Presenter: Borries Demeler

Topic:
Sedimentation IV

Copy of Lecture at:

https://demeler.uleth.ca/biophysics/archive/Demeler/

Reversible Interactions - Oligomerization

Reversible reaction (Le Chatelier's principle):

$$\sum_{i=1}^{n} M \rightleftarrows M_n \qquad \sum_{i=1}^{n} M \rightleftarrows M_n$$

Equilibrium Constant:

$$K_a = \frac{[M_n]}{[M]^n} \qquad K_d = \frac{[M]^n}{[M_n]}$$

Kinetics:

$$K_a = \frac{k_{on}}{k_{off}}$$

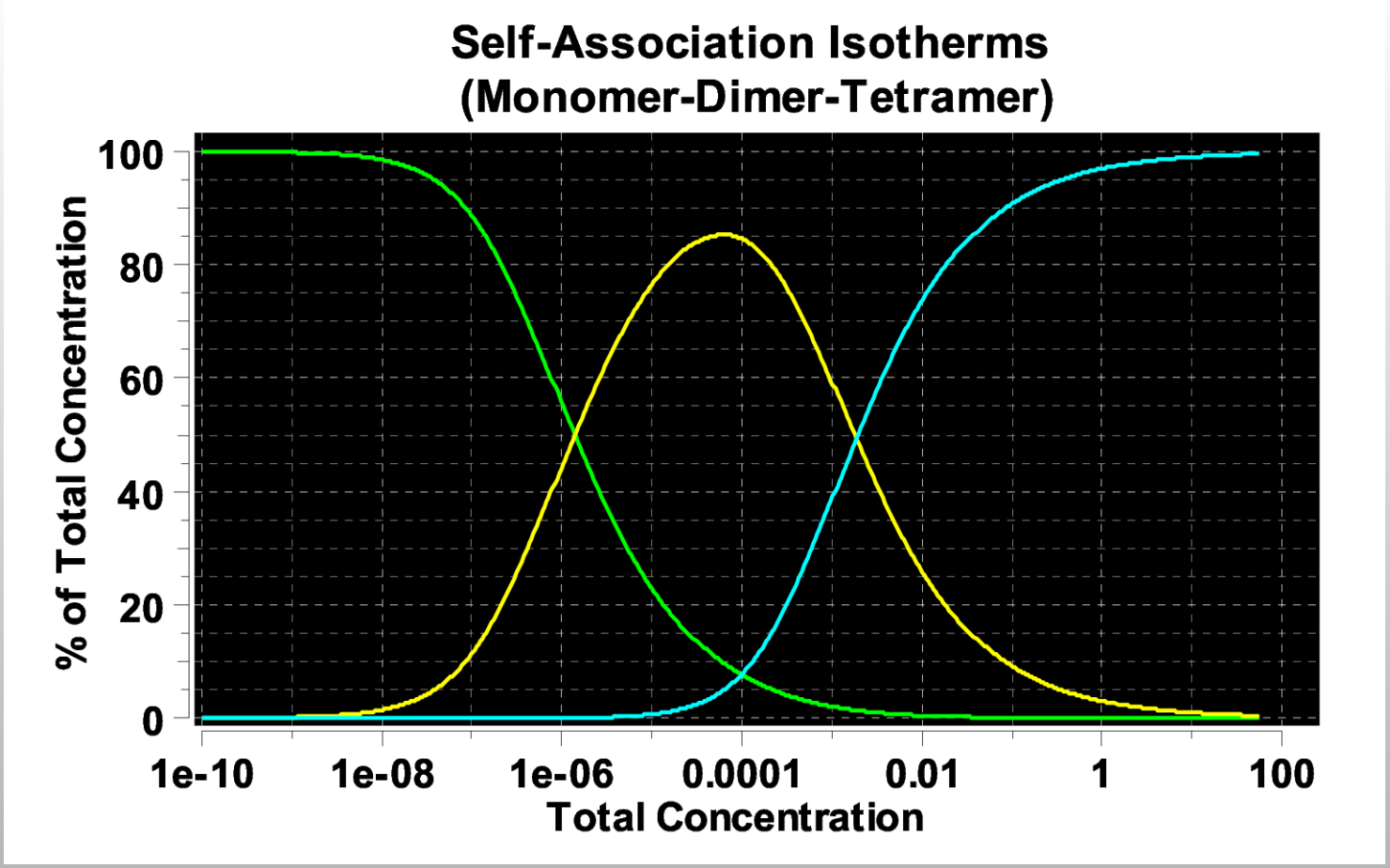
Solve Polynomial

$$[M] + [M_n] = C_{total}$$
$$[M] + K_A[M]^n - C_{total} = 0$$

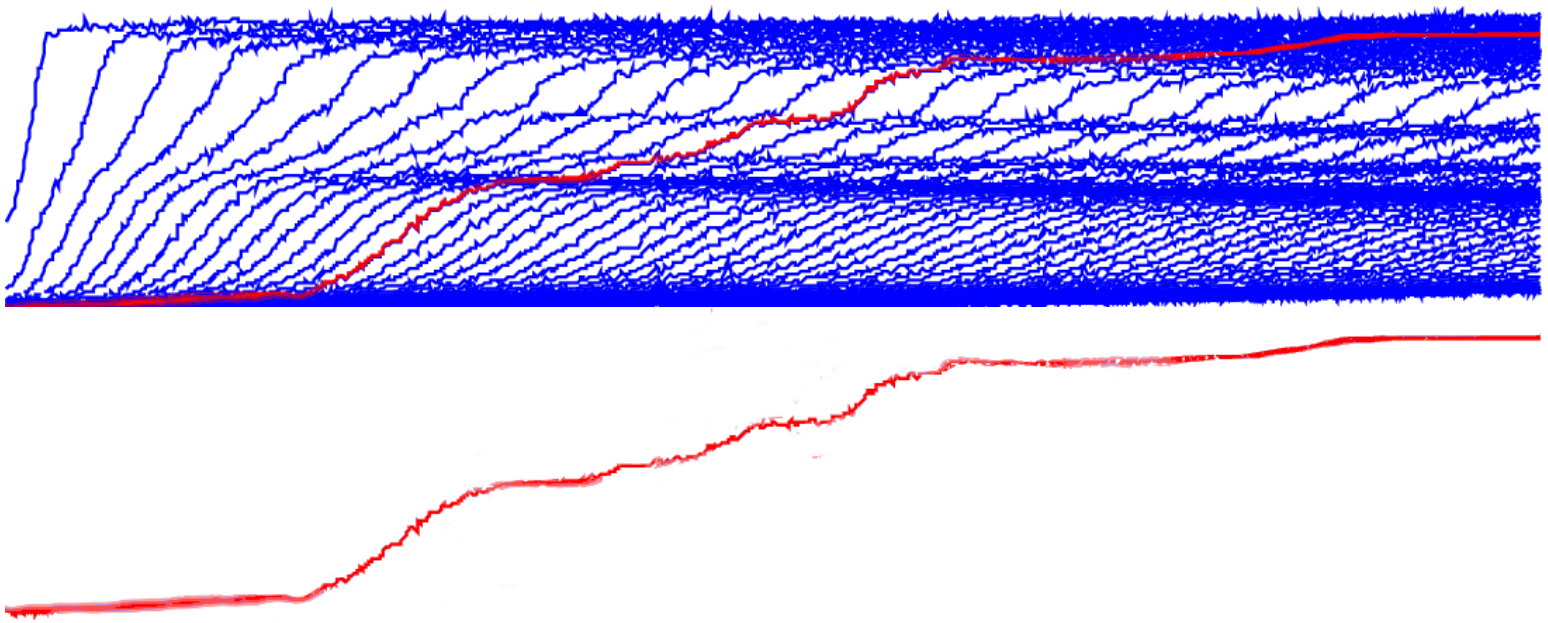
Example, a monomer-dimer equilibrium:

$$A + A \leftrightarrows A_2$$
$$[A] + K_a[A]^2 - C_{total} = 0$$

Background - Reversible Associations



A Model for Reversible Reactions



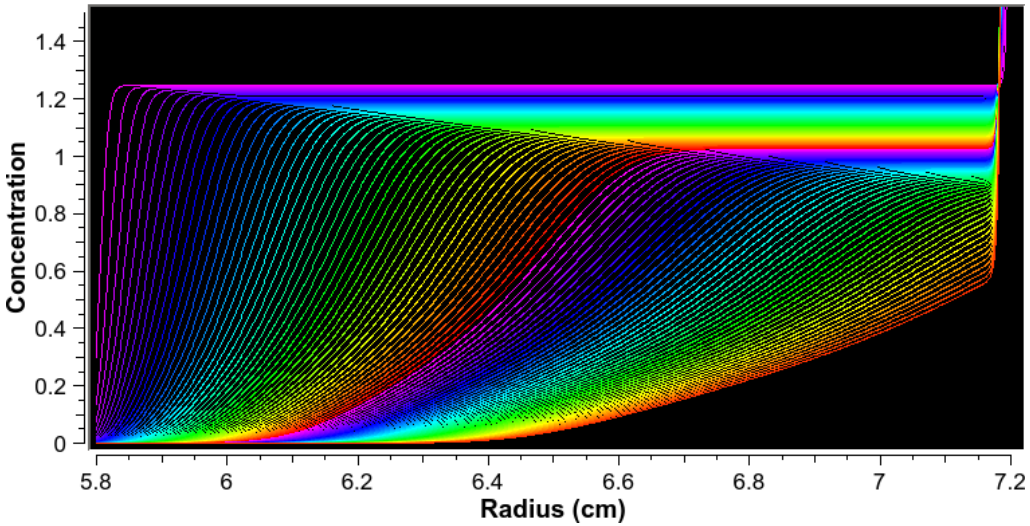
In the gradient, the *weight-average* sedimentation coefficient and the *gradient-average* diffusion coefficient are observed:

$$\bar{s} = \frac{\sum_{j=1}^{m} s_j C_j}{C_T} = \frac{\sum_{j=1}^{m} s_j K_j C_1^j}{C_T} \qquad \bar{D} = \frac{\sum_{j=1}^{m} D_j (\partial C_j / \partial r)}{\sum_{j=1}^{m} (\partial C_j / \partial r)} = \frac{\sum_{j=1}^{m} j D_j K_j C_1^{j-1}}{\sum_{j=1}^{m} j K_j C_1^{j-1}}$$

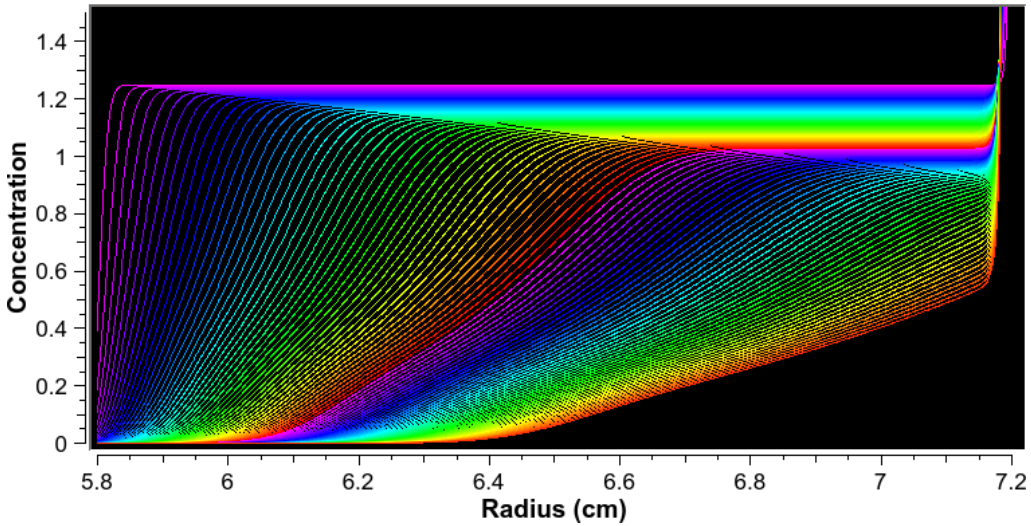
Effect of Kinetics on the Boundary Shape

