNP formation is needed. Current methods for characterization of CRISPR/Cas components are limited and do not typically allow for simultaneous characterization of apo-Cas, apo-sgRNA, and RNP species.

Here, we exploit the differing charges of apo-Cas, apo-sgRNA, and RNP to achieve separations of CRISPR components by ion exchange (IEX) high-performance liquid chromatography (HPLC) paired with ultraviolet (UV) detection. In this work, we have used a chromatography system equipped with high performance surfaces to mitigate the loss of metal-sensitive analytes. Anion exchange and cation exchange columns were coupled, allowing for simultaneous retention of Cas9 on the cation exchange column and sgRNA on the anion exchange column.

Benefits

- Coupled cation/anion exchange chromatography can probe charge states of the components in a single method, revealing information about the components not seen in ion pairing reversed-phase or size exclusion chromatography
- The method allows for stoichiometric evaluation of the RNP formation from the individual components

Introduction

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) are part of most prokaryote genomes and contribute to acquired immunity against phages for bacteria and archaea. CRISPR gene editing systems in nature consist of tracrRNA (trans-activating CRISPR-RNA) and crRNA. In research settings, tracrRNA, and crRNA can be combined into a single guide RNA (sgRNA). Cas protein, an endonuclease, forms a ribonucleoprotein (RNP) complex with sgRNA. The RNP complex introduces double stranded breaks in target DNA first by recognizing the target site and cleaving the two strands of DNA, followed by repair.[6]

To ensure success with CRISPR gene editing experiments, it is critical to understand RNP complex formation. To this end, the differing charge states of Cas9 and sgRNA can be leveraged. Under native conditions, Cas9 carries a positive charge, while sgRNA is highly negatively charged; consequently, the RNP complex exhibits an intermediate charge state (Figure 1).[7–8] Therefore, by coupling cation and anion exchange chromatography, Cas9, sgRNA, and RNP complexes can be separated in a single run, allowing for the stoichiometry of the overall mixture to be monitored. In addition, charge variants for Cas9 and sgRNA single components can be characterized. In this work, we utilize a strong cation exchange (SCX) column (BioResolve™ SCX mAb Column) coupled to a strong anion exchange (SAX) column (Protein-Pak™ Hi Res Q Column) for stoichiometric analysis of CRISPR RNP complexes.

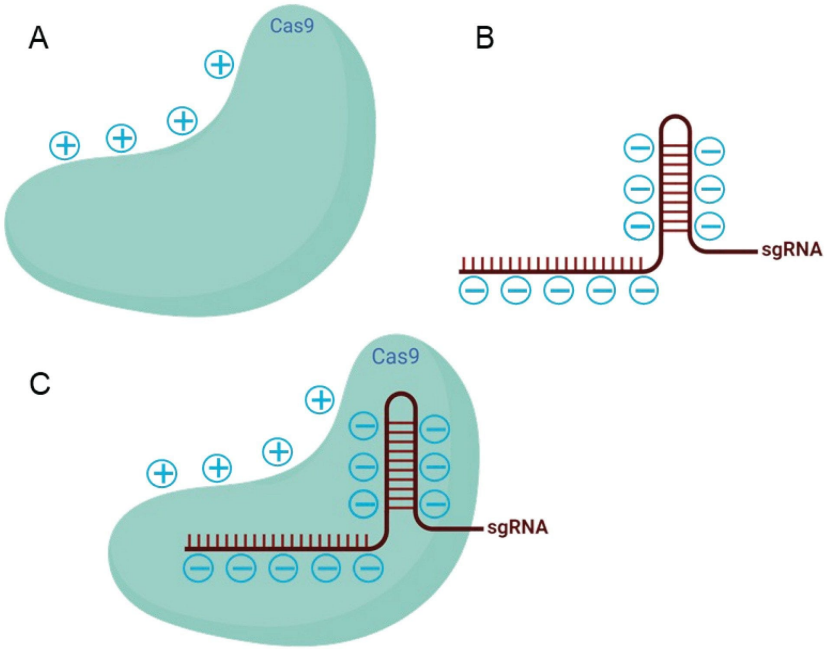

Figure 1. A schematic representation of (A) positively charged apo-Cas9, (B) negatively charged sgRNA, and (C) ribonucleoprotein (RNP) complex. The RNP complex exhibits an intermediate charge state. Coupled SCX and SAX columns retain and separate the unbound apo-Cas9, unbound sgRNA, and RNP complex.

