Understanding CRISPR RNA Secondary Structure Impact on Ribonucleoprotein (RNP) Behavior by SEC-PAGE

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OBJECTIVE

- Understand RNP complexation based on secondary structure of long CRISPR guides

INTRODUCTION

- CRISPR guide RNA (gRNA) complexes with Cas proteins to form non-covalent ribonucleoproteins (RNP) that are essential for targeted genome editing
- It is critical to understand the structure-function relationship of these RNPs (e.g., the impact of structure on editing efficiency)

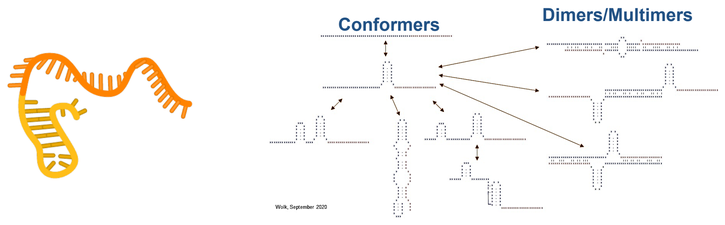


Figure 1. Simple Vs Real View of Long gRNA

- The additional secondary structure observed with longer guides appears to impact complexation of RNPs used in CRISPR application
- Herein we describe the impact of guide secondary structure on RNP formation using size-exclusion chromatography (SEC) and gels

METHOD PARAMETERS

Size-Exclusion Chromatography (SEC) Parameters

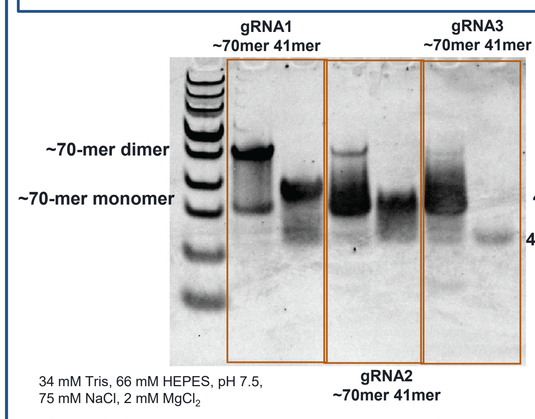
- Column: XBridge BEH200Å SEC 3.5µm 7.8 x 300mm
- Buffer: 100mM Sodium Phosphate pH 7.4 150mM NaCl
- Flow rate = 1mL/min ; Detection at 280nm
- Fractionation parameters
  - Start time: 5 min; End time: 12 min
  - Collection by time: 30 sec; Delay Time: 5 sec

Gel Parameters

- RNA gels are 10% and RNP gels are 4% PAC
- Running buffer composition: 340mM Tris, 660mM HEPES, 75mM NaCl, 2mM $MgCl_2$
- RNP Gel: Run gel at 85V for ~45 min on ice
- OLI Gel: Run gel at 85V for ~60 min on ice
- Sample prep: ~10% glycerol added to all fractionated samples prior to loading

RESULTS

Dimerization of gRNA is Sequence-Dependent



guide	41mer	~70mer
gRNA1	dimer	dimer
gRNA2	dimer	monomer
gRNA3	monomer	monomer

Figure 2. Impact on secondary structure based on sequence dependence of same length guides

- Both the 41mer and ~70mer guides from each set belong to the same target
- gRNA1 is predominantly a dimer
- gRNA2 is a dimer as 41mer and monomer as ~70mer
- gRNA3 is predominantly a monomer
- Target sequence affects the range of secondary structures observed

RESULTS

Impact of gRNA Multimers on Corresponding RNPs by SEC

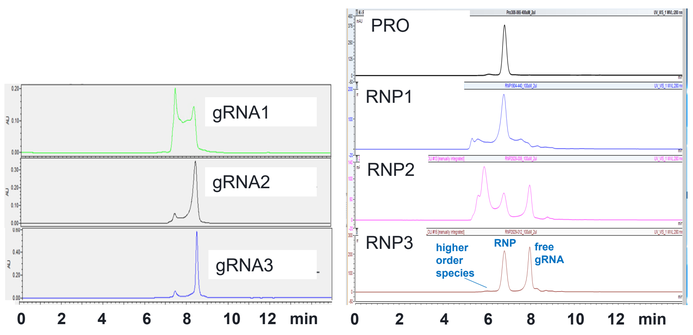


Figure 3. Impact of same length gRNA secondary structure on RNP complexation

- Correlation between SEC and gel-shift data for guides observed
- gRNA1 is mostly dimer as ~70mer whereas gRNA2 and gRNA3 are predominantly monomer
- RNPs from same length guides have different separation profile on SEC

