Characterization of gRNAs and Ribonucleoproteins for CRISPR Applications

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Editas Medicine

Gene Therapy Analytical Development Europe 2022

Disclosures

- Steven Wolk is an employee and shareholder of Editas Medicine, Inc.

Outline

Introduction to CRISPR

- Brief Introduction to CRISPR
  - gRNAs and RNPs

Characterization of Secondary Structure and Multimer Formation

- gRNAs
  - methods for characterizing intramolecular folding and multimer formation
  - secondary structure predictions, PAGE, SEC, Ion Mobility

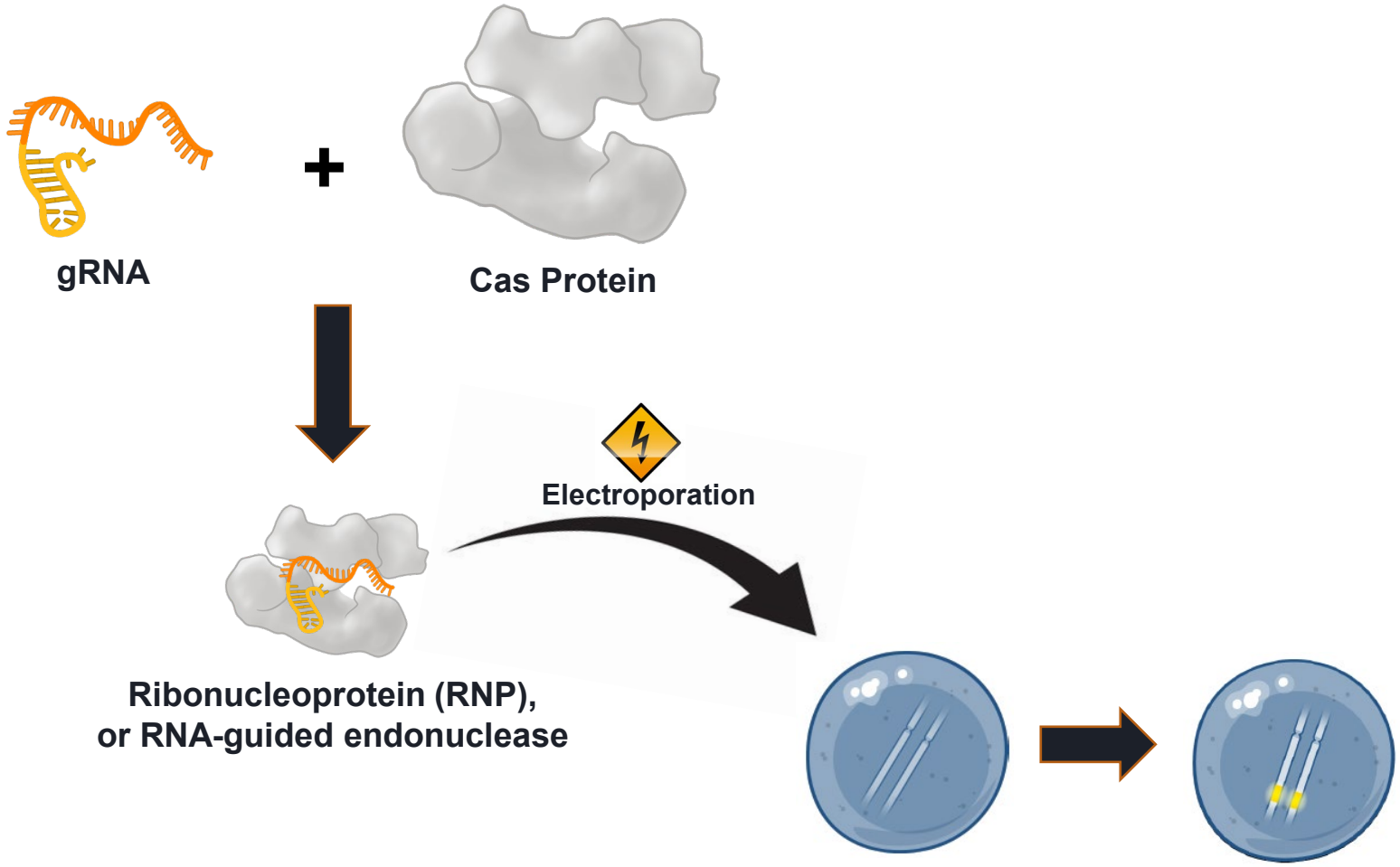
- CRISPR enzyme complex (RNP)
  - impact of these structures on complex formation
  - PAGE, SEC, Ion Mobility, IEX, and Intrinsic Fluorescence

Concluding Remarks and Acknowledgements

CRISPR Basics

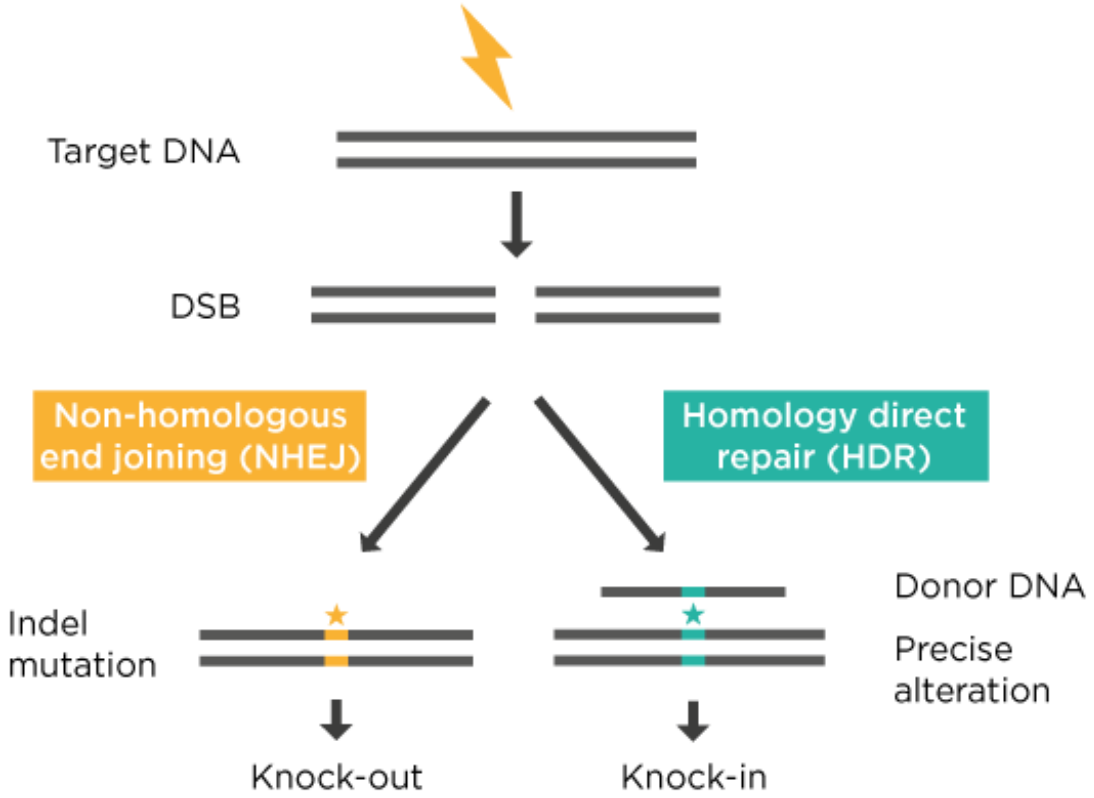
Subtitle

RNP: The Simple View of a Cell Based Medicine



CRISPR Editing

Knock-outs and Knock-ins

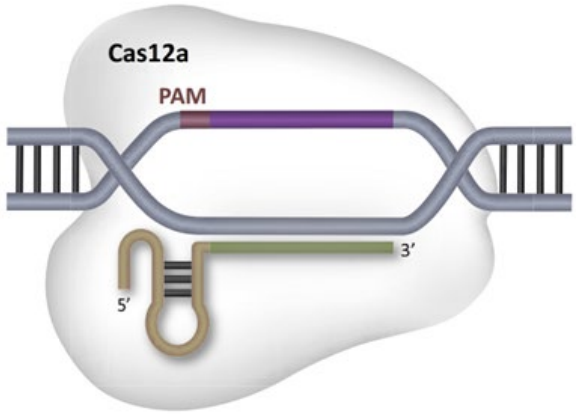


Cox et al. (2015) Nature Medicine 21(2): 121-131

Advantages of AsCas12a gRNA

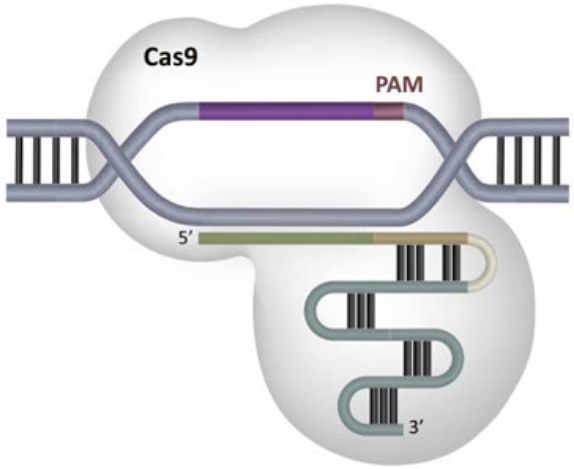
Oligos are synthesized stepwise 3'→5'

Cas12a



- Shorter 41mer gRNA (higher purity and yield)
  - e.g., $(0.99)^{40}$ = 67% crude yield
- Targeting sequence on the 3'-terminus of molecule
  - 3'-terminus: beginning of synthesis cycle → highest fidelity
- Optional short proprietary 5' DNA extensions increase activity of editing in certain difficult to edit cell types

Cas9



- Longer 100mer gRNA (lower purity & yield)
  - e.g., $(0.99)^{99}$ = 37% crude yield
  - more impurities, harder to separate chromatographically
  - pegRNAs (for prime editing based on Cas9 sgRNA) are even longer (~140mers)
- Targeting sequence on the 5'-terminus of molecule
  - 5' terminus: end of synthesis cycle → lowest fidelity

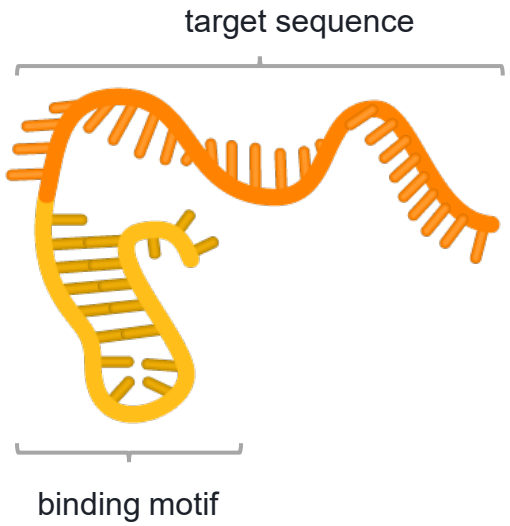
Images adapted from Moon et al. (2019) Trends in Biotechnology 37(8): 870-881.

gRNA Characterization

It’s complicated…….