Monomer – Trimer Equilibrium, Monomer MW = 66 kDa

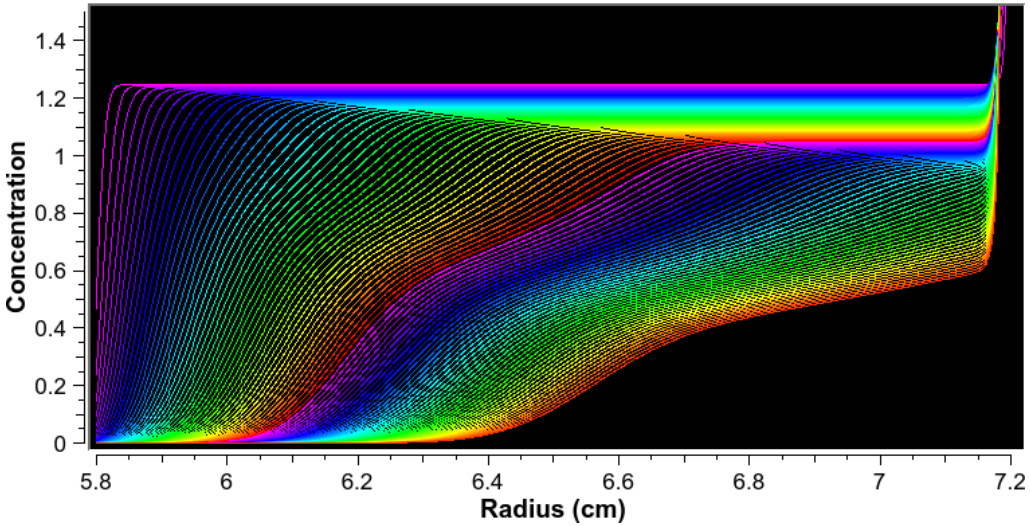
k-off = 1.0/sec



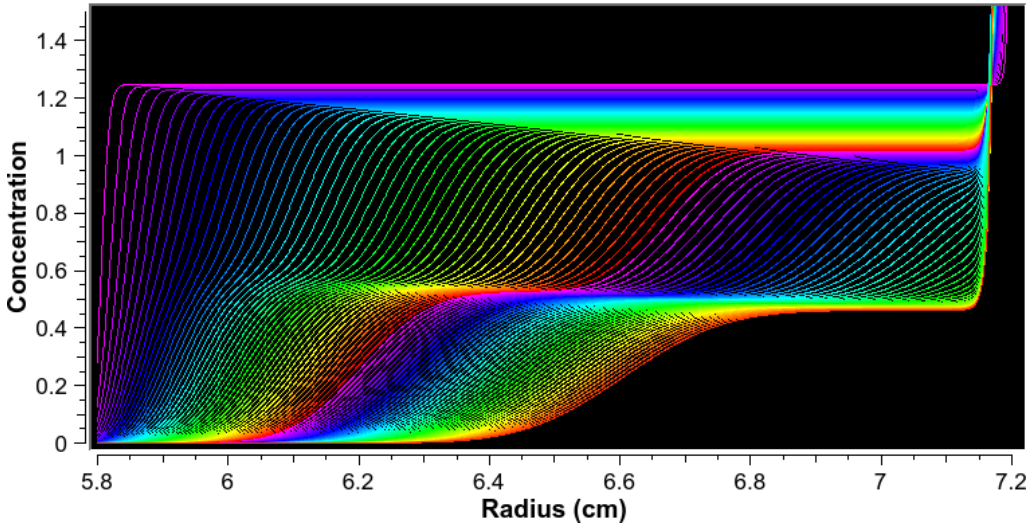
k-off = 0.001/sec



k-off = 0.0001/sec



non-interacting



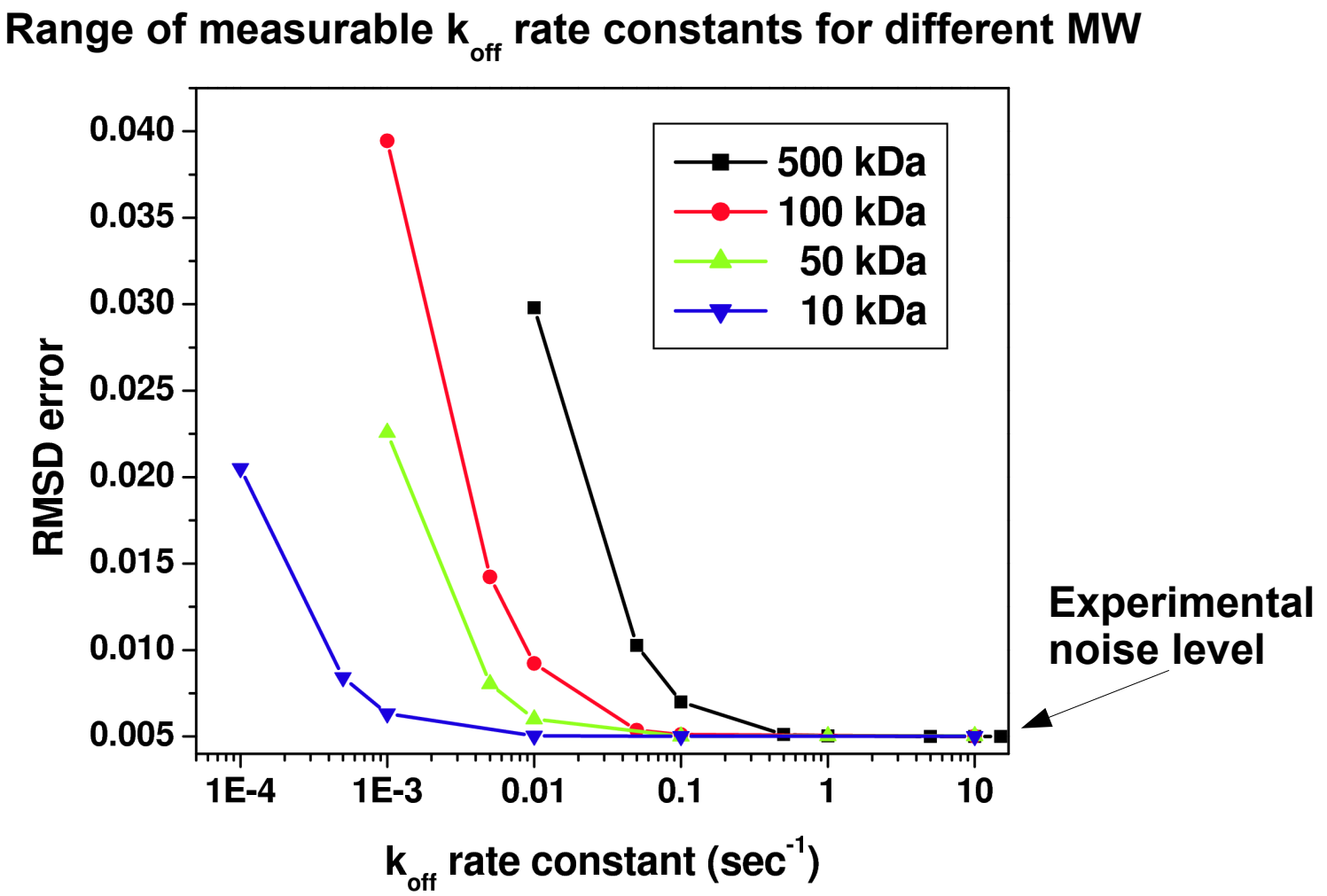
Models for Reacting Systems:

The magnitude of the measurable off-rate depends on rotor speed and sedimentation coefficient:

$$s \sim \frac{M}{f}$$

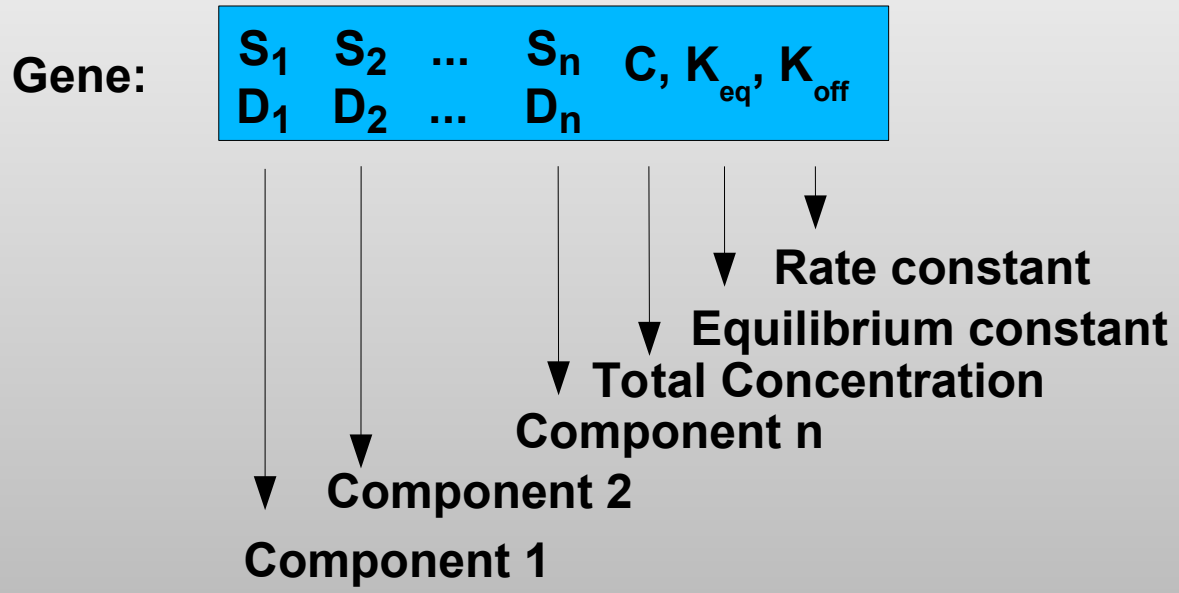
Faster rotor speed, higher molecular weight and globular shape will favor the measurement of faster rate constants.

Models for Reacting Systems:

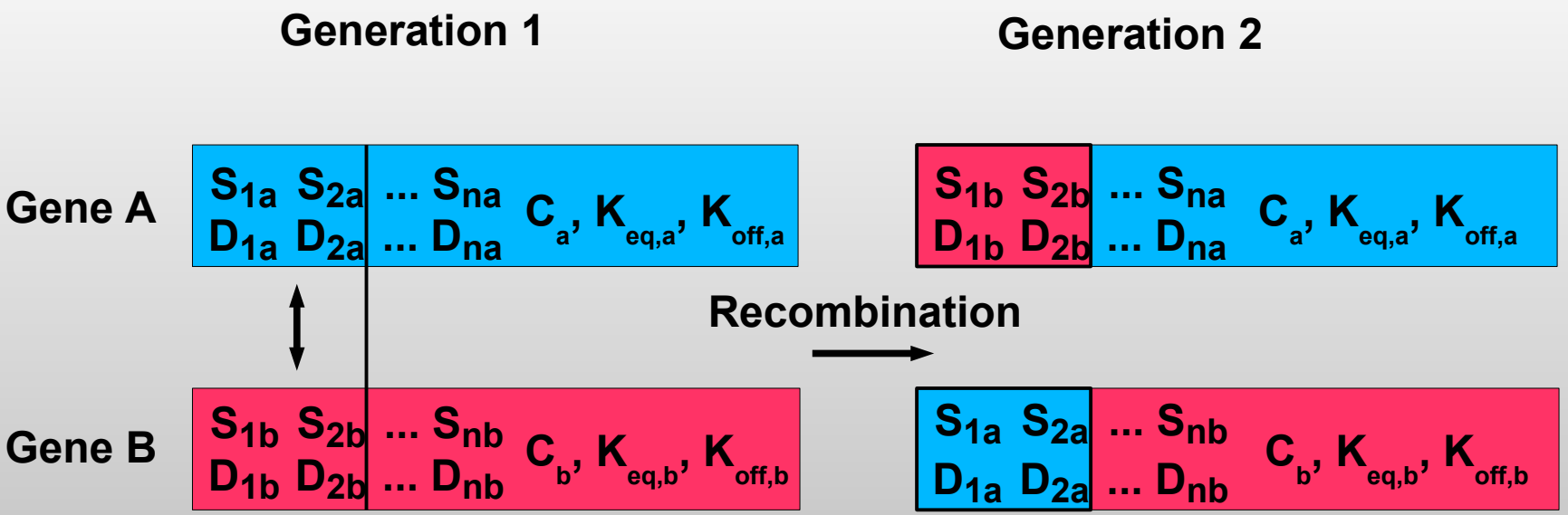


Genetic Algorithm Optimization:

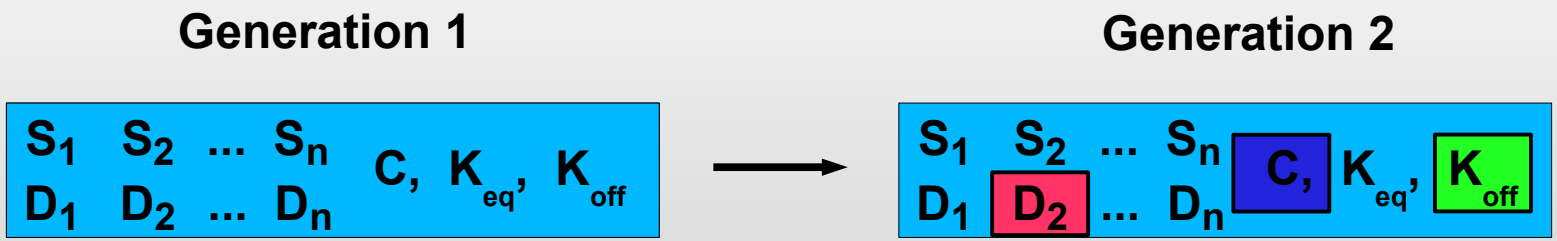
Genes are strings of parameters, each gene consists of a pair of corresponding sedimentation and diffusion coefficients, loading concentration, equilibrium constants and rate constants.



Crossover/Recombination



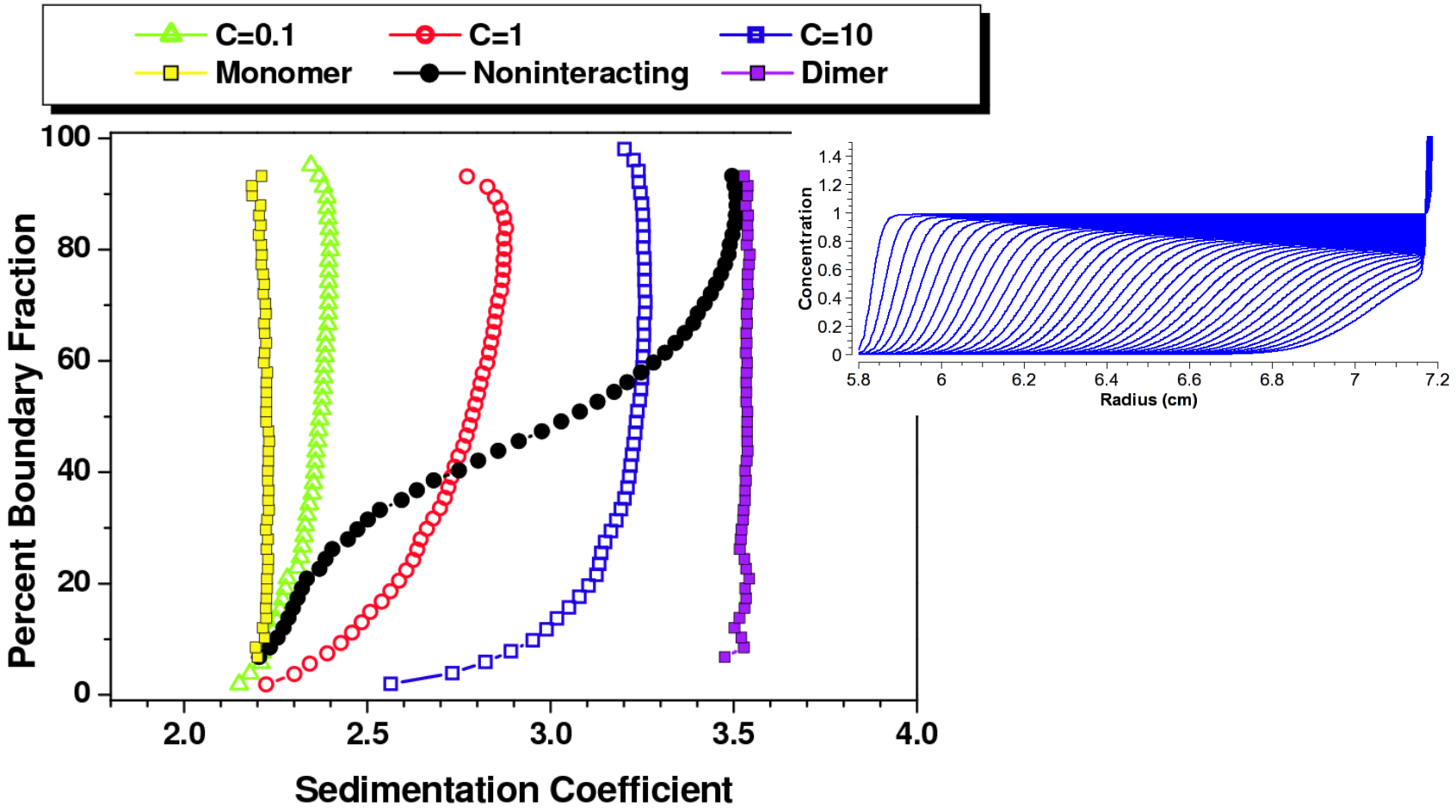
Mutation



Mutation Event
(within linear constraints)
maintaining molecular weight, shape constraints