SEC Fractionation of RNPs

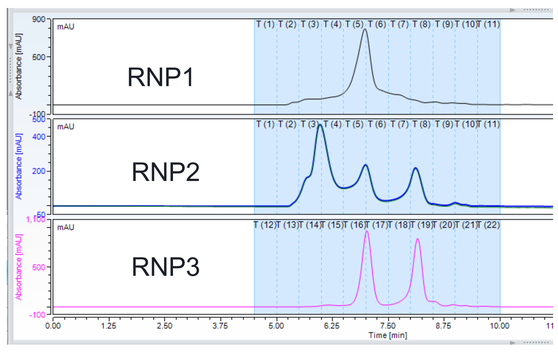
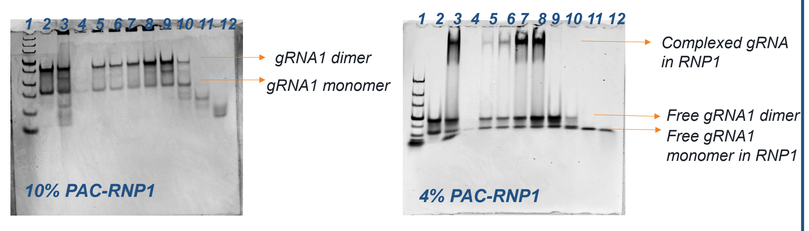


Figure 4. Fractionation of RNPs at regular time intervals to access the individual peaks by gel

- To understand the behavior of RNP formation, SEC fractionation with peak collection was performed on these three ~70mer guides
- 10% PAC gels inform us about the OLI behavior in these fractions within the RNP
- 4% PAC gels inform us about the amount of gRNA complexed within the RNP

4% and 10% PAC gels for RNP1

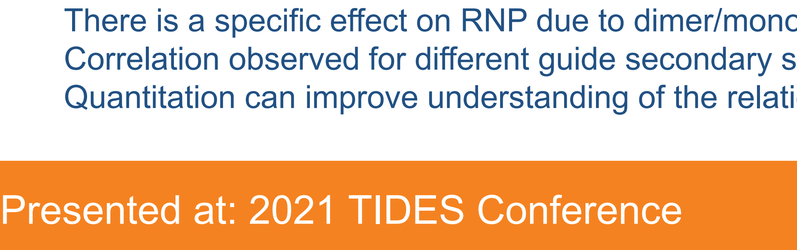


- Lane 1: LMW DNA ladder
- Lane 2: gRNA1 (unfractionated)
- Lane 3: RNP1 (unfractionated)
- Lane 4-12:SEC-Fractions of RNP1 (F2-F10)

Figure 5. Fractionated samples of RNP1 on 4% and 10% PAC gel

- 10% gel indicates gRNA1 dimer/monomer distribution in F3-F8 (Lane 5-10)
- 4% gel fractions F5-F6 (Lane 7-8) indicate RNP1 - the single main peak in SEC
- Protein cannot run into gel due to its isoelectric point (pI)

4% and 10% PAC gels for RNP2



- Lane 1: LMW DNA ladder
- Lane 2: gRNA2 (unfractionated)
- Lane 3: RNP2 (unfractionated)
- Lane 4-12:SEC-Fractions of RNP2 (F2-F10)

Figure 6. Fractionated samples of RNP2 on 4% and 10% PAC gel

- 10% gel indicates gRNA2 monomer distribution mainly in F7-F8 (Lane 9-10)-third peak in SEC
- 4% gel fractions F3-F6 (Lane 5-8) indicate RNP2 - the second peak in SEC
- The first peak might be protein aggregation/some higher order structures

4% and 10% PAC gels for RNP3

- Lane 1: LMW DNA ladder
- Lane 2: gRNA3 (unfractionated)
- Lane 3: RNP3 (unfractionated)
- Lane 4-12:SEC-Fractions of RNP3 (F2-F10)

Figure 7. Fractionated samples of RNP3 on 4% and 10% PAC gel

- 10% gel indicates gRNA3 monomer distribution
- F7-F9 (Lane 9-11) show predominantly gRNA3 peak in 4% gel
- 4% gel fractions F5-F6 (Lane7-8) indicate RNP3 - the first peak in SEC

RNP Formation: A Less Simple View

- RNP formation is impacted by both guide and protein secondary structures
- Protein truncation and aggregation can complicate RNP formation
- Multiple stoichiometries of RNP can be formed based on the nature of guide and protein

CONCLUSIONS

- Longer guides in CRISPR application tend to show enhanced editing efficiency and seem to show an impact of guide secondary structure on RNP complexation
- There is a specific effect on RNP due to dimer/monomer distribution seen in these gRNAs
- Correlation observed for different guide secondary structures between SEC and gels
- Quantitation can improve understanding of the relationship of secondary structure and RNP complexation

DISCLOSURES

Pranjali Ghude is an Employee and a stock holder of Editas Medicine, Inc.

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