RNA Secondary Structure

- This is how we like to think the gRNA looks:
- Which it does in the RNP crystal structures



RNA Secondary Structure

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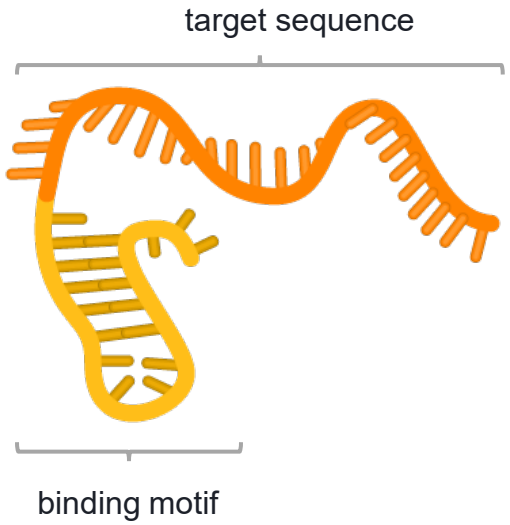
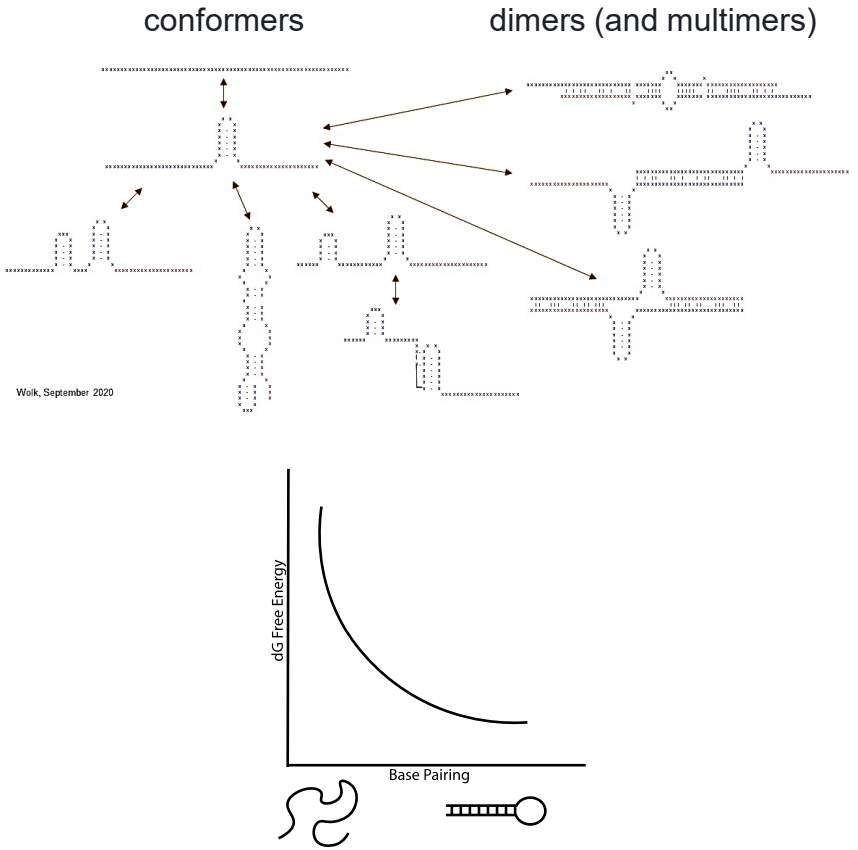


- The situation is likely much more complicated
- Gets more complicated with increasing length



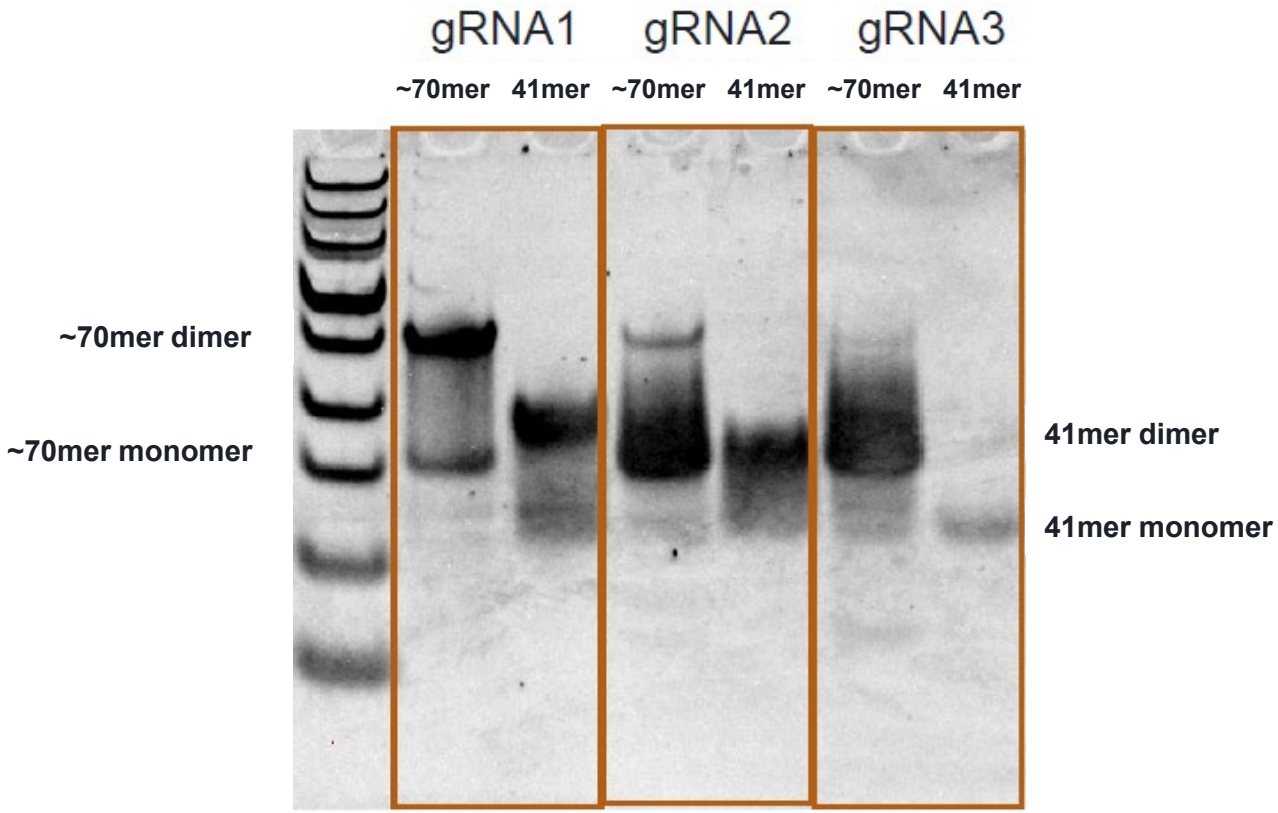
gRNA Characterization

Characterization Platform

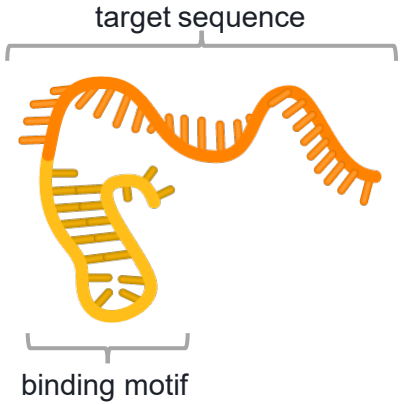
- Purity/Impurity Profile by LC/MS
  - Industry std reverse-phase, ion pairing chromatography coupled to electrospray ionization mass spectrometry (RPIP-LCMS)
  - not addressed today
- Conformational Analysis
  - secondary structure predictions
  - PAGE
  - SEC
  - ion mobility

Sequence Dependent gRNA Properties: Page Analysis

PAGE analysis of three guides that differ in the target sequence region (34 mM Tris, 66 mM HEPES, 75 mM NaCl, 2 mM $MgCl_2$, pH 7.5)