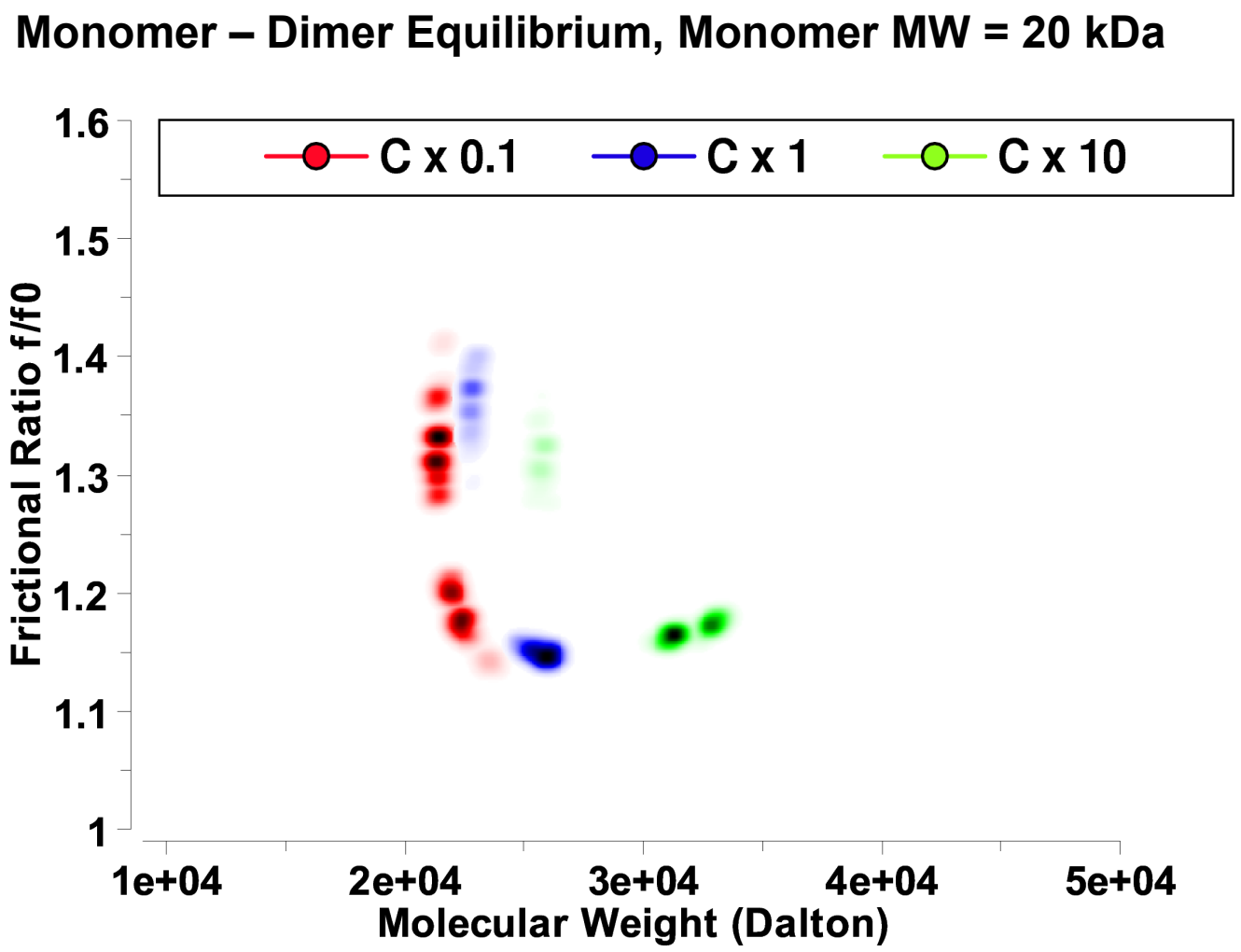
Diagnostics: van Holde – Weischet Analysis

Example 1: Simulated Monomer – Dimer Equilibrium

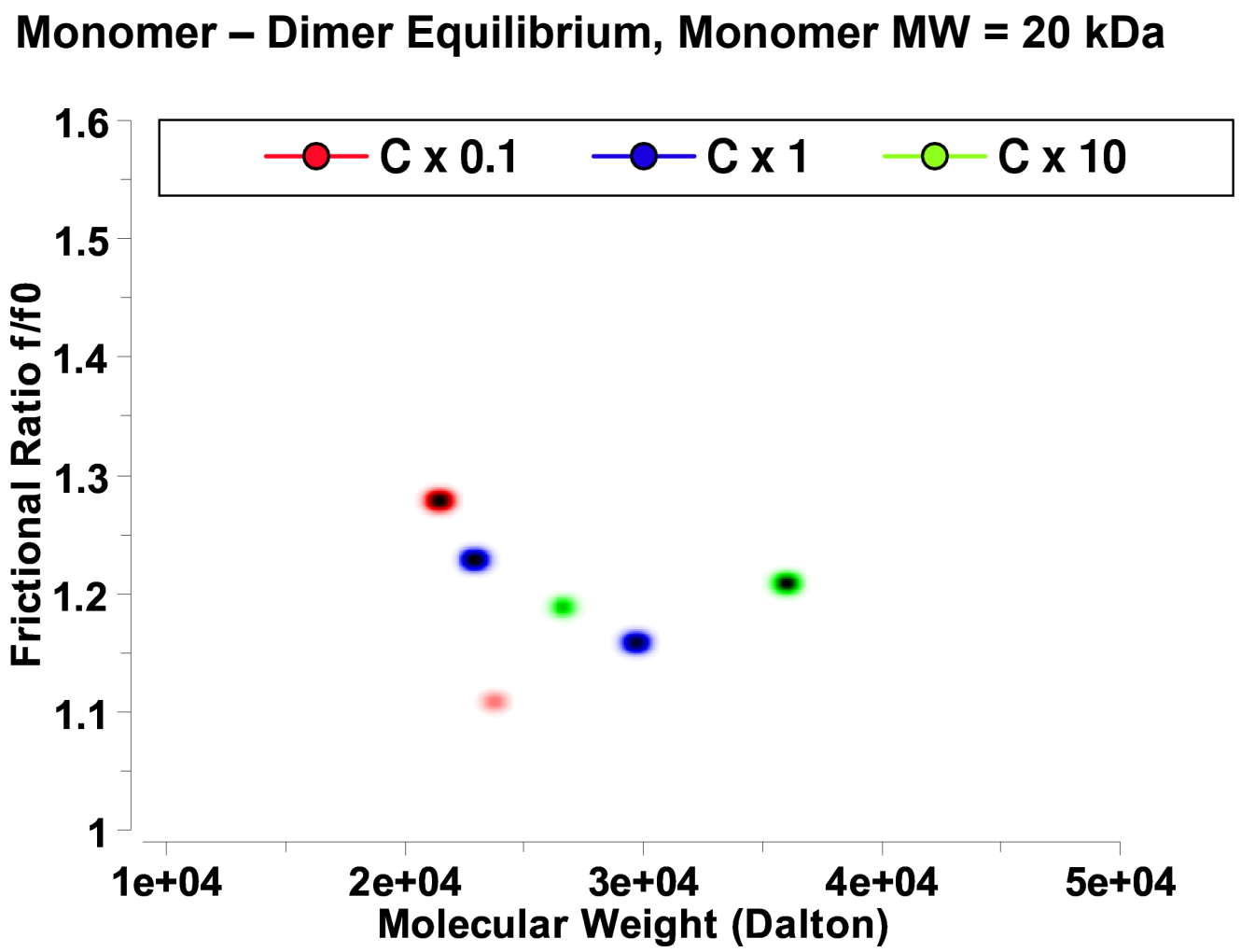


Monomer MW = 20 kDa, K_d = C x 1, k_{off} = 1x10^{-3}/sec, f/f0 = 1.25 (both)

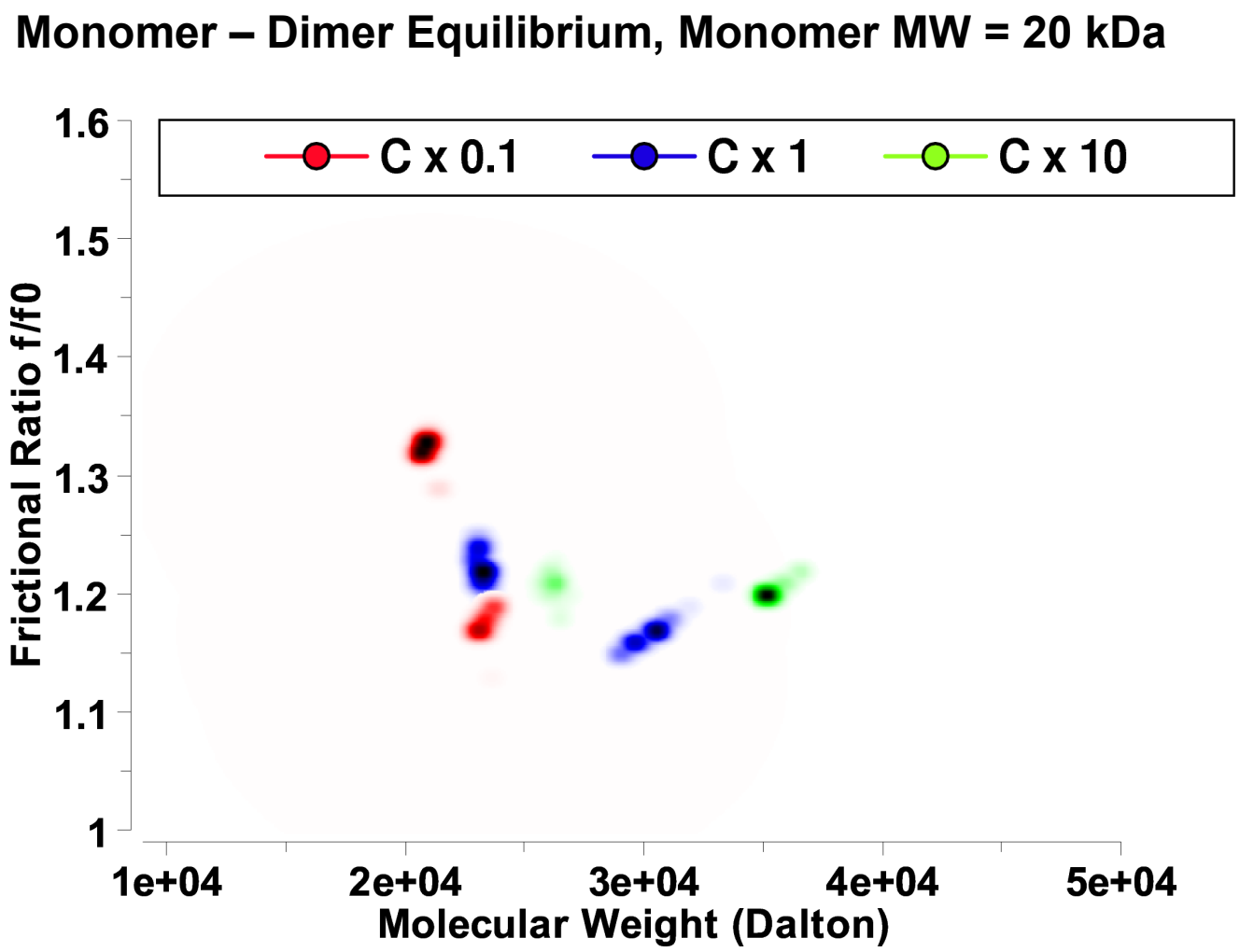
2DSA Monte Carlo Analysis



Genetic Algorithm Analysis

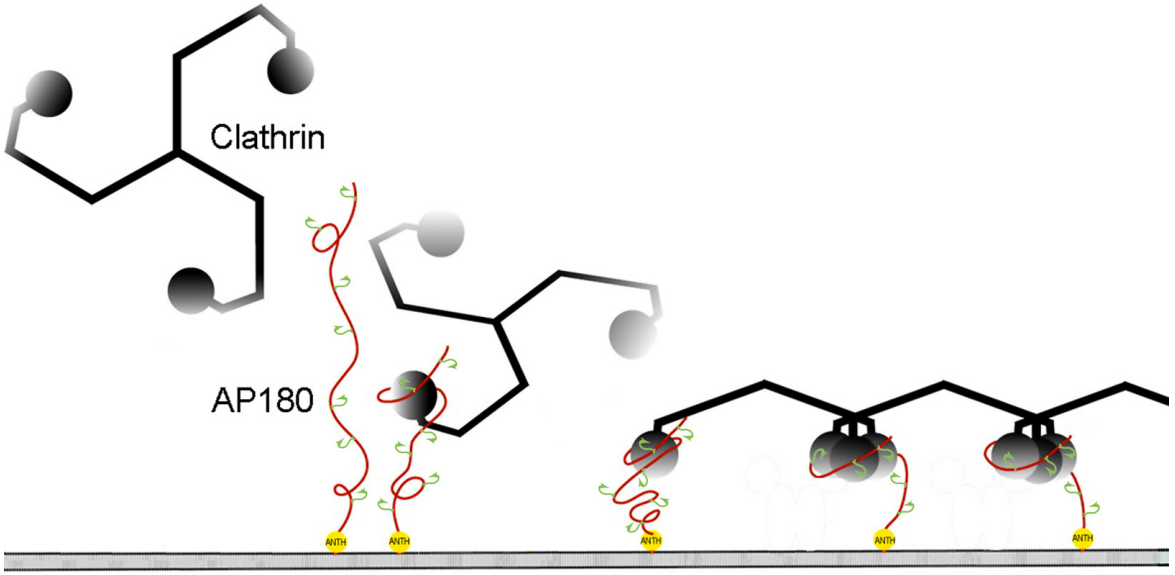


Genetic Algorithm Monte Carlo Analysis



Hetero-associations:

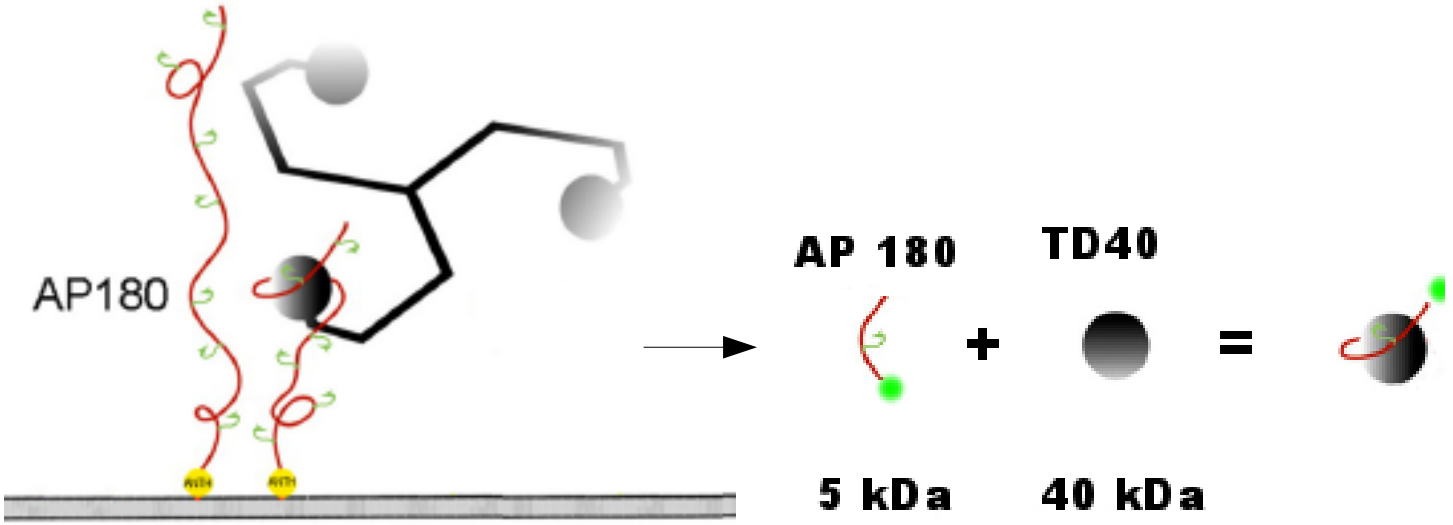
Example Application: Clathrin assembly/Ligand Binding



The line fishing model for assembling the endocytic apparatus. After docking to the plasma membrane via interactions between the N-terminal ANTH domain of AP180 (yellow) and membrane bound PIP_2, the long and flexible C-terminal domain of AP180 (red) can bind and recruit clathrin (black) from a large volume of cytosol to initiate the formation of a clathrin coated pit. The large number of clathrin binding sites (green) recruit multiple clathrin heavy chains together to form the vertexes of the clathrin lattice (adapted from Kalthoff et al. JBC, 2002 with permission from the Journal of Biological Chemistry).

Hetero-associations:

Find conditions where the *free* larger component does not contribute to the observed sedimentation signal

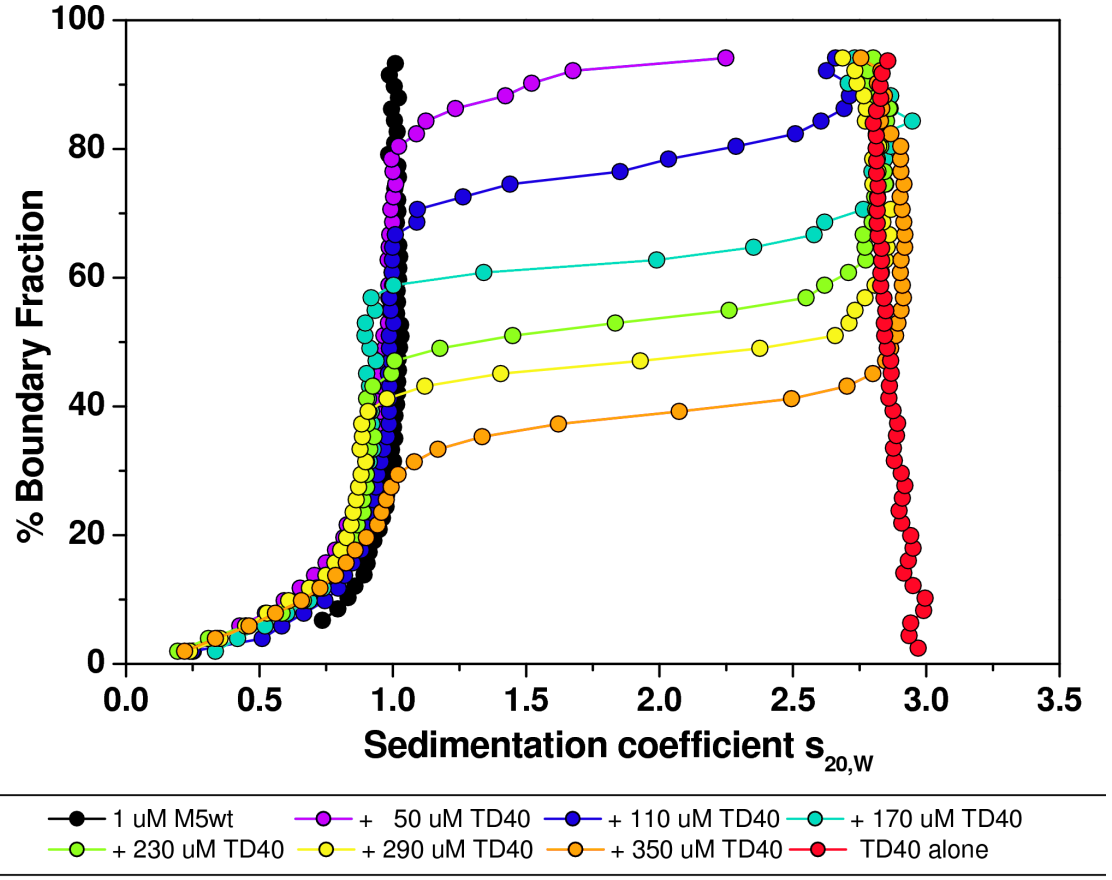


Strategy:

Always label the smaller component, since the complex will have a larger percentage change in sedimentation value. Measure under conditions where there is no background from the free substrate.

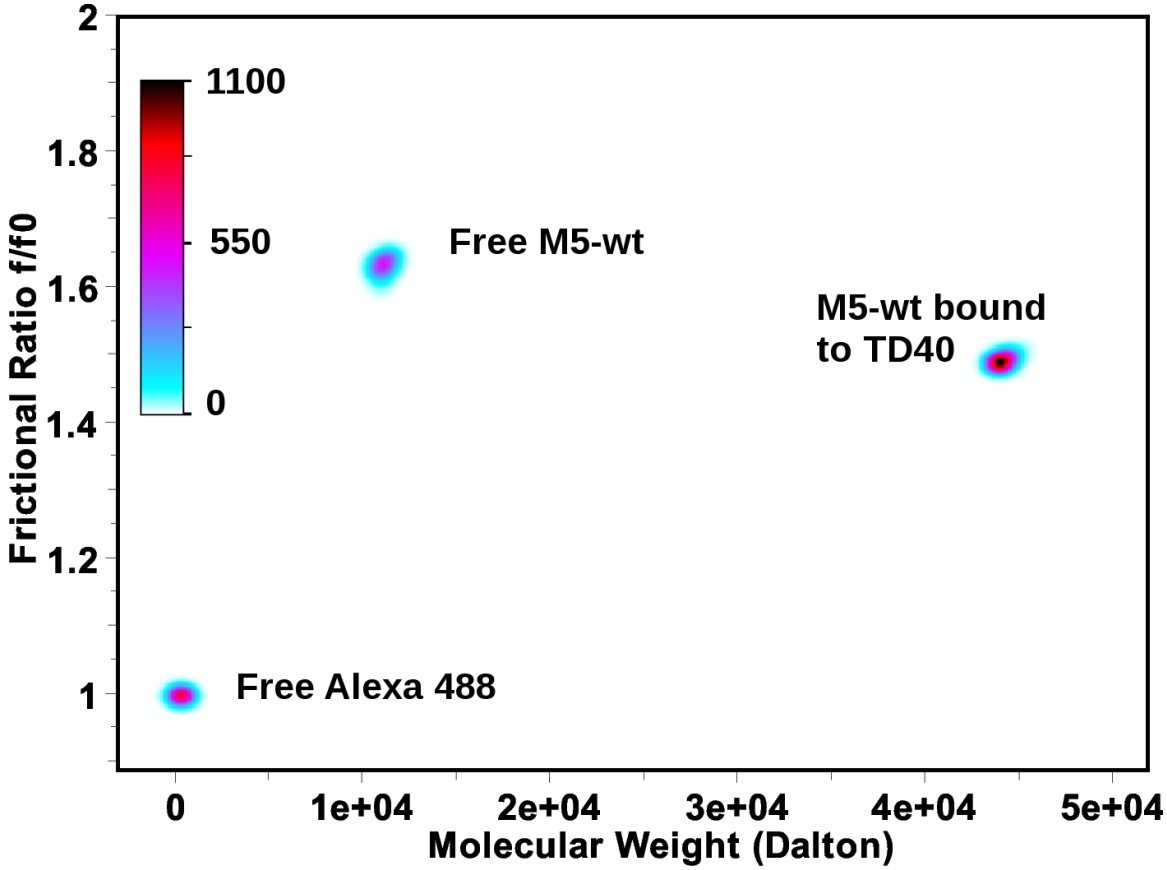
AUC Applications

Titrate free TD40 against fixed amount of free M5 ligand and watch complex TD40*M5 appear. Measure amounts of free M5 and complex TD40*M5 and use that to calculate the Kd



AUC Applications

Quantification of ligand/protein equilibrium concentrations by genetic algorithm Monte Carlo analysis



$M5 + TD40 \Leftrightarrow M5 \cdot TD40$

$Kd = [M5 \cdot TD40]/[M5][TD40]$

Measure:

fraction *f* of free M5

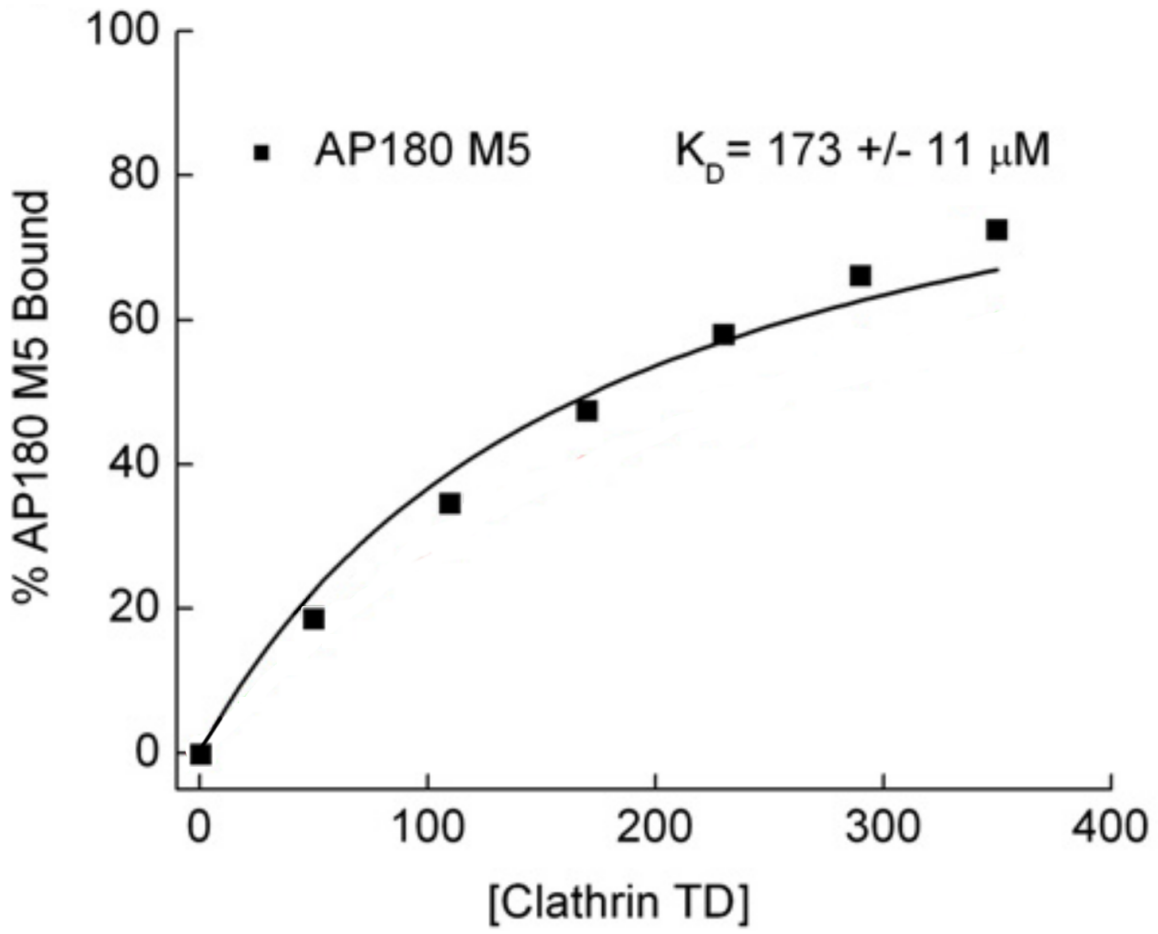
$[M5] = f * [M5_{loading}]$

$[M5:TD40] = (1 - f)\,[M5_{loading}]$

$[TD40] = [TD40_{loading}] - [M5:TD40]$

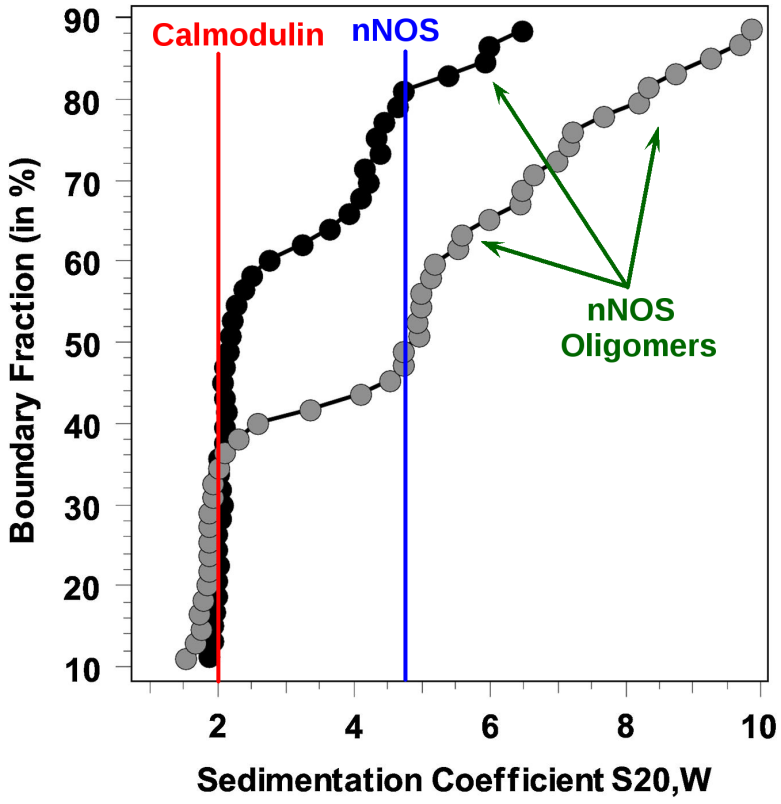
AUC Applications

Binding Curve for M5 and TD40:



Kd measurements for hetero-associations

Qualitative comparison of binding strengths between wildtype and mutant forms of neuronal Nitric Oxide Synthase with Calmodulin

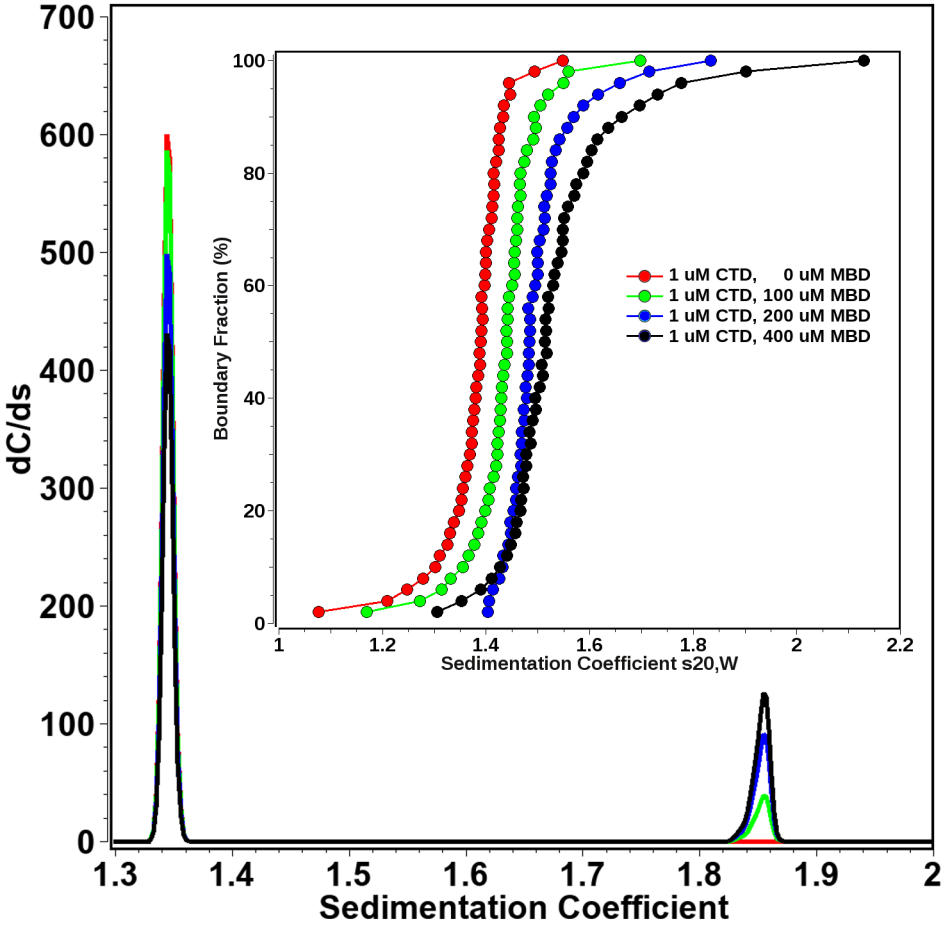


van Holde–Weischet integral distribution graphs for CaM (2S) binding to nNOS-reductase domain (5S and above) for a 1:0.5 M ratio of CaM to nNOS. A clear difference in binding strength is evident from the relative amount of free CaM for wild type nNOS (grey circles, about 40% free CaM) versus mutant R753A nNOS (black circles, about 65% free CaM). The loading concentration of CaM was 250 nM.

Panda SP, Polusani SR, Kellogg DL 3rd, Venkatakrishnan P, Roman MG, Demeler B, Masters BS, Roman LJ. Intra- and inter-molecular effects of a conserved arginine residue of neuronal and inducible nitric oxide synthases on FMN and calmodulin binding. Arch Biochem Biophys. 2013 Mar 15.

Kd measurements for hetero-associations

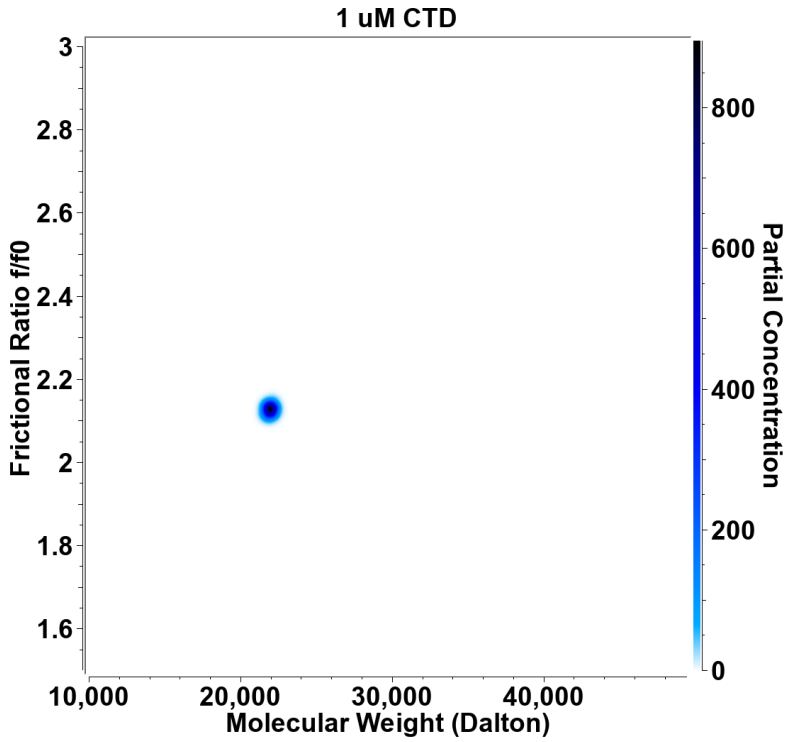
Domain Interactions of MeCP2
Intrinsically disordered protein associated with Rett syndrome

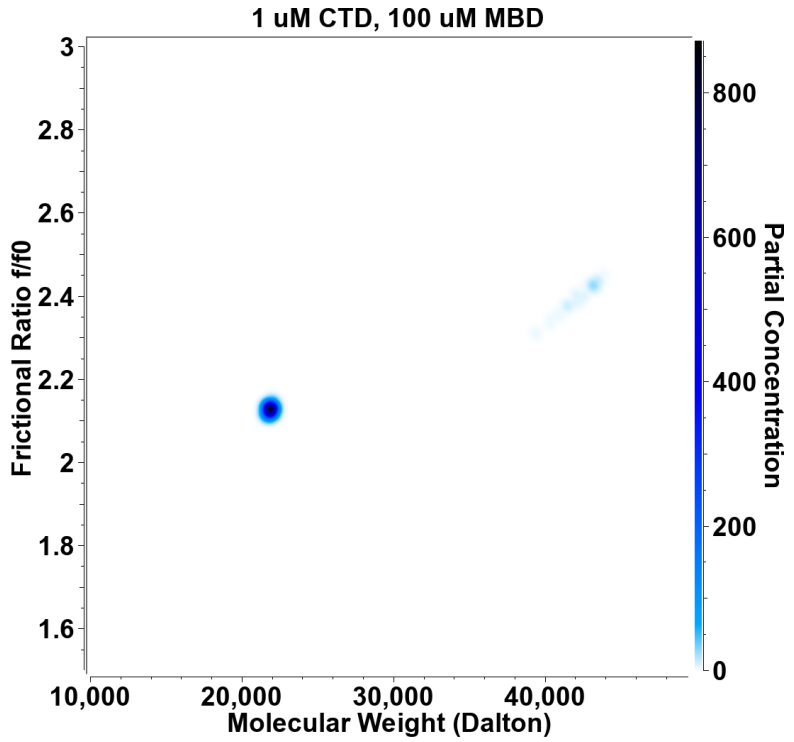


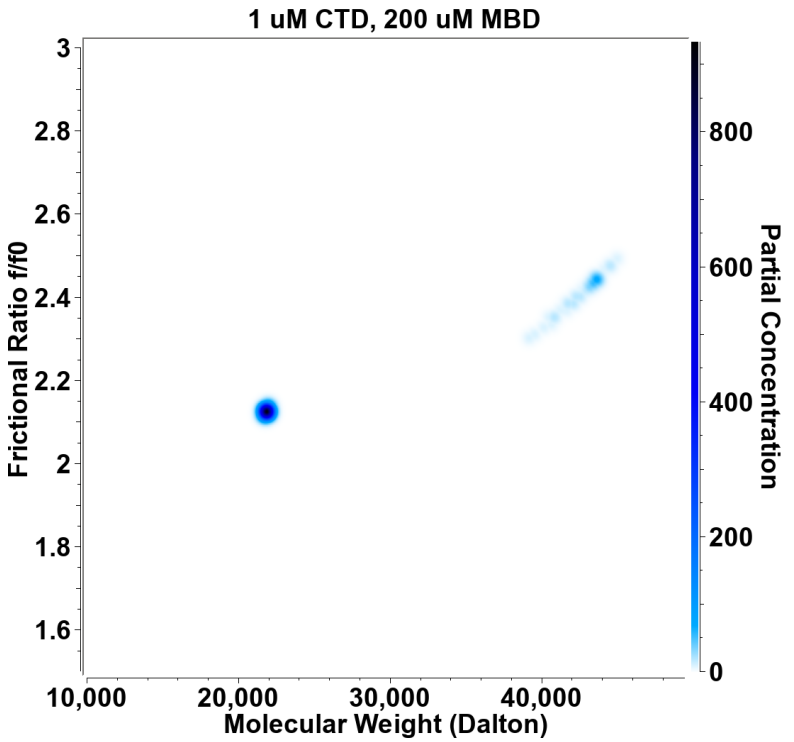
Measurement of interaction between 2 binding domains, MBD (11.2 kDa) and CTD (20.4 kDa). In this case, CTD (the larger molecule) is labeled. So the expected change in s is going to be small and easily missed. Earlier experiments showed no binding when MBD was labeled.

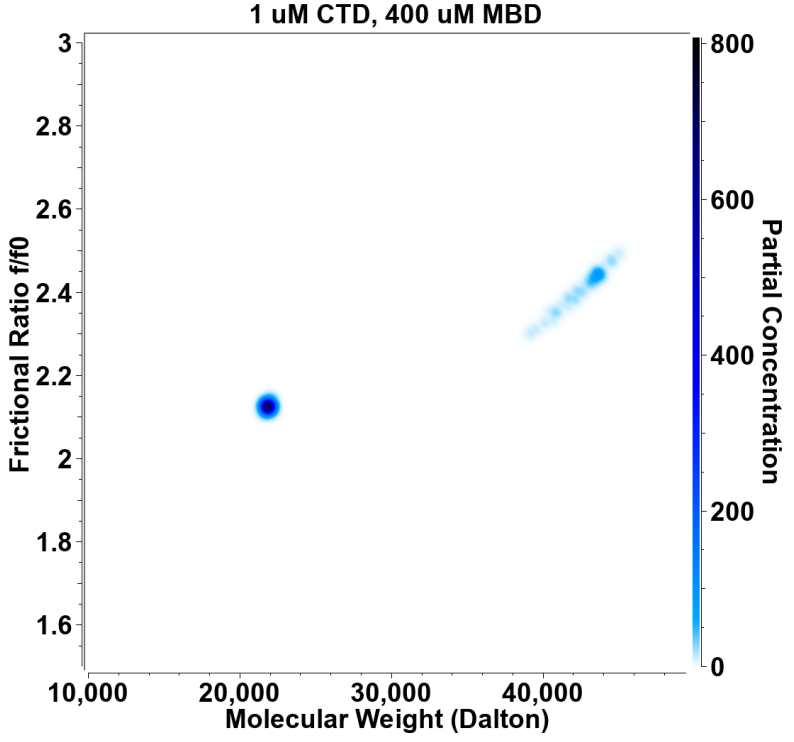
Caveat: Fluorescence label may interfere with binding!

