Characterization of Ribonucleoproteins by Intrinsic Fluorescence and Ion-Exchange Chromatography

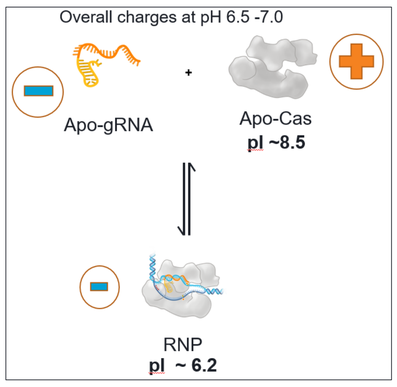
Bryant Chica[1], Jean-Noel Lemercier [1], Pranjali Ghude[1], Chrysa Latrick[1], Bridget Moffet[1], Steven Wolk[1]

[1]Analytical Chemistry Department, Editas Medicine, Boulder, CO, USA.

INTRODUCTION

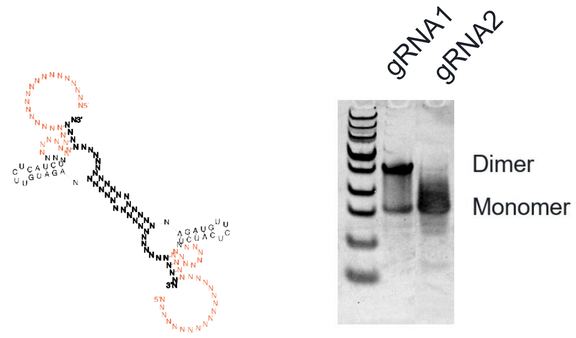
- CRISPR gene editing for therapeutic applications is carried out by ribonucleoproteins (RNPs) consisting of Cas protein and guide RNA (gRNA). We present combined methods of analysis for characterization of RNPs and examination of the effect of gRNA secondary structure on RNP complex formation. A two-pronged approach is used.
- Combined weak cation and weak anion exchange chromatography separate and resolve gRNA, Cas12a protein and RNPs based on their differing charge states in solution.
- Further, an intrinsic fluorescence method is developed that exploits the sensitivity of aromatic amino acid residues to their local environment to probe the specific binding of gRNA to Cas12a. This enables the label free interrogation of RNP complexation free from external perturbations and in various conditions, including those relevant to therapeutic RNP formulations. These combined methodologies can be used to interrogate the effect of gRNA secondary structural characteristics on RNP complexation

Ribonucleoprotein (RNP) Complex Formation



mode	gRNA	RNP	Cas
WCX	void	in between	retain
WAX	retain	in between	void*
WCX-WAX	retain	retain	retain

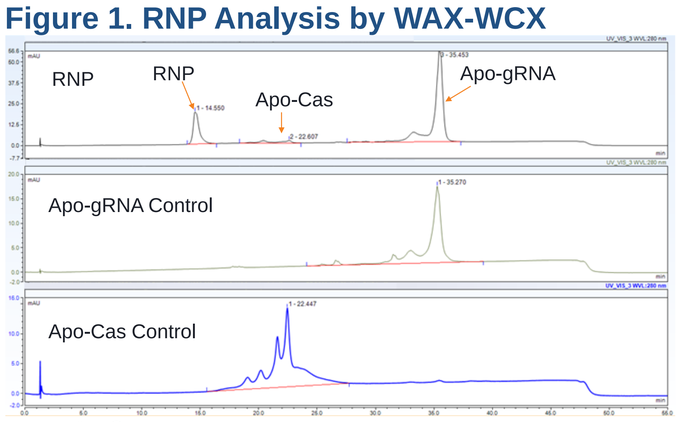
Potential for Secondary Structure Formation in Cas12a gRNA



- gRNAs for Cas nucleases have potential to form secondary structure in a sequence dependent manner.
- Above is shown the predicted dimeric secondary structure for gRNA1 (~70mer), the presence of which is confirmed by native gel EMSA
- See poster by Chrysa Latrick for more details on effect of secondary structure on RNP behavior

RESULTS

Figure 1. RNP Analysis by WAX-WCX



All 3 species are retained and resolved

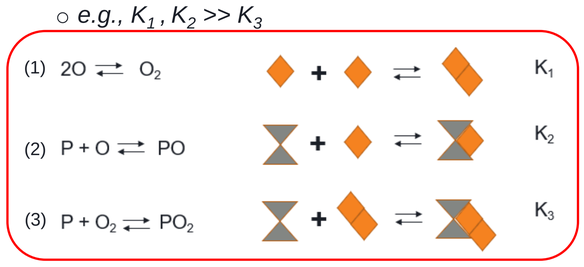
Method Parameters

- Columns: Agilent BioWAX 3 µm, 4.6 x 50 mm
  Agilent BioWCX 3 µm, 4.6 x 50 mm
- Column Temperature = 25 °C Flow Rate = 0.5 mL/min
  Detection Wavelength: 280 nm
- MP A = 10 mM HEPES, 150 mM NaCl, pH 6.5
- MP B = 10 mM HEPES, 1 M NaCl, pH 6.5
- Gradient: 10 %B to 100%B in 42 min