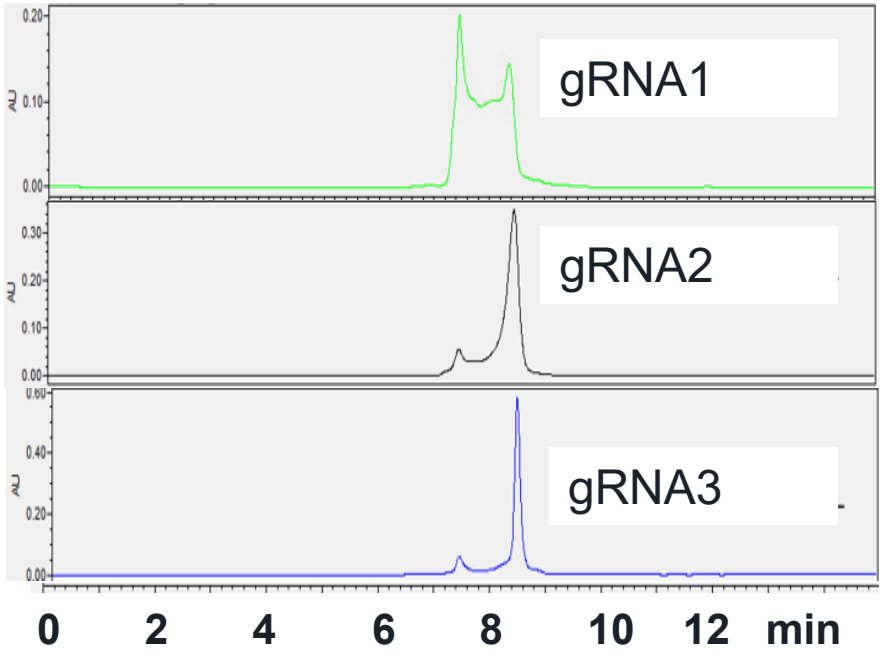


guide	41mer	~70mer
gRNA1	dimer	dimer
gRNA2	dimer	monomer
gRNA3	monomer	monomer



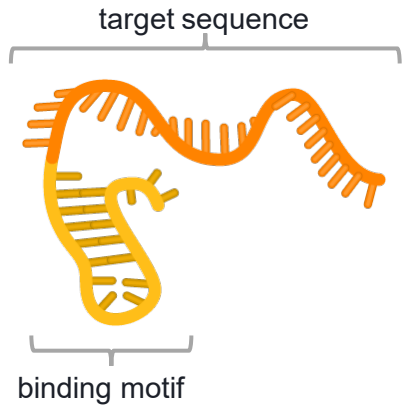
Sequence Dependent gRNA Properties: SEC Analysis

SEC analysis of three guides (~70mer versions) that differ in the target sequence region (Waters UPLC-SEC column, 150 mM NaCl, 100 Na-Phos, pH 7.4)

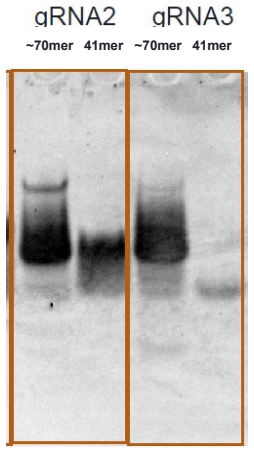


PAGE results for comparison:

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



Sequence Dependent gRNA Properties: IM Analysis



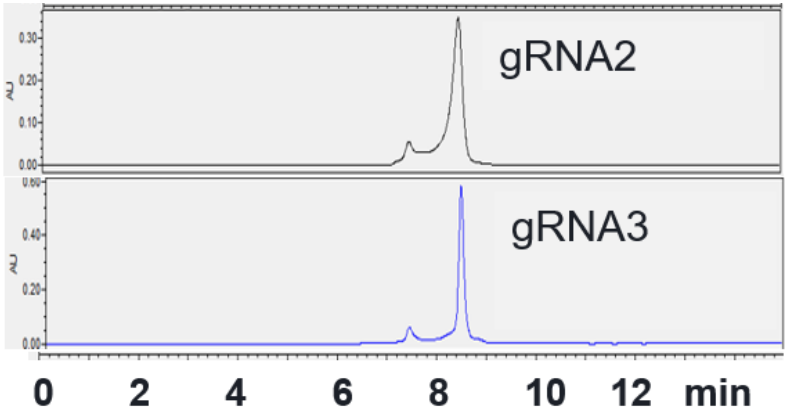
~70mer dimer

~70mer monomer

41mer dimer

41mer monomer

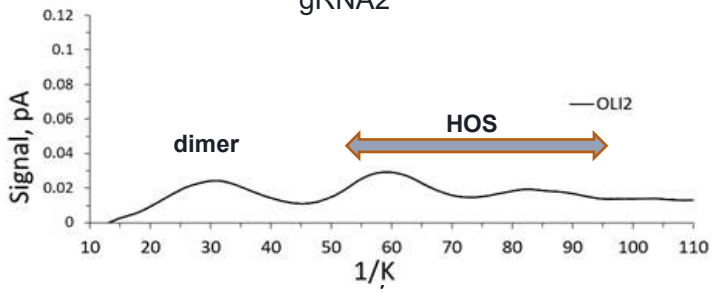
34 mM Tris, 66 mM HEPES, 75 mM NaCl, 2 mM $MgCl_2$, pH 7.5



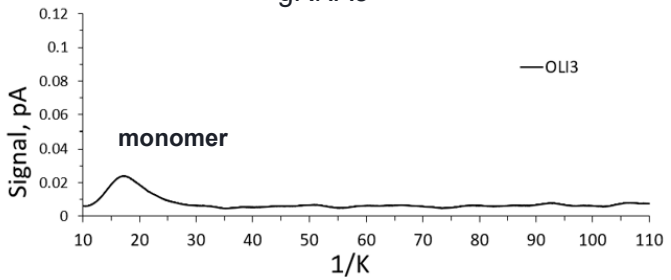
150 mM NaCl, 100 Na-Phos, pH 7.4

Ion Mobility Data (~70mers), 5 mM $(NH_4)OAc$, pH 7.4 [IonDX Imgenius]

gRNA2



gRNA3

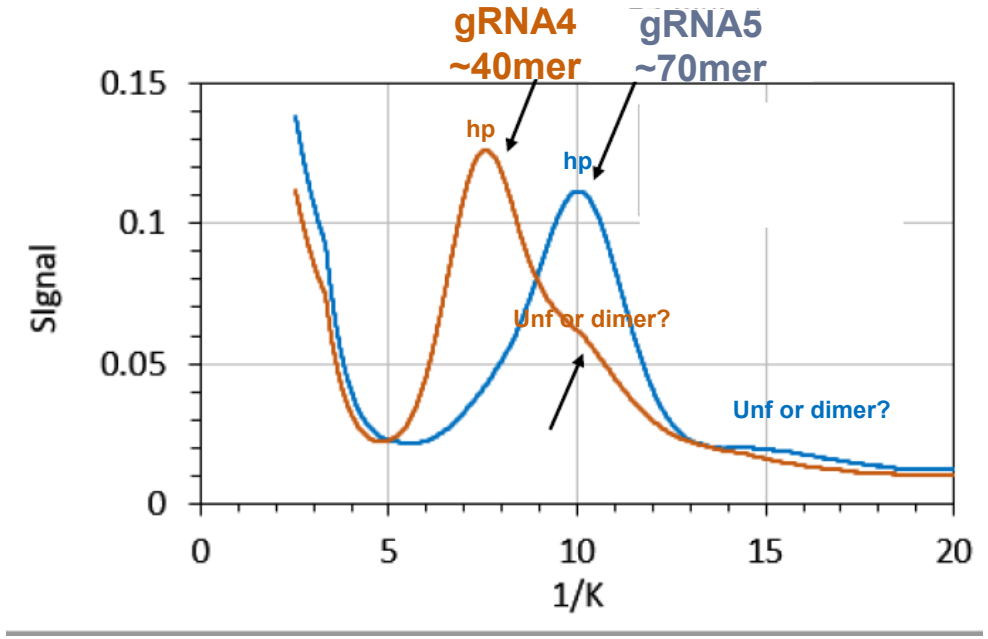


1/K ∝ collisional cross-section (size)

Sequence Dependent gRNA Properties: IM Analysis

Detection of gRNA Folding via IM

Two additional gRNAs (40mer and ~70mer)
- 25 mM $(NH_4)_2CO_3$, pH 8.5
- earlier IM hardware parameters (resulting in different 1/K values)



1/K ∝ collisional cross-section (size)

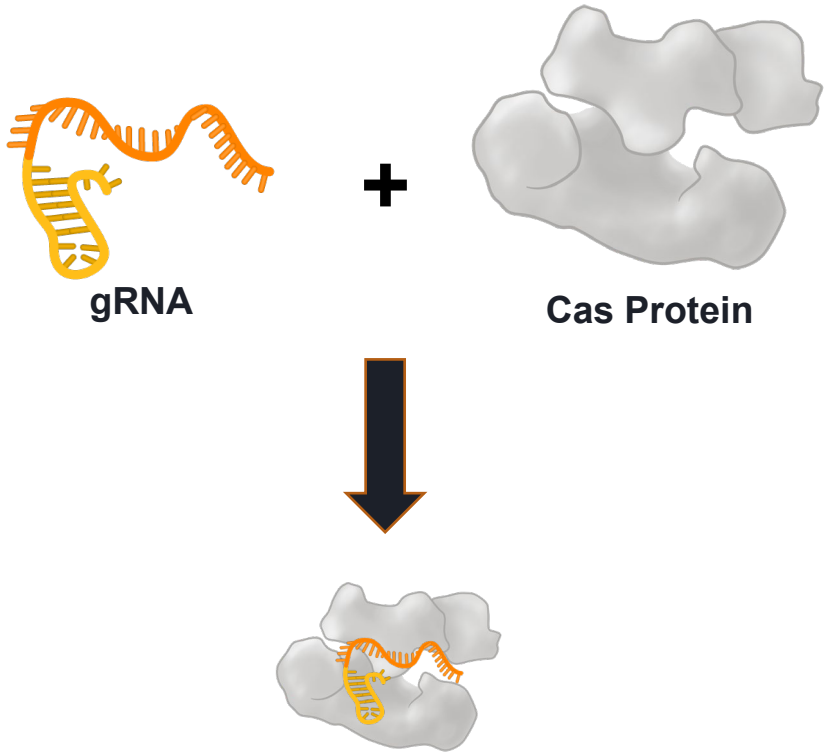
Summary of gRNA Data

guide	Secondary structure prediction	PAGE	SEC	IM
	Vienna RNA-fold	75 mM NaCl 34 mM Tris 66 mM HEPES 2 mM $MgCl_2$ pH 7.5	150 mM NaCl 100 Na-Phos pH 7.4	5 mM $(NH_4)OAc$ pH 7.4
gRNA2	prone to multimer formation	mostly monomer	mostly monomer (exchange observed)	all HOS
gRNA3	not prone to multimer formation	monomer	mostly monomer	monomer