Other caveat: Binding may be missed if change in s is too small to be detected.

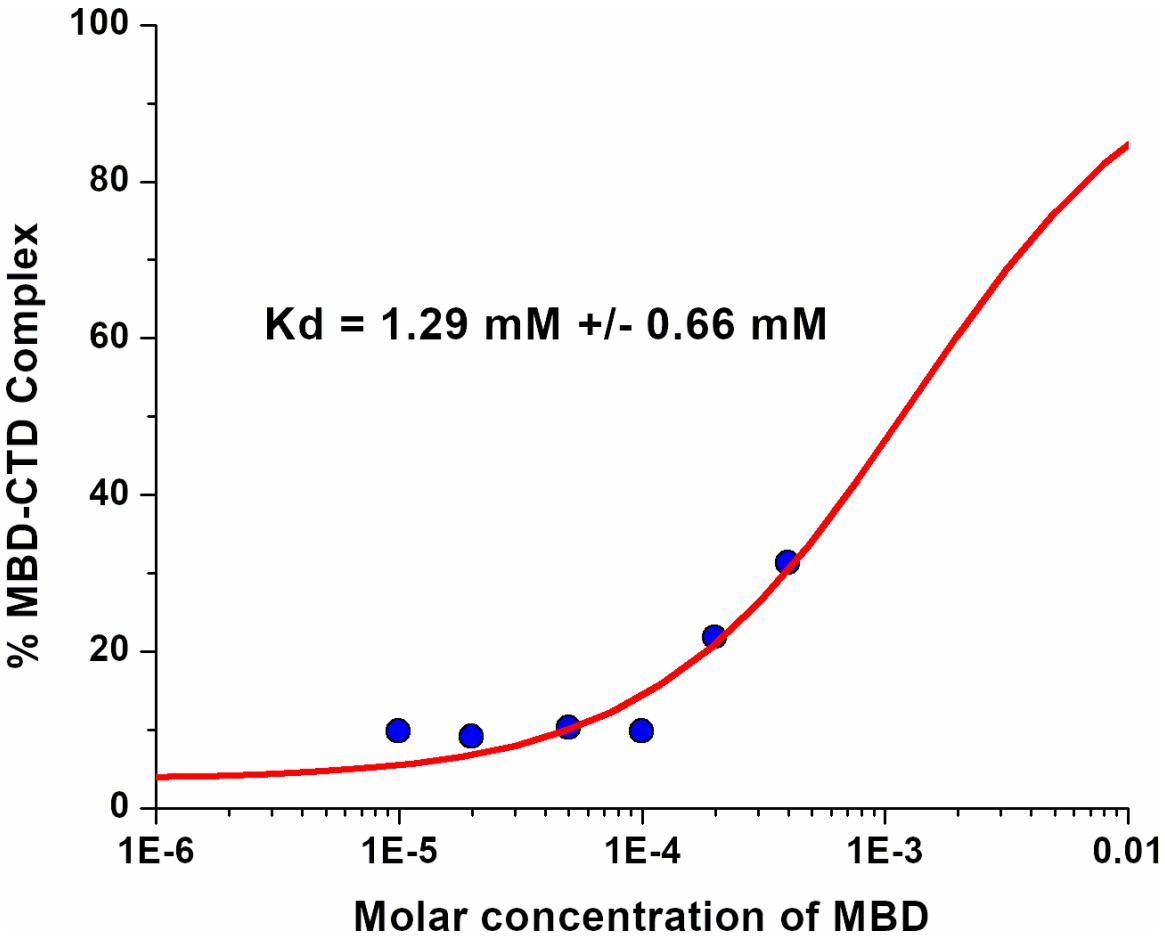

1 uM CTD
Frictional Ratio f/f0
3
2.8
2.6
2.4
2.2
2
1.8
1.6
10,000
20,000
30,000
40,000
Molecular Weight (Dalton)
Partial Concentration
800
600
400
200
0


1 uM CTD, 100 uM MBD
Frictional Ratio f/f0
3
2.8
2.6
2.4
2.2
2
1.8
1.6
10,000
20,000
30,000
40,000
Molecular Weight (Dalton)
Partial Concentration
800
600
400
200
0


1 uM CTD, 200 uM MBD
Frictional Ratio f/f0
3
2.8
2.6
2.4
2.2
2
1.8
1.6
10,000
20,000
30,000
40,000
Molecular Weight (Dalton)
Partial Concentration
800
600
400
200
0

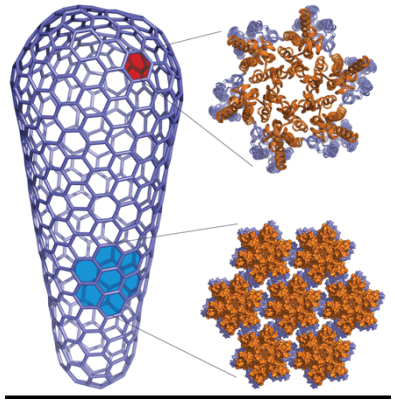

1 uM CTD, 400 uM MBD
Frictional Ratio f/f0
3
2.8
2.6
2.4
2.2
2
1.8
1.6
10,000
20,000
30,000
40,000
Molecular Weight (Dalton)
Partial Concentration
800
600
400
200
0