Experimental

Sample Preparation

Single Component sgRNA Analysis

Human HRPT1 sgRNA (GenScript, Piscataway, NJ; 5'-mG* mA* mU* rG rA rU rC rU rC rU rC rA rA rC rU rU rU rA rA rC rG rU rU rU rU rA rG rA rG rC rU rA rG rA rA rA rU rA rG rC rA rA rG rU rU rA rA rA rA rU rA rA rG rG rC rU rA rG rU rC rC rG rU rU rA rU rC rA rA rC rU rU rG rA rA rA rA rA rG rU rG rG rC rA rC rC rG rA rG rU rC rG rG rU rG rC rU* mU* mU* mU-3', m = 2′-O-methyl, * = phosphorothioate) was resuspended in nuclease-free Tris

EDTA buffer (Integrated DNA Technologies, Coralville, Iowa) to prepare a ~1 µg/µL (24.1 µM) solution.

Single Component Cas9 Analysis

Cas9 Nuclease Protein (Horizon Discovery, Cambridge, UK; 61.8 µM in buffered glycerol) was mixed in a 1:1 (v:v) ratio with mobile phase B. This stock solution was diluted using nuclease free buffer to prepare a ~3.7 µg/µL (22.9 µM) Cas9 solution.

Ribonucleoprotein Complex Solutions

Stock solutions of sgRNA and Cas9 were prepared. Stock sgRNA solution was prepared at a concentration of 93 µM by resuspending sgRNA in nuclease free buffer. Stock Cas9 was prepared at a concentration of 30.9 µM by mixing the glycerol buffered solution in a 1:1 (v:v) ratio with mobile phase B. Stock sgRNA was added to stock Cas9 to achieve a slight stoichiometric excess of sgRNA (final concentrations of 24.1 µM sgRNA and 22.9 µM Cas9). Solutions were complexed at room temperature for 30 minutes prior to analysis.

Chromatographic Conditions

LC system:	ACQUITY™ Premier System with a Binary Pump and FTN-SM
Detector:	ACQUITY™ Premier TUV Detector with titanium flow cell (5 mm, p/n: 205000611)
Wavelengths:	260 and 280 nm
Column:	Waters BioResolve SCX mAb, 3 µm 4.6 × 50 mm Column (p/n: 186009058) coupled to Waters Protein-Pak Hi Res Q, 5 µm 4.6 × 100 mm Column (p/n: 186004931) with column union (p/n: 700009524)
Column temperature:	Ambient

Sample temperature:	8 ˚C
Injection:	10 µL for each injection 229 pmol apo-Cas, 241 pmol apo-sgRNA for single component injections For stoichiometric analysis, theoretical 0 pmol apo-Cas, 12 pmol apo-sgRNA, and 229 pmol RNP
Flow rate:	0.24 mL/min
Mobile phase A:	10 mM BIS-TRIS propane, 150 mM NaCl, pH 6.5 in water, filtered through 0.2 µm nylon membrane filters
Mobile phase B:	10 mM BIS-TRIS propane, 1 M NaCl, pH 6.5 in water, filtered through 0.2 µm nylon membrane filters

Gradient Table

Time (min)	% Mobile phase A	% Mobile phase B	Curve
Initial	90	10	*
52.0	0	100	6
52.1	90	10	6
60.0	90	10	6

Results and Discussion

Single components of sgRNA and Cas9 were injected onto the coupled SAX and SCX columns to evaluate

charge variants using a single chromatography method. This method utilizes a BIS-TRIS propane buffered mobile phase and salt gradient. UV data were collected at 260 and 280 nm. As shown in Figure 2, apo-Cas9 protein was retained on the SCX column, and apo-sgRNA was retained on the SAX column. Some charge variants were observed in the apo-Cas9 sample, while significant charge heterogeneity was revealed in the apo-sgRNA sample.