RNP Characterization

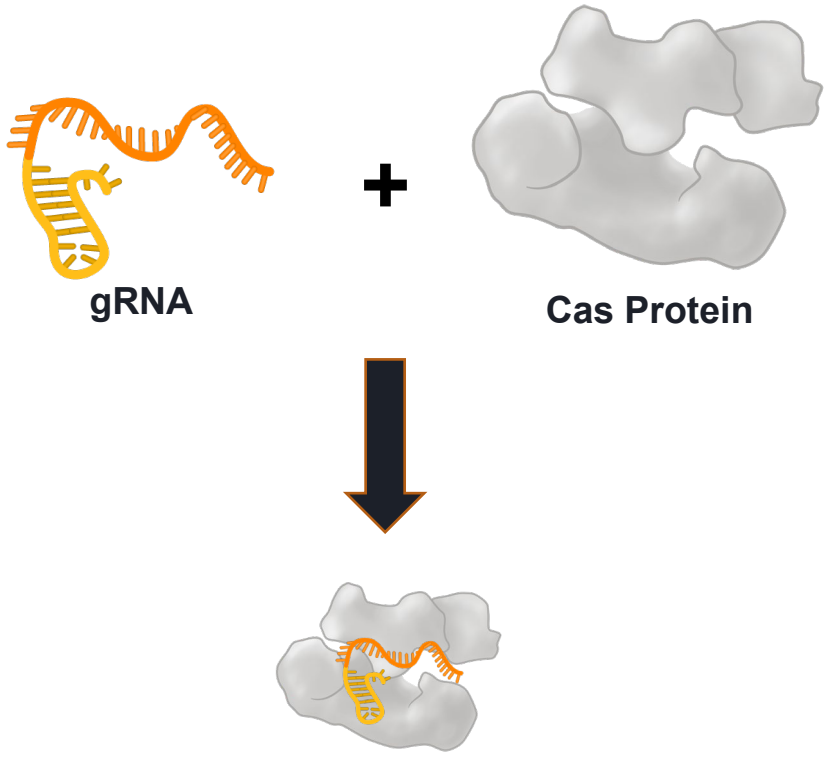
It’s more complicated…….

RNP: The Simple View



RNP: The Less Simple View

Impact of Conformers and multimers

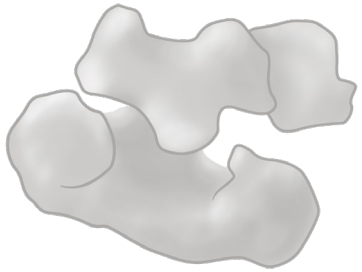


RNP: The Less, Less Simple View

Impact of Conformers and multimers

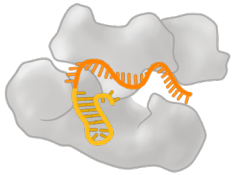
gRNA

+



Cas Protein

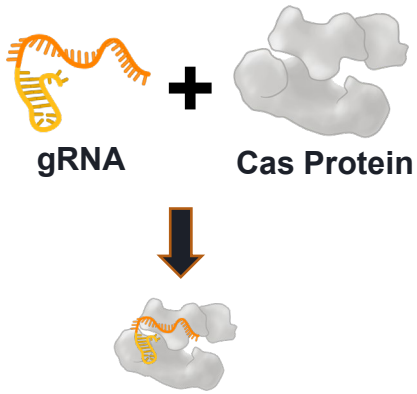
- truncates
- PTMs
- disulfide variants
- aggregates (soluble/insoluble)
- degradants (oxi, deamidation)



Ribonucleoprotein (RNP), or RNA-guided endonuclease

- multiple stoichiometries
- aggregates (soluble/insoluble)
- dynamics/exchange

Characterization of RNPs



Ribonucleoprotein (RNP), or RNA-guided endonuclease

Complexities of Analyzing a Noncovalent Complex of Large Biomolecules

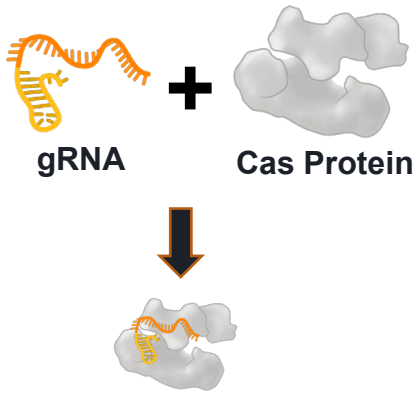
Chromatographic Methods

- well established
- more robust/easier to validate/transfer
- more perturbing to eq.

Spectroscopic Methods

- less routine
- less robust/harder to validate
- often more expensive hardware
- less perturbing to eq.

Characterization of RNPs



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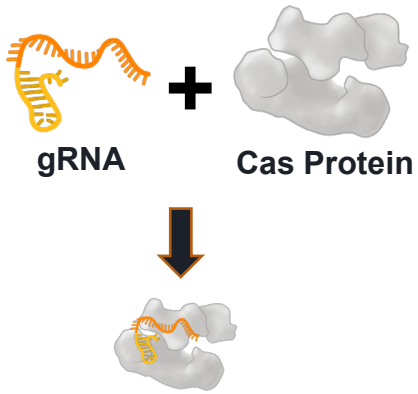
Spectroscopic Methods
- less routine
- less robust/harder to validate
- often more expensive hardware
- less perturbing to eq.

QC/Release ⟷ Characterization/Confirmation (orthogonal)