Kd measurements for hetero-associations

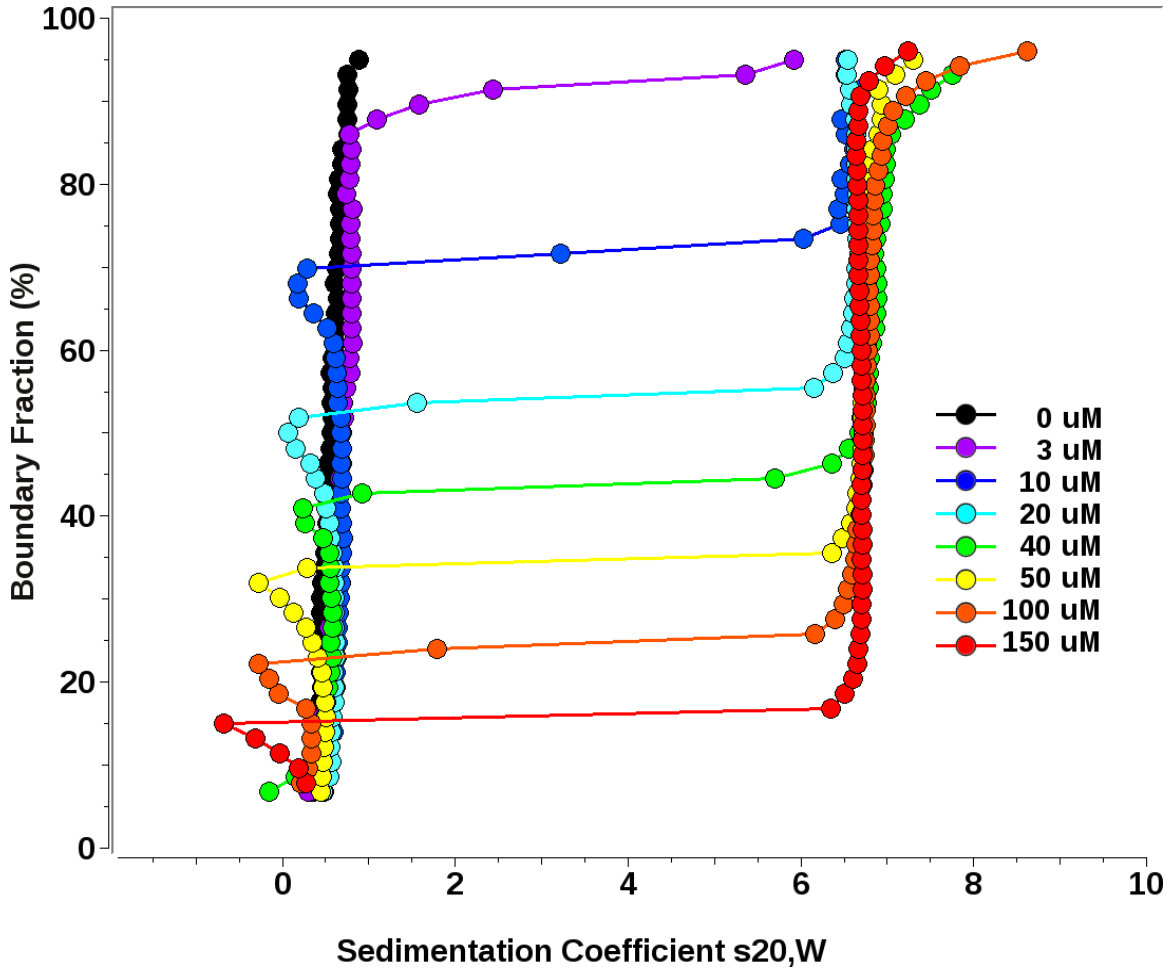


Kd measurements for hetero-associations

Binding of protein domain to HIV1 capsid

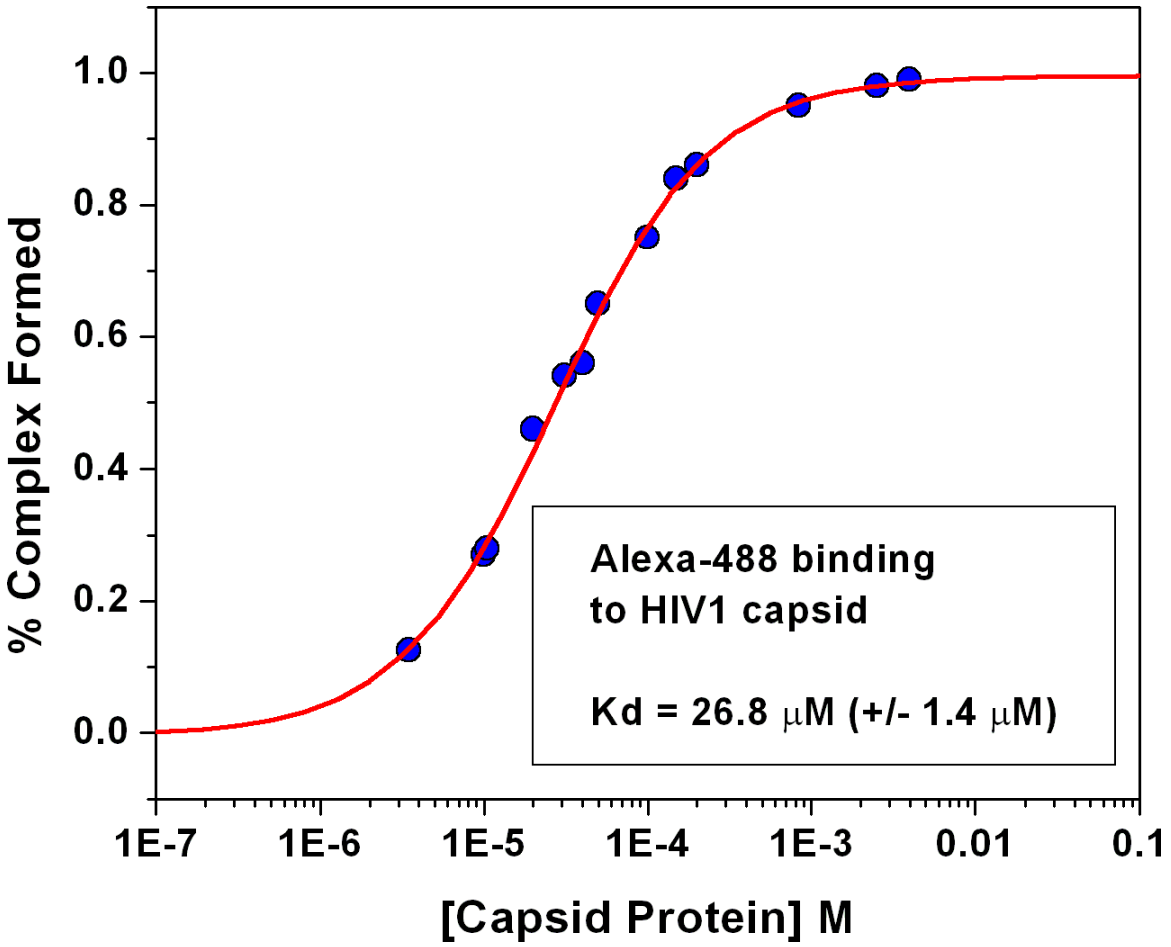


+ Alexa-488 labeled binding protein



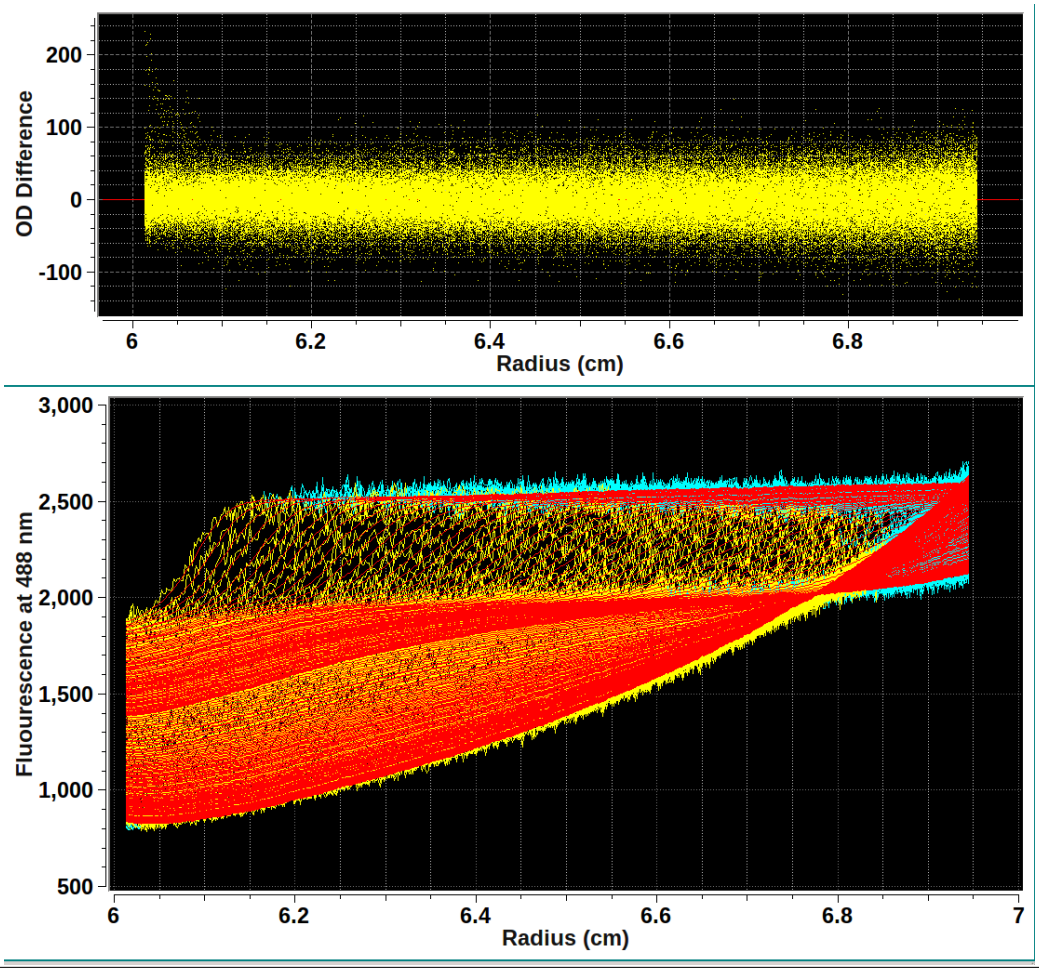
Kd measurements for hetero-associations

Binding of protein domain to HIV1 capsid



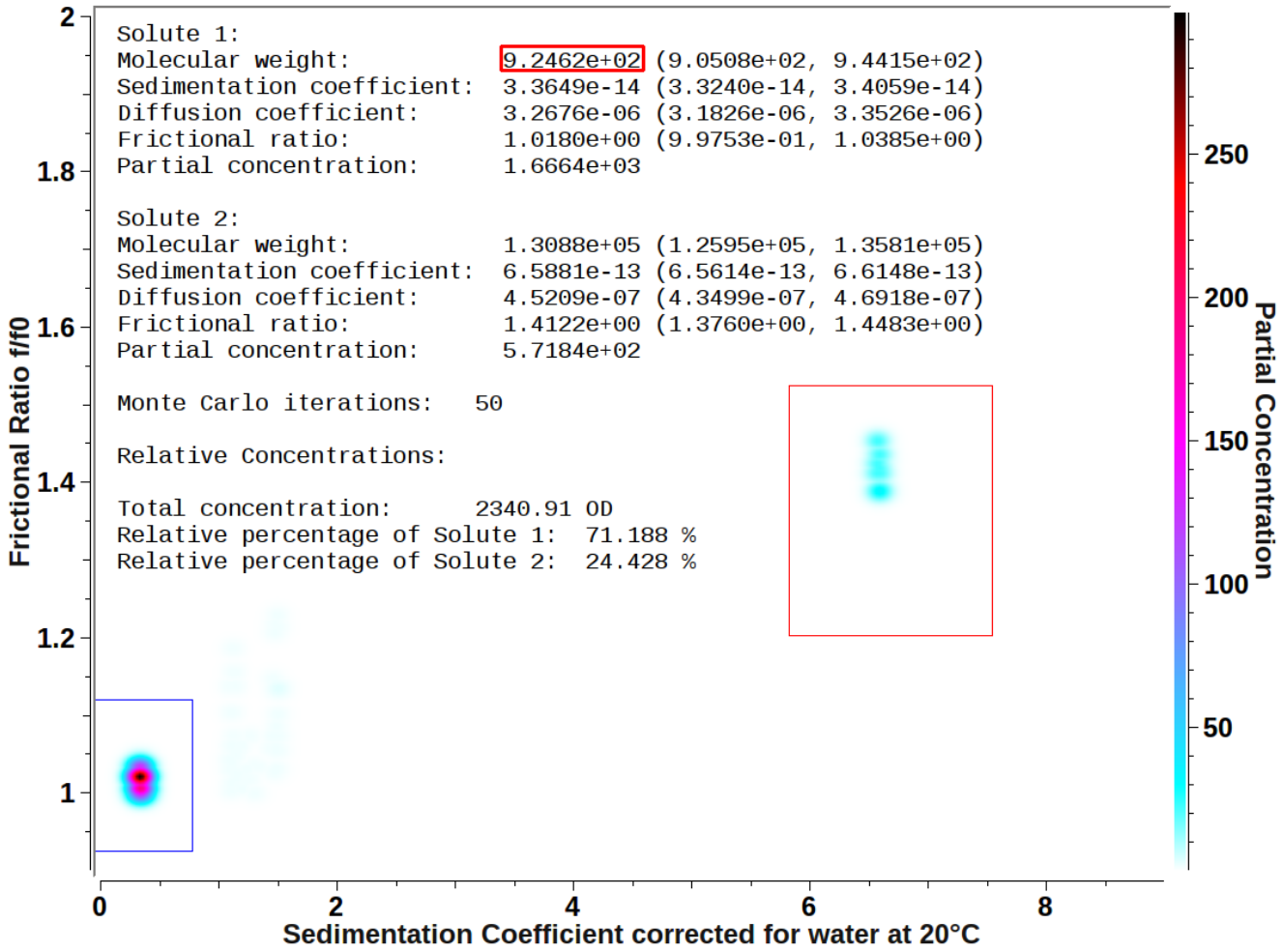
Kd measurements for hetero-associations

Binding of protein domain to HIV1 capsid (10 µM binding protein)



Kd measurements for hetero-associations

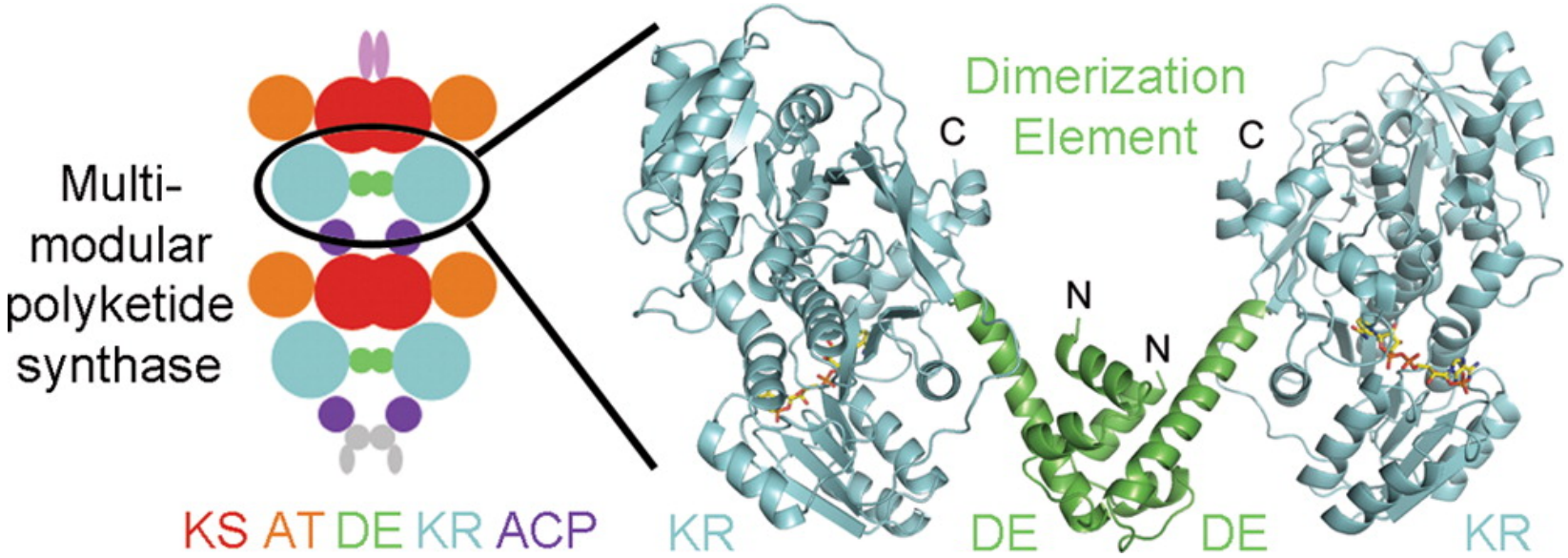
Binding of protein domain to HIV1 capsid



Caveat:

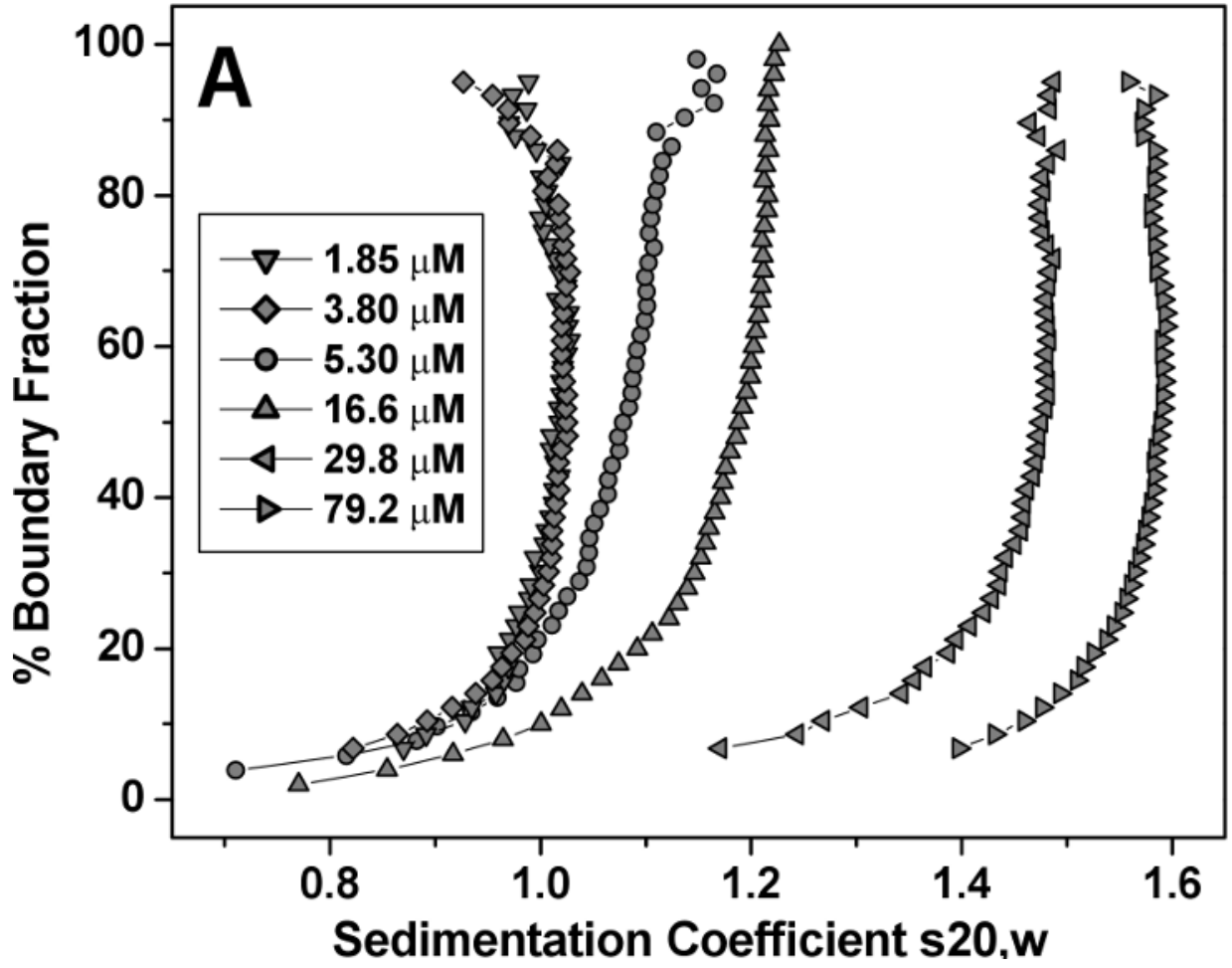
Always check to make sure molecular weights or sedimentation coefficients make sense!

Reversible self-association of Polyketide Synthase Dimerization Motif



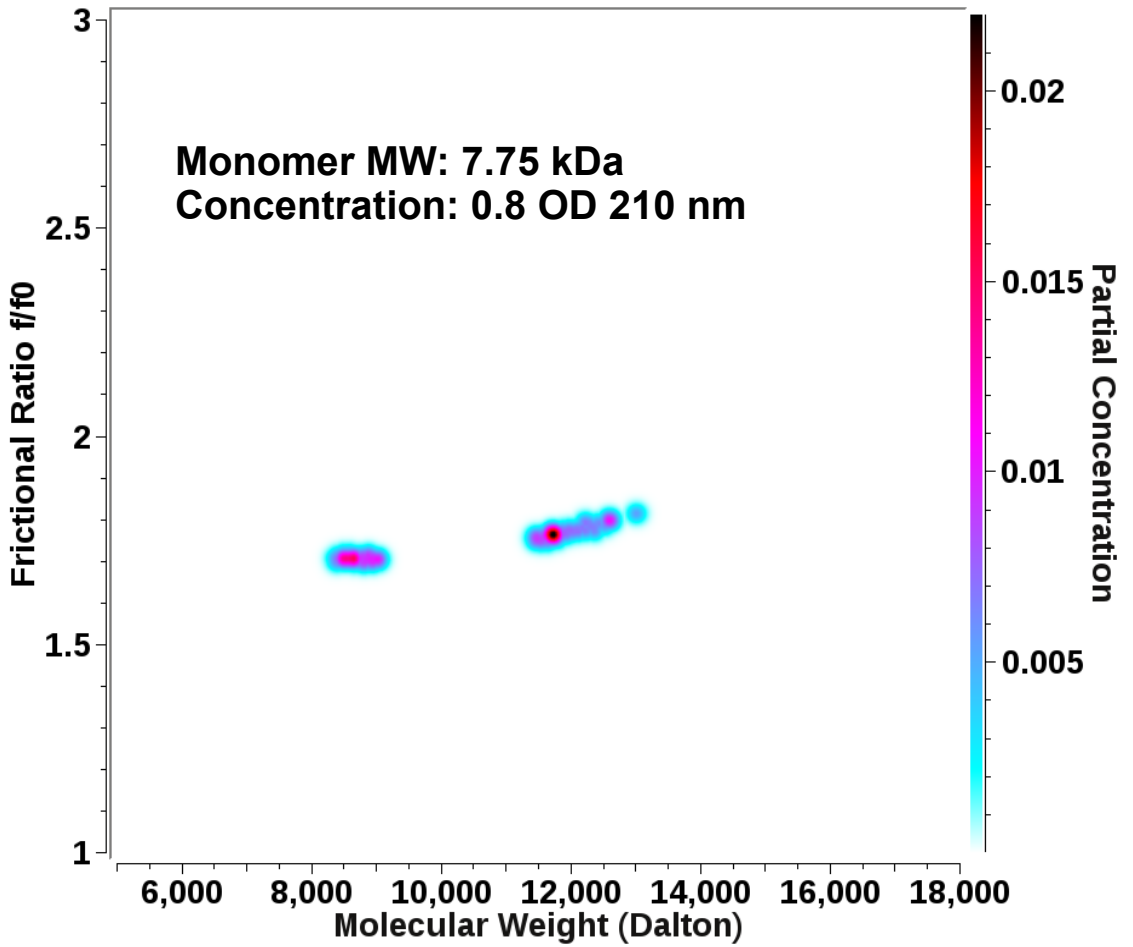
The dimerization of multimodular polyketide synthases is essential for their function. Motifs that supplement the contacts made by dimeric polyketide synthase enzymes have previously been characterized outside the boundaries of modules, at the N- and C-terminal ends of polyketide synthase subunits.