Figure 2. Modeling % Uncomplexed Cas12a vs gRNA:Cas12a Ratio

Possible Explanations:

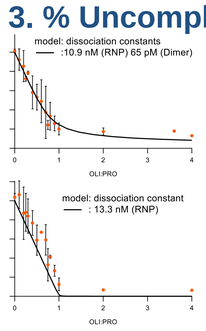
- kinetically driven
  - *on-column dissociation* $k_{-3} > k_{-2}$
- thermodynamically driven
  - *e.g.*, K_1 , $K_2 >> K_3$



Initial kinetic modeling:

- Modeling done through numerical integration of above kinetic equations (eqs (1) and (2)) and non-linear least squares fitting to data
- Formation of a strong dimer can partially account for plateau formation but requires very low dimer k_D
- Formation of plateau for monomeric gRNA 2 suggests additional effects must be modeled to account for observed results (e.g. eq. (3), on-column dissociation, incomplete sample equilibration etc.)

Figure 3. % Uncomplexed Cas12a vs gRNA:Cas Ratio

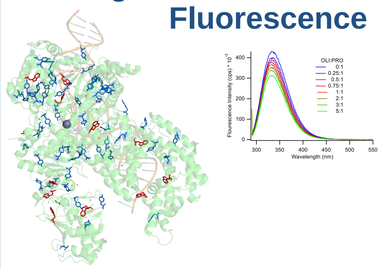


~20%

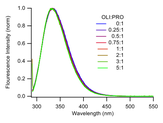
~6%

Different plateaus observed at gRNA:Cas >1 for gRNA1 (Dimer, ~70mer), top vs. gRNA2 (monomer, ~70mer) bottom

Figure 5. Intrinsic Protein Fluorescence



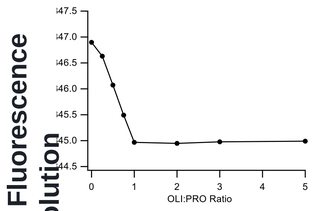
- Cas12a contains many fluorescent residues (11 Tryptophans & 56 Tyrosines)



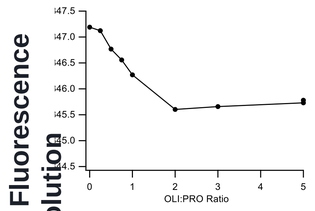
- local electronic environments for some of these residues change upon gRNA binding, modifying the fluorescence spectrum

Spectral changes upon titrating Cas12a with gRNA3 (monomer ~40mer

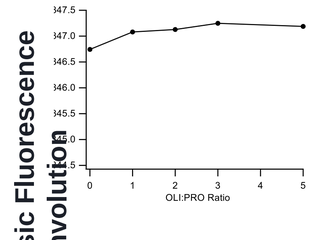
Figure 5. Deconvolution of Fluorescence Spectra for gRNA Complexation with Cas12a



gRNA3 (~40mer)
Monomer



gRNA1 (~70mer)
Dimer



RNA4 (21mer) (non-specific binding control sequence)

Intrinsic Protein Fluorescence Spectroscopy:

- 400 nM Cas12a was excited at 285 nM and fluorescence spectra were deconvoluted to extract the average change in wavelength of fluorescence spectrum
- Fluorescence spectra show specific response to OLI biding with an intact direct repeat sequence (pseudoknot) while a sequence with a disrupted pseudoknot (RNA4) does not give characteristic response of binding of competent gRNA

CONCLUSIONS

- We here describe the development of ion-exchange chromatography and intrinsic fluorescence analytical methods to help characterize RNP complexation
- The amount of uncomplexed protein reached different plateaus for monomeric vs. dimeric apo-gRNAs at gRNA:Cas ratios ≥ 1. Kinetic modeling suggests that dimerization influences the level of the plateau, but may not fully account for the observed plateau behavior
- Intrinsic protein fluorescence spectroscopy can probe gRNA complexation in a label free, manner and show distinctions between monomeric and dimeric gRNAs
- Understanding the relationship between the structure and function of these non-covalent RNP complexes is key to optimizing the cell editing process as well as characterizing these compounds as therapeutics

DISCLOSURES

Bryant Chica is an employee and a stock holder of Editas Medicine, Inc.

Acknowledgments:
Process Chemistry Team at Editas Boulder
Jamaica Siwak
Pallavi Gambhire

Presented at: 2022 Genome Engineering: CRISPR Frontiers