Characterization of RNPs – SEC Analysis

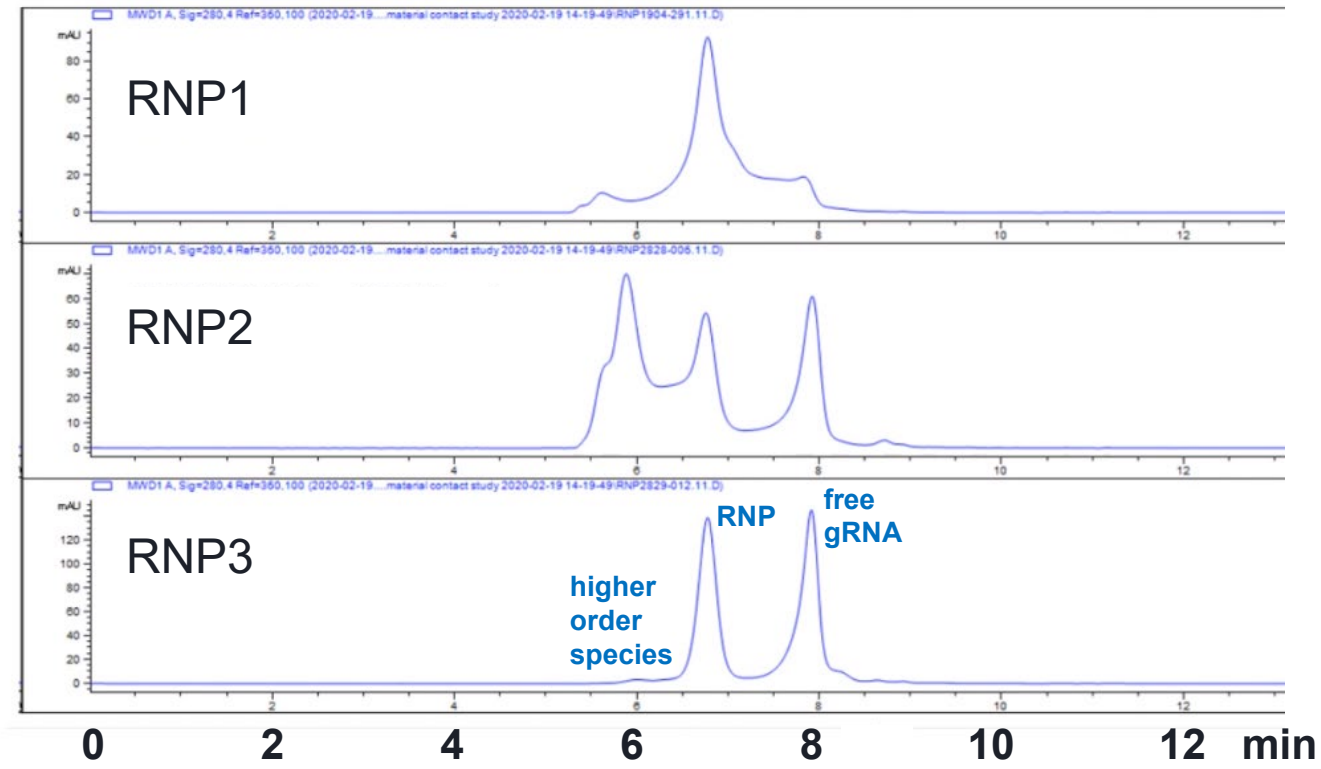


SEC of 3 RNPs

The only difference is the target sequence within the gRNA

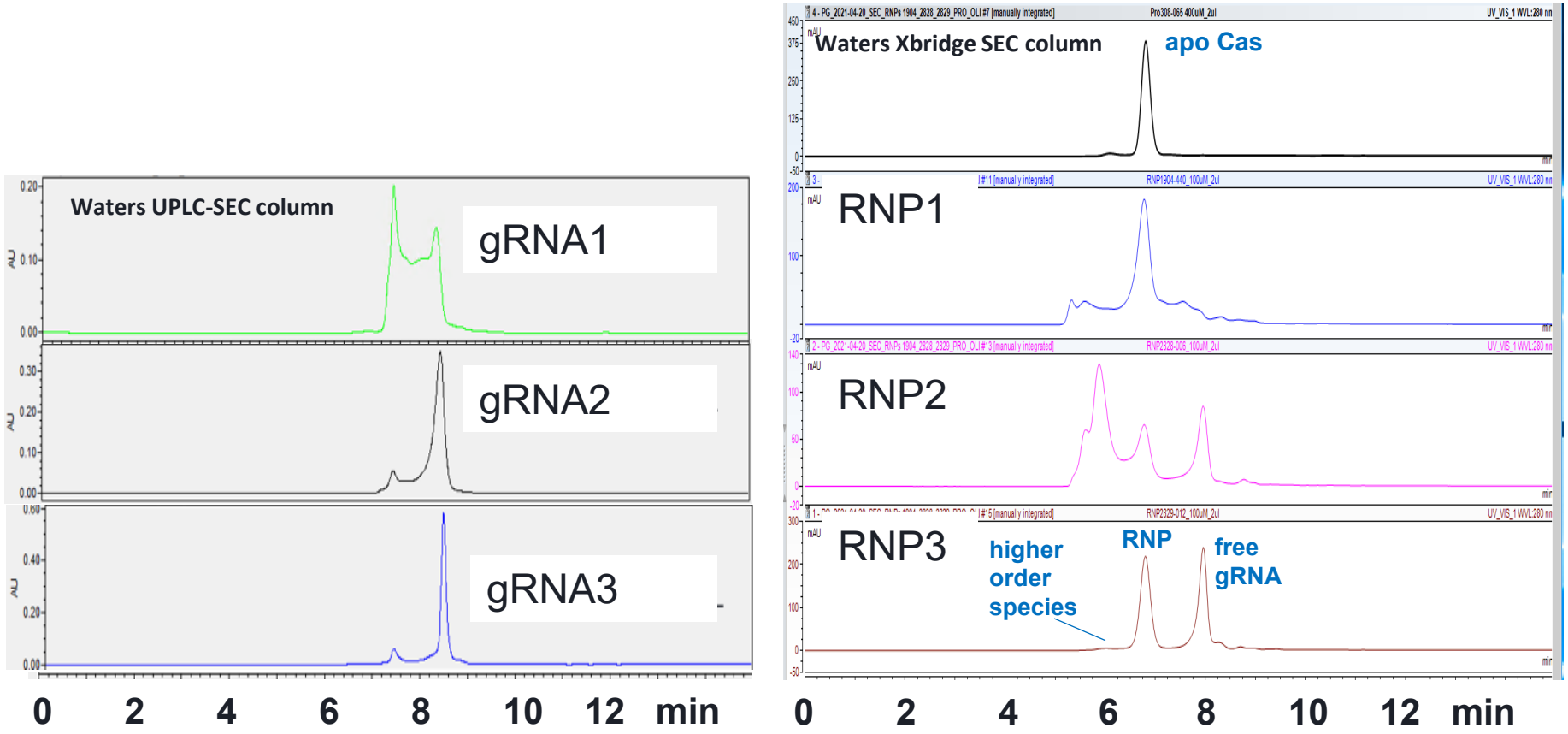
Waters XBridge BEH SEC 3.5 µm, 7.8 x 300 mm