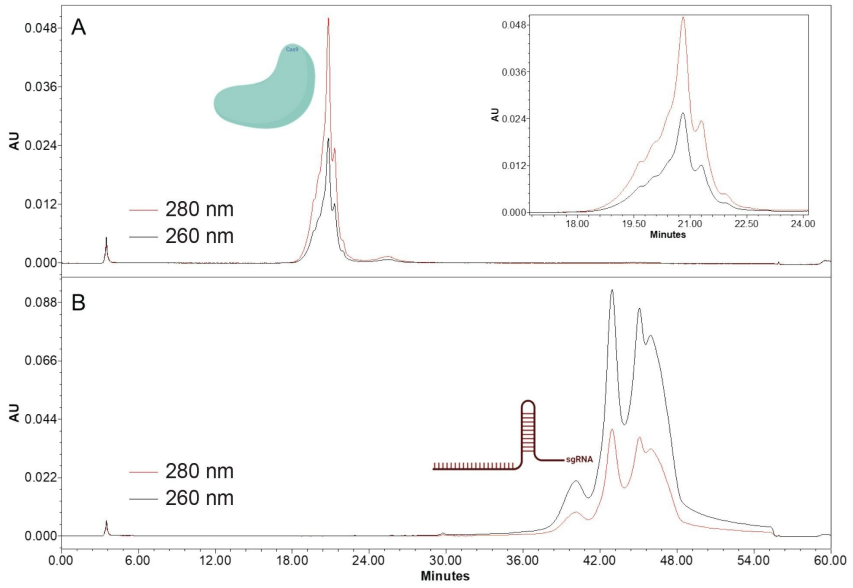

Figure 2. Representative chromatograms for coupled SCX and SAX columns. (A) single component injection of apo-Cas9. Inset: Scaled to show Cas9 charge variants. (B) Single component injection of apo-sgRNA. SAX HPLC reveals significant charge heterogeneity.

After apo-Cas9 and apo-sgRNA were analyzed as single components, the two components were complexed at approximately a 1:1 molar ratio with a slight excess of sgRNA. The resulting solution was analyzed using the coupled columns (Figure 3). A sharp, homogenous peak was present at approximately 12 minutes, indicating RNP complexation. The peak corresponding to apo-Cas9 at approximately 20 minutes was not detected, indicating complete complexation of Cas9. The heterogeneous apo-sgRNA eluting from approximately 40 to 48 minutes was not completely consumed, as is expected given the slight excess of sgRNA in solution.

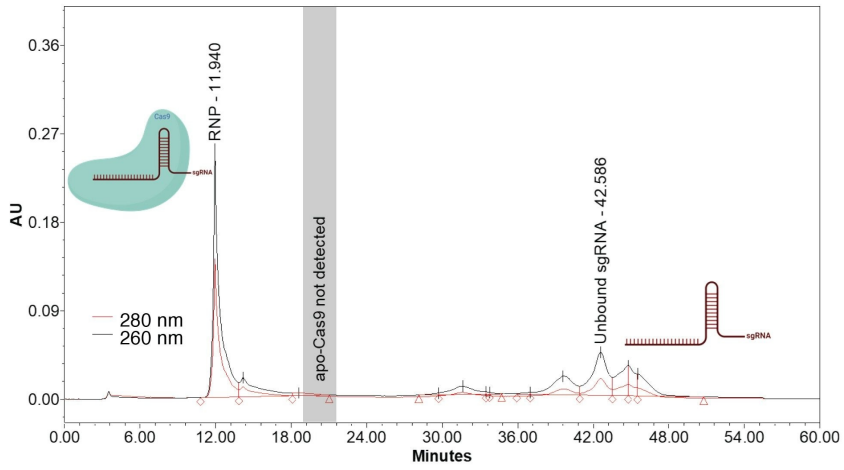

Figure 3. After incubation of apo-Cas9 with sgRNA, Cas9 is fully complexed with a stoichiometric excess of unbound sgRNA remaining. Complexation was monitored at both 260 (black) and 280 nm (red).

As an orthogonal separation method to both ion pairing reversed phase chromatography and size exclusion chromatography, native IEX chromatography highlights charge heterogeneity unseen by other methods.[9,10] Separately, cation exchange and anion exchange can be used to evaluate CRISPR components from a quality perspective. When coupled, the two column chemistries can serve as an all-in-one method for an analysis of CRISPR components and RNPs that gives valuable insights into RNP complexation efficiency and optimization of CRISPR reagent ratios.

Conclusion

This application note demonstrates the capability of coupled SAX/SCX-HPLC to serve as an all-in-one method to evaluate charge heterogeneity in individual CRISPR components and perform stoichiometric analysis of RNP formation. This will be a versatile technique for a variety of applications, including evaluation of critical quality attributes of CRISPR-based therapeutics and optimization of CRISPR reagents.

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