150 mM NaCl, 100 mM Na-Pho pH 7.4, 1 mL/min



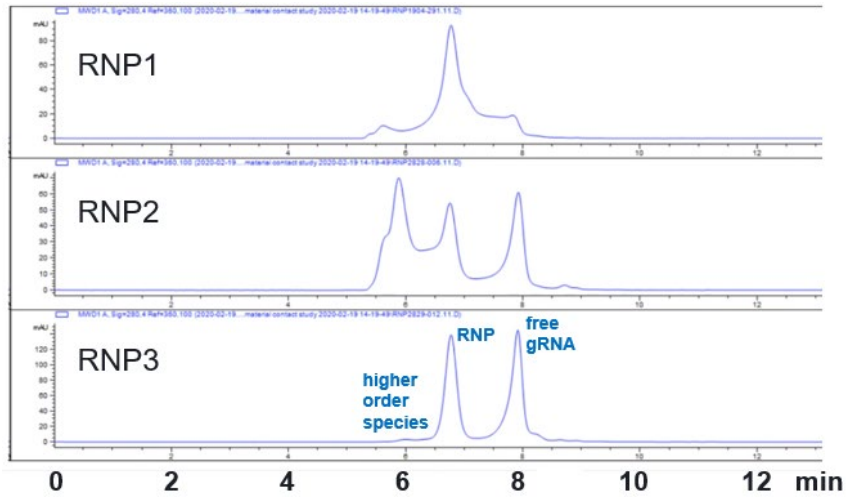
Characterization of RNPs – SEC Analysis

Impact of gRNA multimerization on RNP Complexation

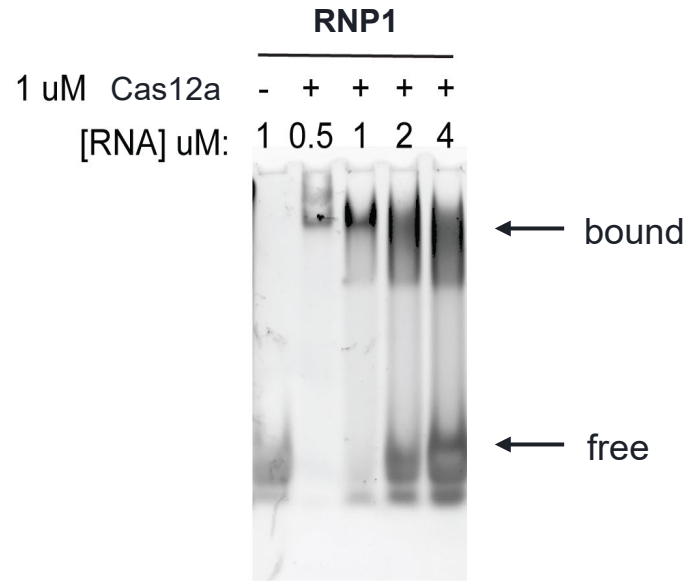


Characterization of RNPs – Correlation of SEC & PAGE Data

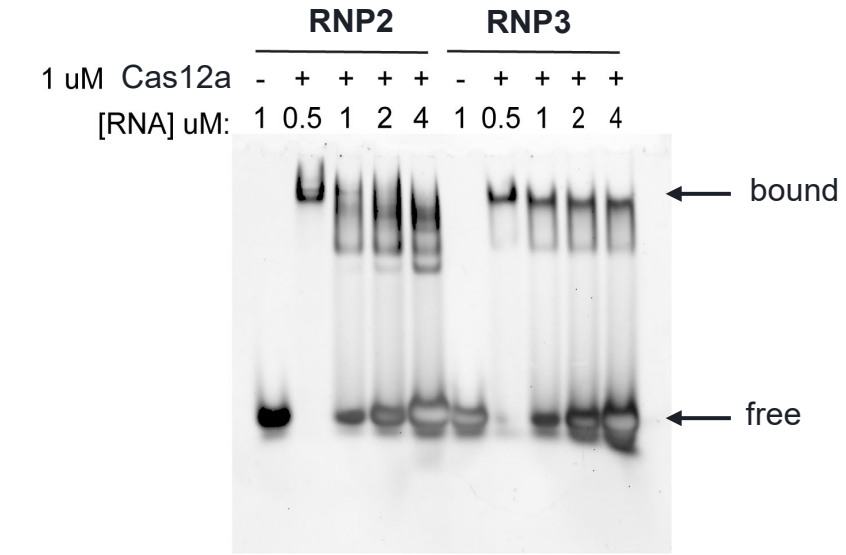
SEC



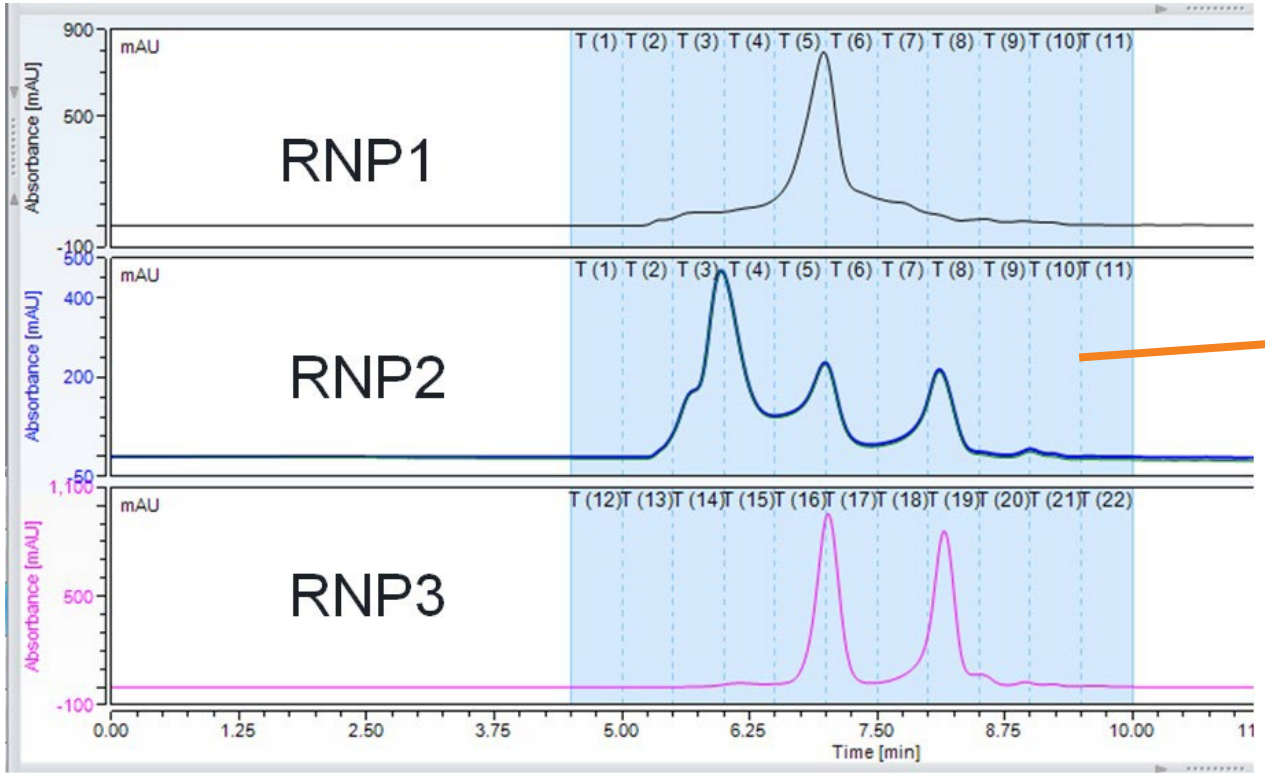
SEC analysis of 2:1 gRNA:PRO

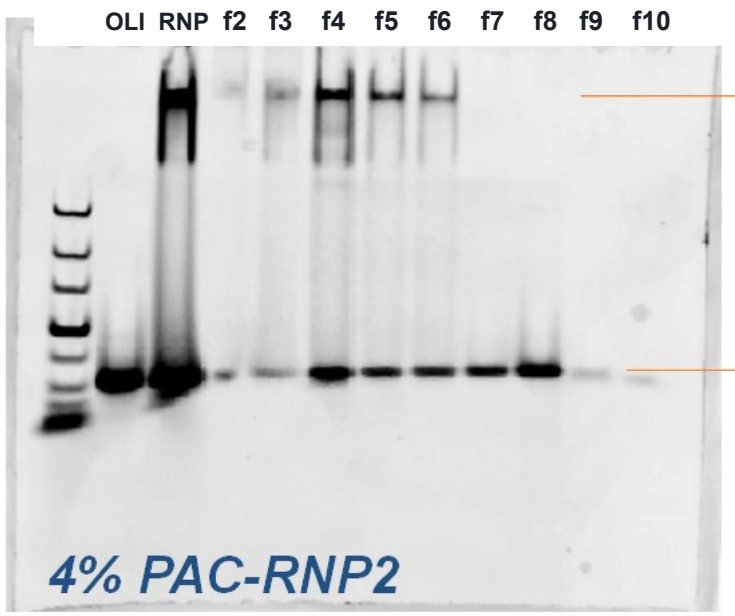


10% gels, 340 mM Tris, 660 mM HEPES, 75 mM, 2 mM MgCl2



Characterization of RNPs – 2D Analysis (SEC + PAGE)



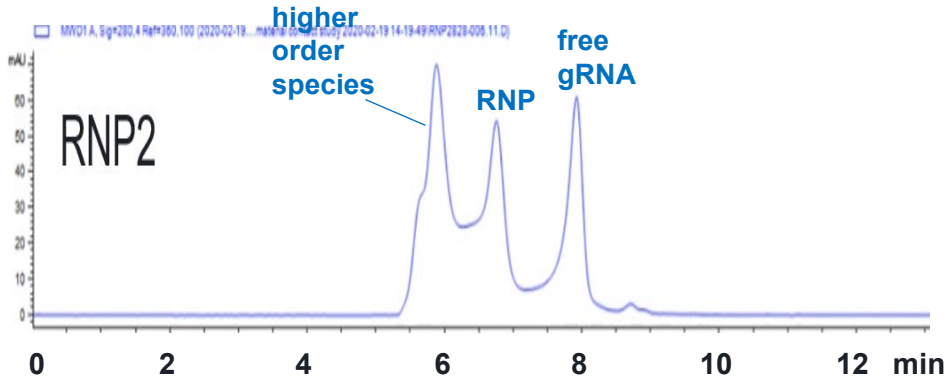


Complexed gRNA in RNP2

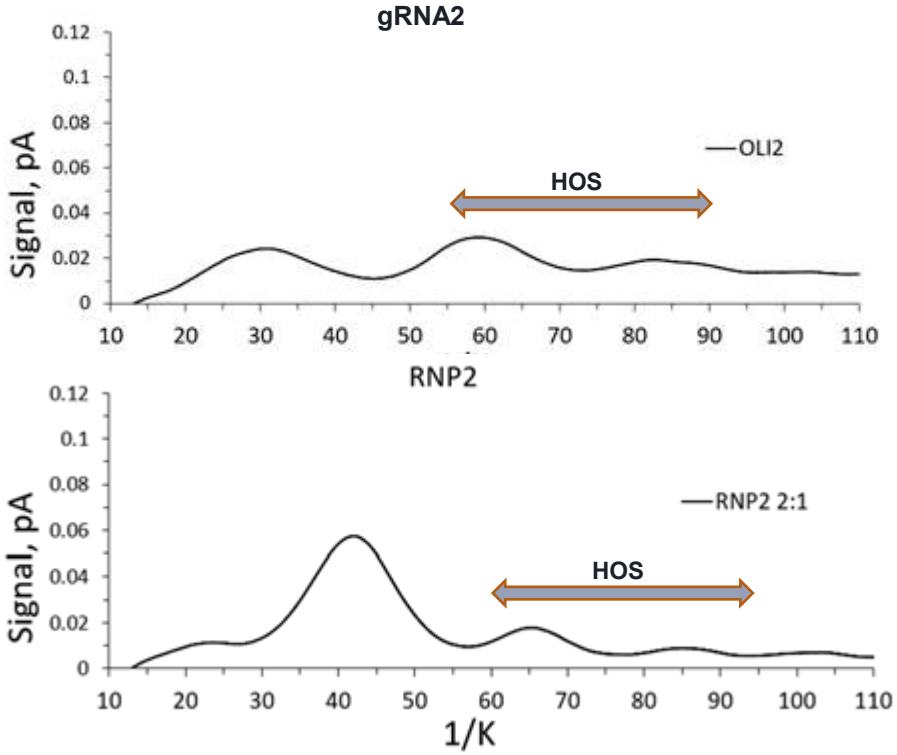
Free gRNA monomer in RNP2

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

Characterization of RNPs – SEC vs. IM for RNP2

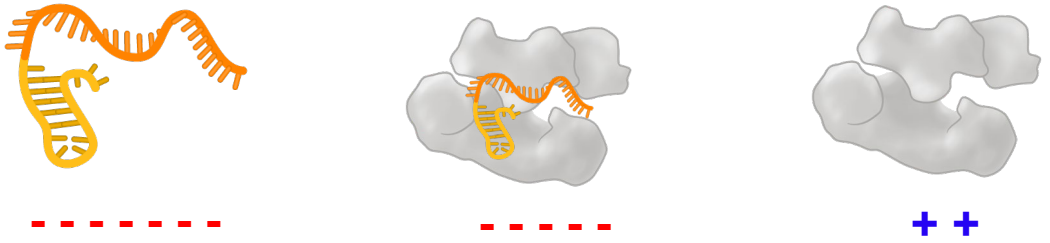


IM data in 5 mM NH_4OAc, pH 7.4



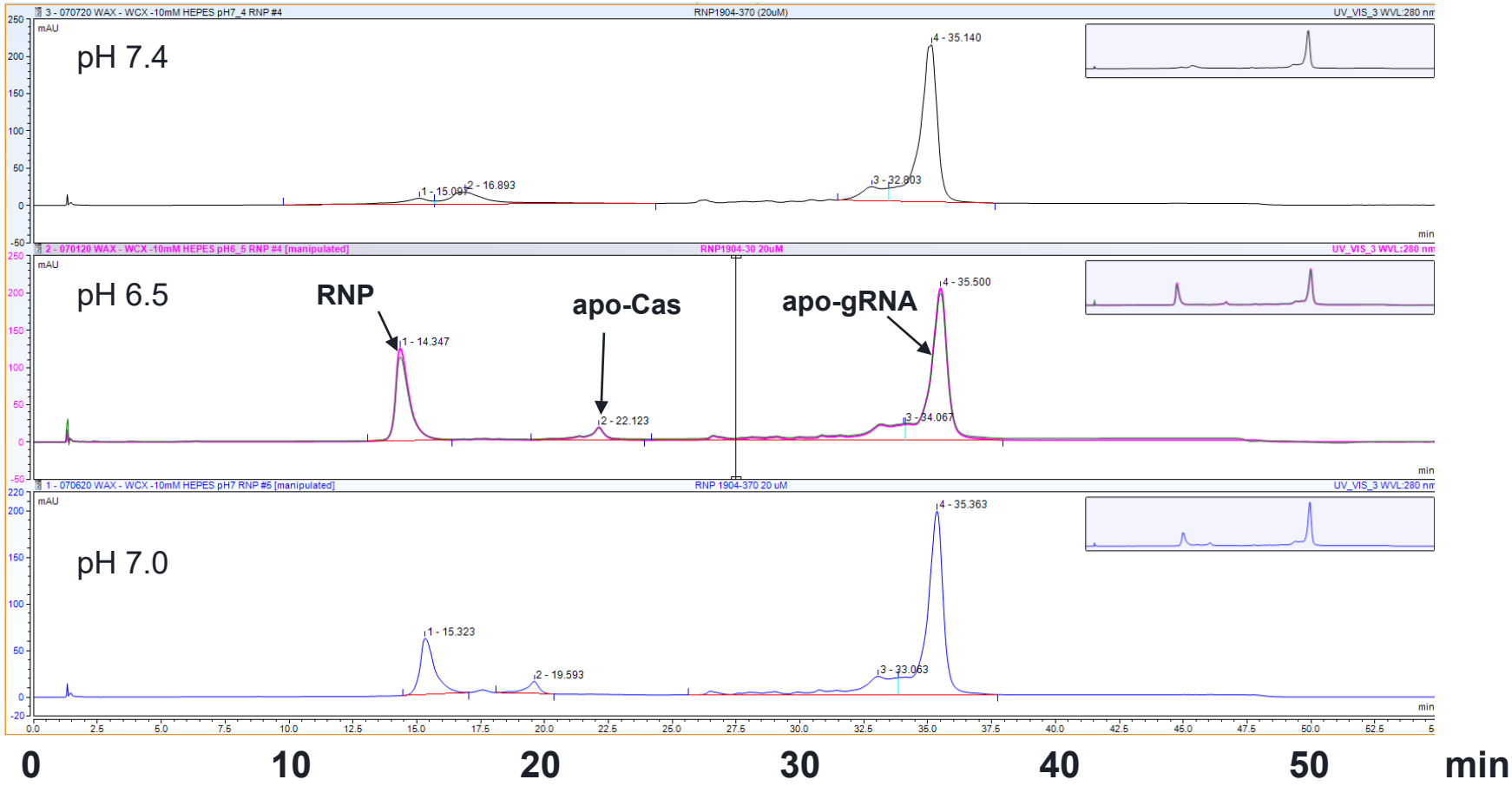
1/K	MW_{calc}
41.97	159 kDa
65.08	332 kDa
85.15	519 kDa

Characterization of RNPs – Ion Exchange Analysis

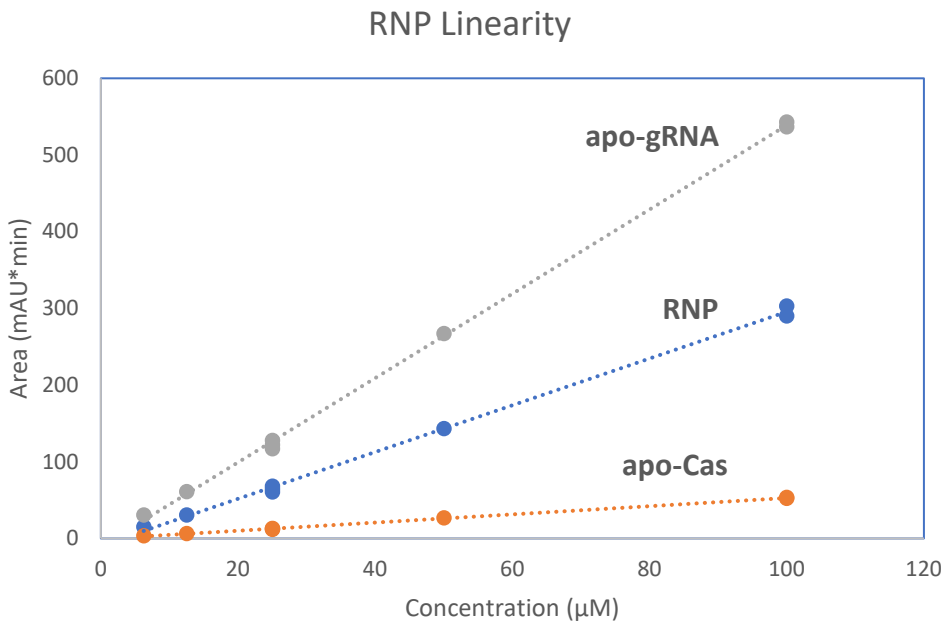


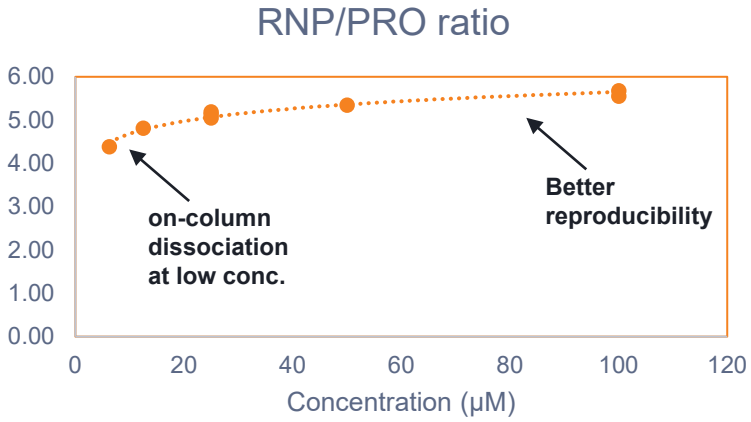
mode	gRNA	RNP	Cas
WCX	void	in between	retain
WAX	retain	in between	void
WCX-WAX	retain	retain	retain

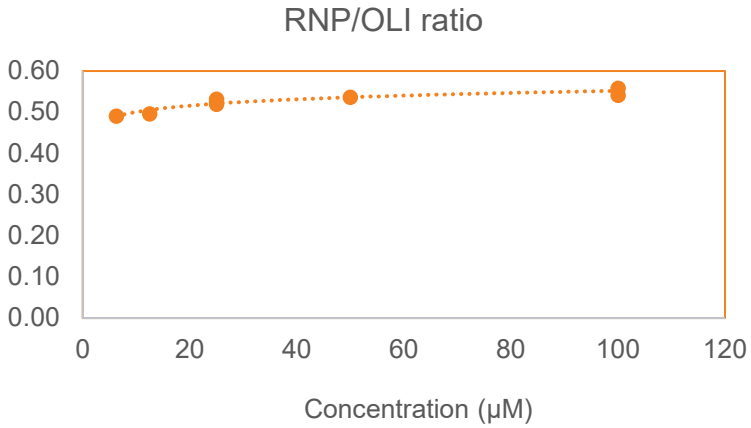
Characterization of RNPs – Analysis by WCX-WAX



Characterization of RNPs – WCX-WAX Linearity

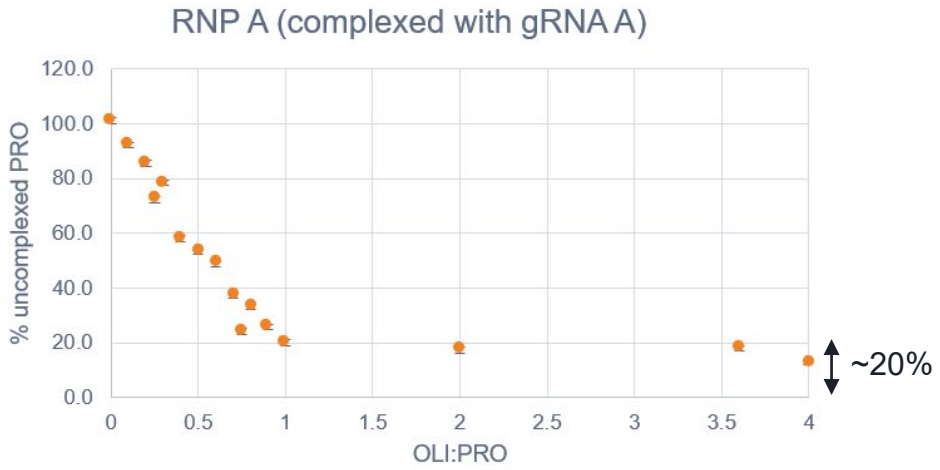


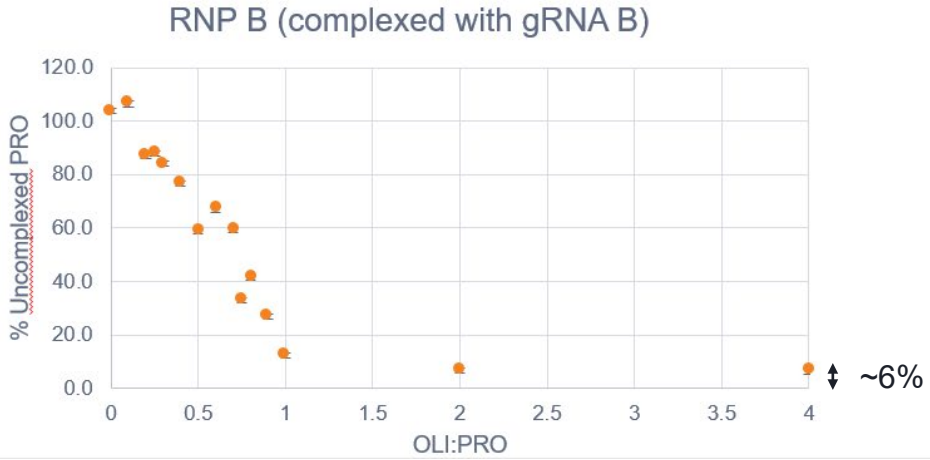


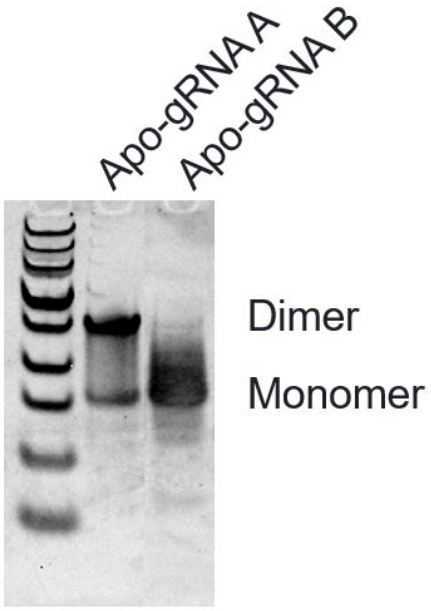


Characterization of RNPs – WCX-WAX Accuracy

% Uncomplexed Cas12a as a function of gRNA:Cas12a ratio and gRNA Dimerization





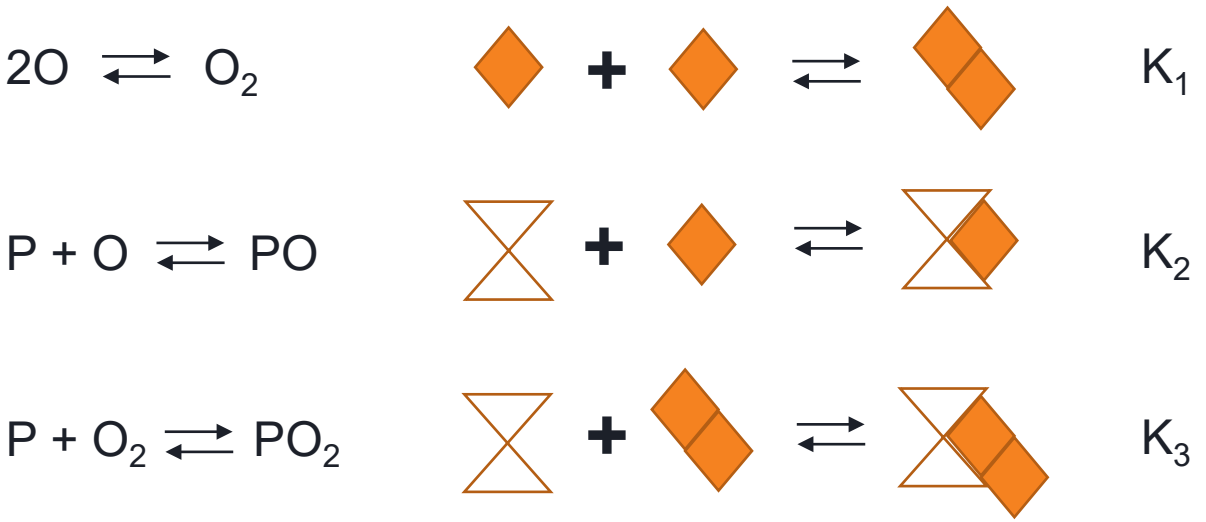


Characterization of RNPs – WCX-WAX Accuracy

Different Plateaus - Influence of competing equilibria?

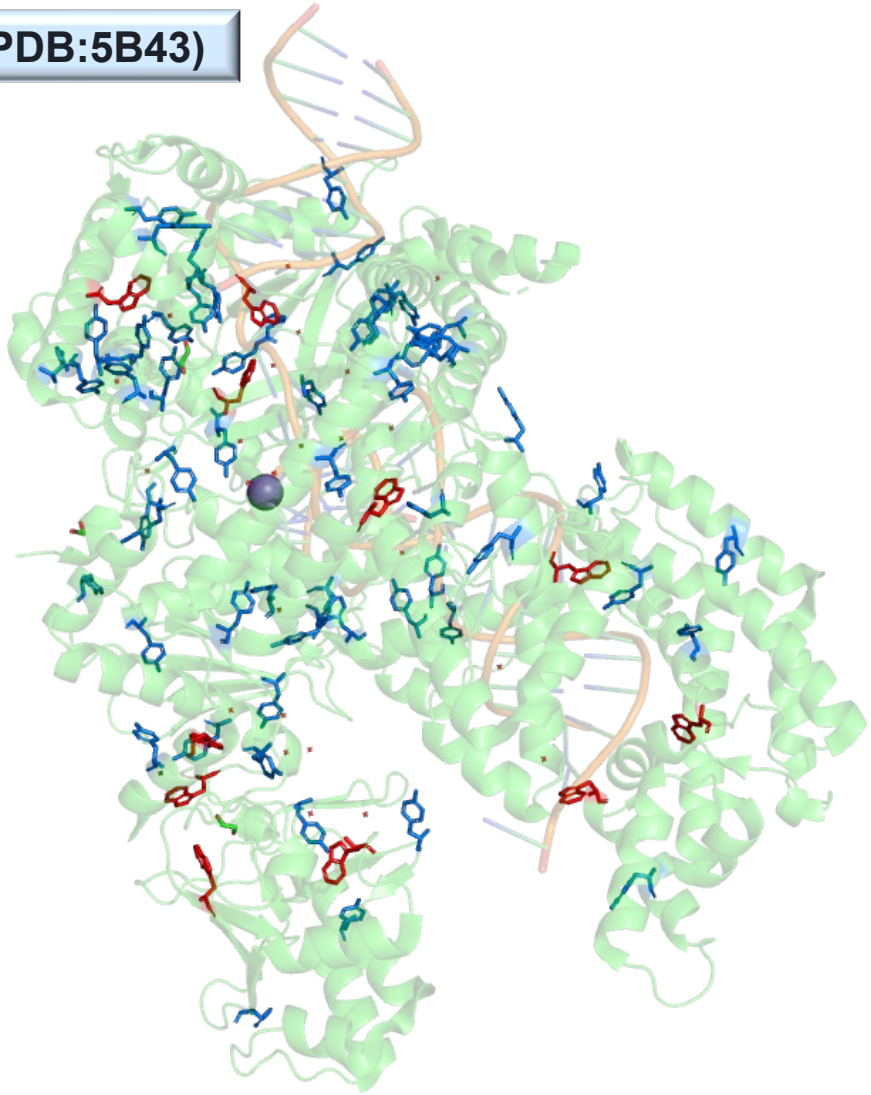
Possible Explanations:

- kinetically driven
  - *on-column dissociation $k_{-3} > k_{-2}$*
- thermodynamically driven
  - *e.g., K_1 , K_2 >> K_3*
- *modeling not yet adequate to reproduce data*



Spectroscopic Characterization of RNPs – Intrinsic Fluorescence

AsCas12a (PDB:5B43)

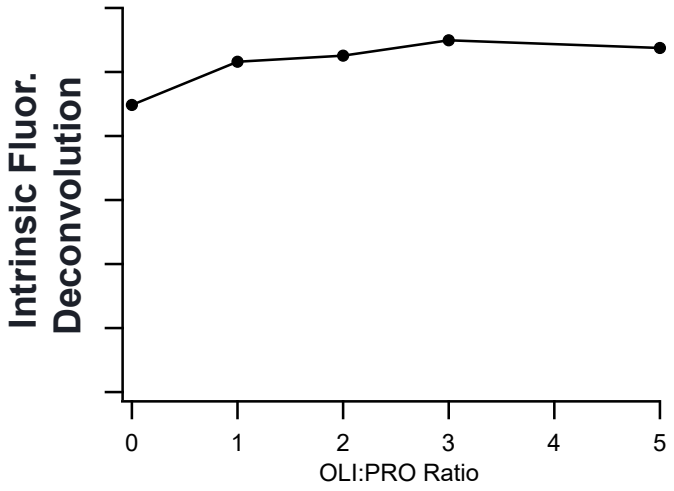


AsCas12a

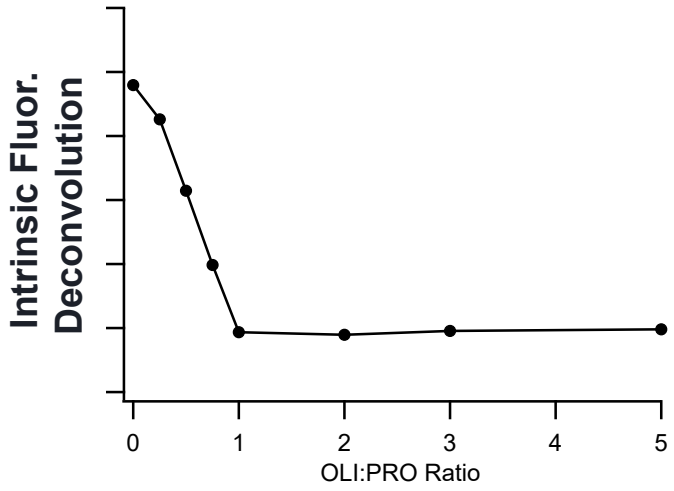
- many fluorescent residues
- (11 Tryptophans & 56 Tyrosines)
- local electronic environments for some of these will likely change upon gRNA binding

Spectroscopic Characterization of RNPs – Intrinsic Fluorescence

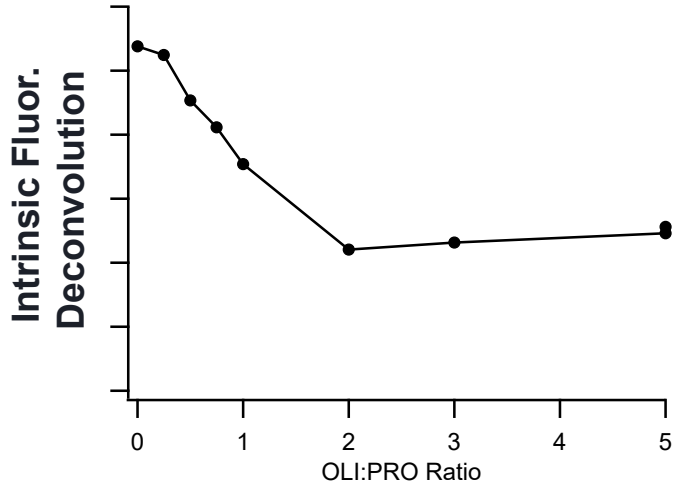
Titration of Cas12a with gRNAs
Comparison of gRNAs with different secondary structure



gRNA6 (21mer)
(non-binding
control sequence)



gRNA3 (41mer)
(predominantly
monomer)



gRNA1 (~70mer)
(predominantly
dimer form)

Summary & Concluding Remarks

gRNA Conformers and Multimers

- formation is sequence dependent, and more likely for longer sequences
- manifestation is condition dependent (gRNA conformers, stoichiometries, dynamics, and analysis method)

RNP Complexation

- another level of complexity due to noncovalent nature of complex (conformers, stoichiometries, dynamics, and method-dependent results)
- SEC, IEX, PAGE, IM data shows that the secondary structures formed by the guides impact the complexation
  - this can potentially impact characterization assays and editing
  - some method dependence of results, which is not surprising for noncovalent complexes of large biomolecules
- Promising new methods:
  - “Combo” WCX/WAX method
  - intrinsic fluorescence
  - Characterization vs. QC/release methods

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