Reversible self-association of Polyketide Synthase Dimerization Motif



Van Holde - Weischet integral sedimentation distributions from reaction boundaries for SpnDE1 at different concentrations

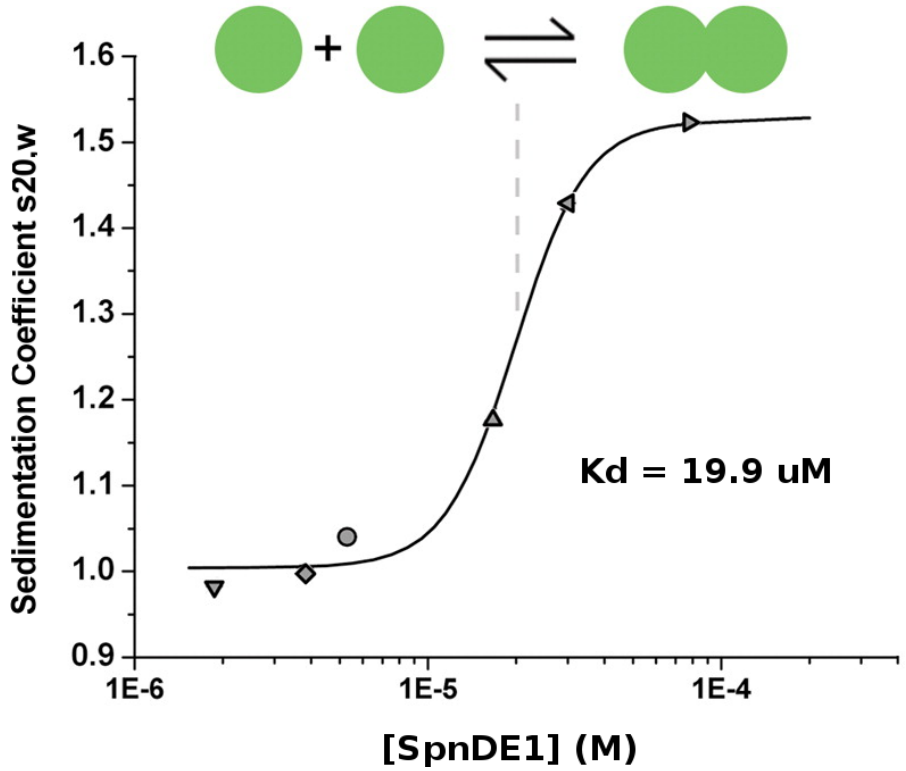
Reversible self-association of Polyketide Synthase Dimerization Motif



$$\bar{s} = \frac{\sum_{j=1}^{m} s_j C_j}{C_T}$$

$$\bar{D} = \frac{\sum_{j=1}^{m} D_j \left(\partial C_j / \partial r\right)}{\sum_{j=1}^{m} \left(\partial C_j / \partial r\right)}$$

Reversible self-association of Polyketide Synthase Dimerization Motif

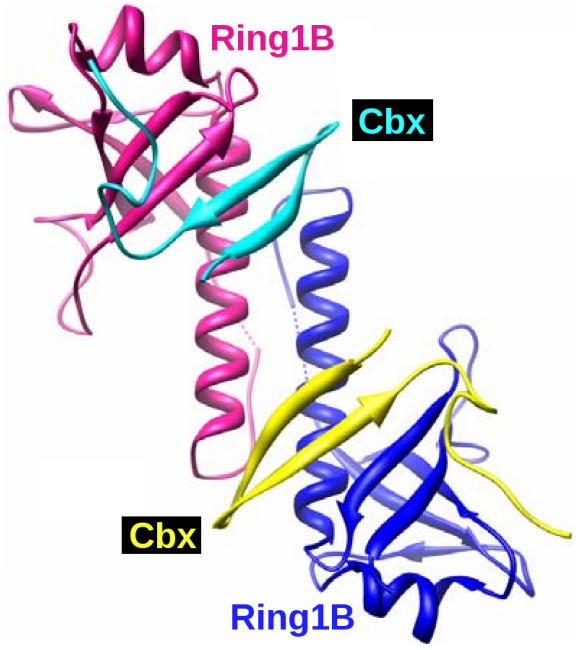


Jianting Zheng, Chris D. Fage, Borries Demeler, David W. Hoffman & Adrian T. Keatinge-Clay. The missing linker: a dimerization motif located within polyketide synthase modules. ACS Chem. Biol. (2013)

Monomer-Dimer Interface Mutation Analysis

Example 2: Ring1B mutation analysis (Dr. Chong Kim, UTHSCSA)

Assembly of Polycomb Repression Complex 1 (PRC1) (Wang et al., 2009)
- involved in chromatin packaging and responsible for gene silencing during differentiation



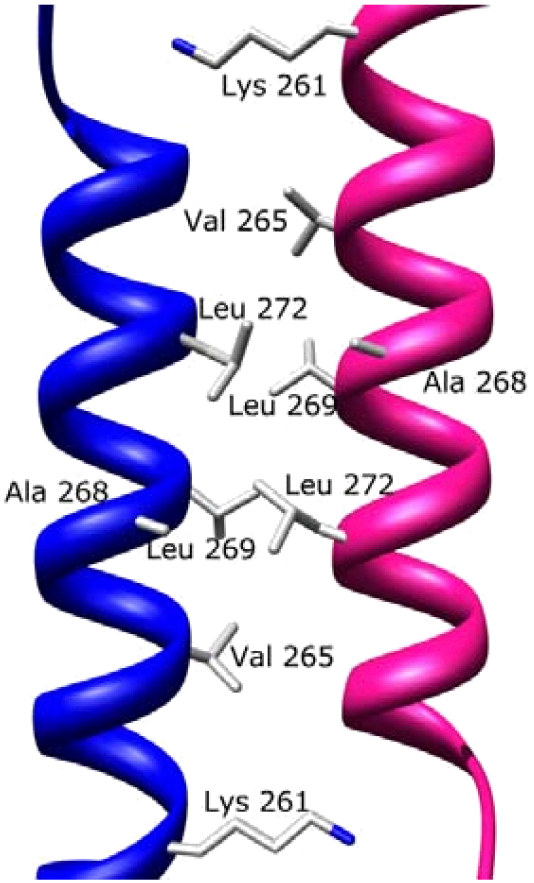
PRC1 contains 4 proteins: Ring1B, Polyhomeotic, Polycomb, and BMI1.
What is the stoichiometry in PRC1?
It is thought to be 1:1:1:1

Observations:

Ring1B binds the C-terminal domain of Polycomb, but crystallizes as a hetero-dimer. In solution without c-polycomb, Ring1B is a dimer. Is the crystal dimer interface the same observed in solution?

Monomer-Dimer Interface Mutation Analysis

Example 2: Ring1B mutation analysis (Dr. Chong Kim, UTHSCSA)

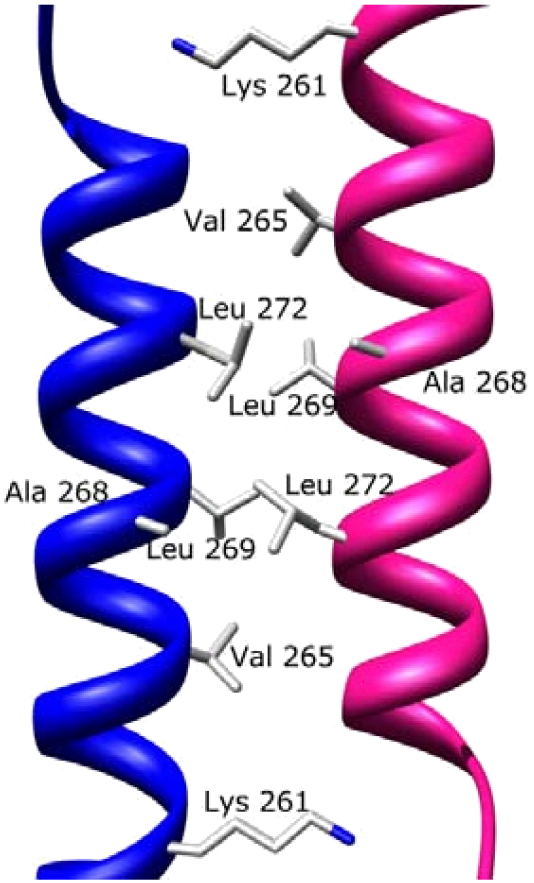


Question: Is the dimerization interface observed in crystal structure responsible for dimerization in solution?

Approach: mutate non-polar residues to charged residues to see if the dimer interface is disrupted.

Monomer-Dimer Interface Mutation Analysis

Example 2: Ring1B mutation analysis (Dr. Chong Kim, UTHSCSA)

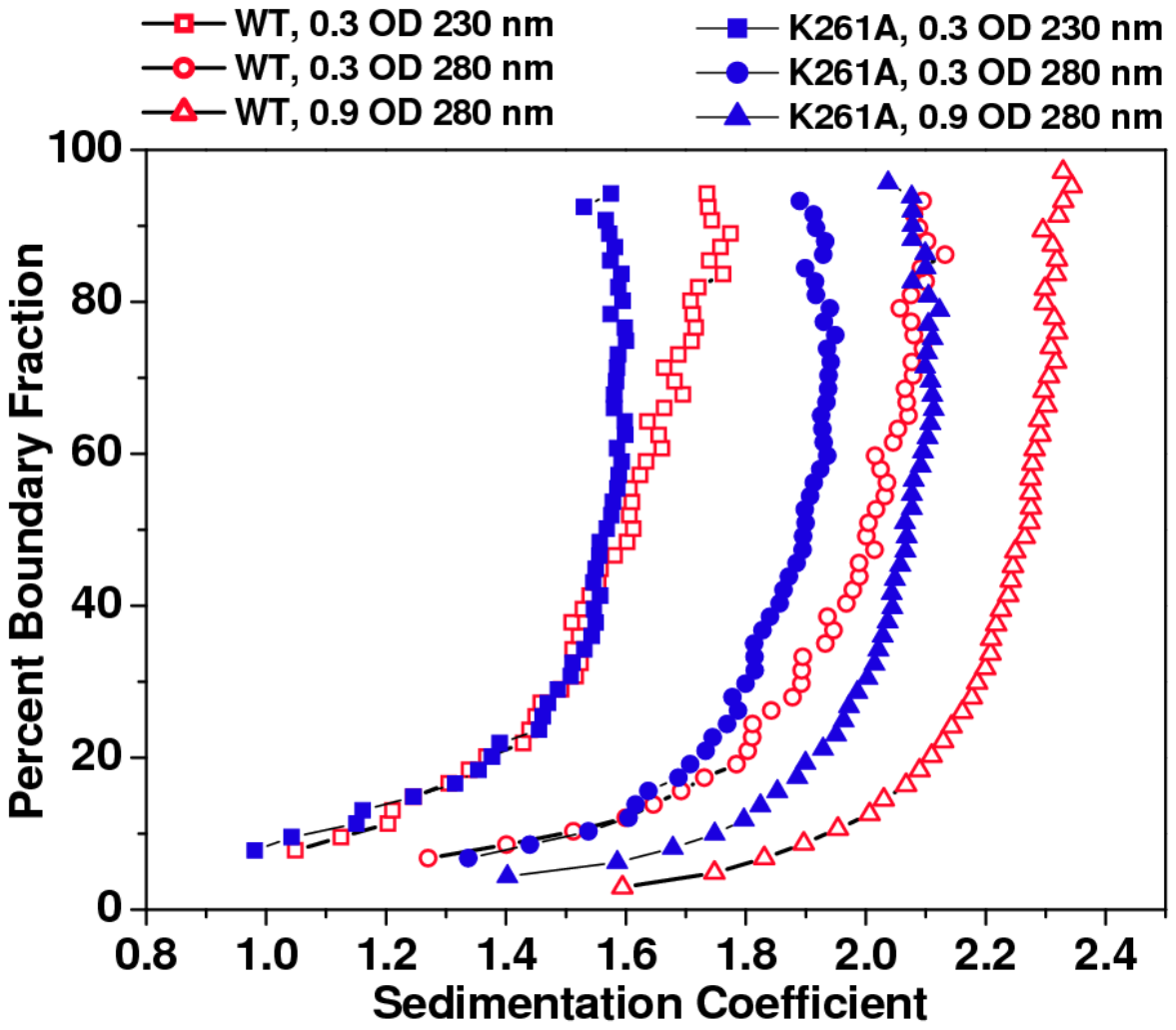


Hydrophobic residues were replaced by polar residues in dimerization study:

	Dimerizes?
Wildtype	yes
Val 265 Glu	no
Leu 269 Glu	no
Leu 272 Arg	no
Lys 261 Ala	yes

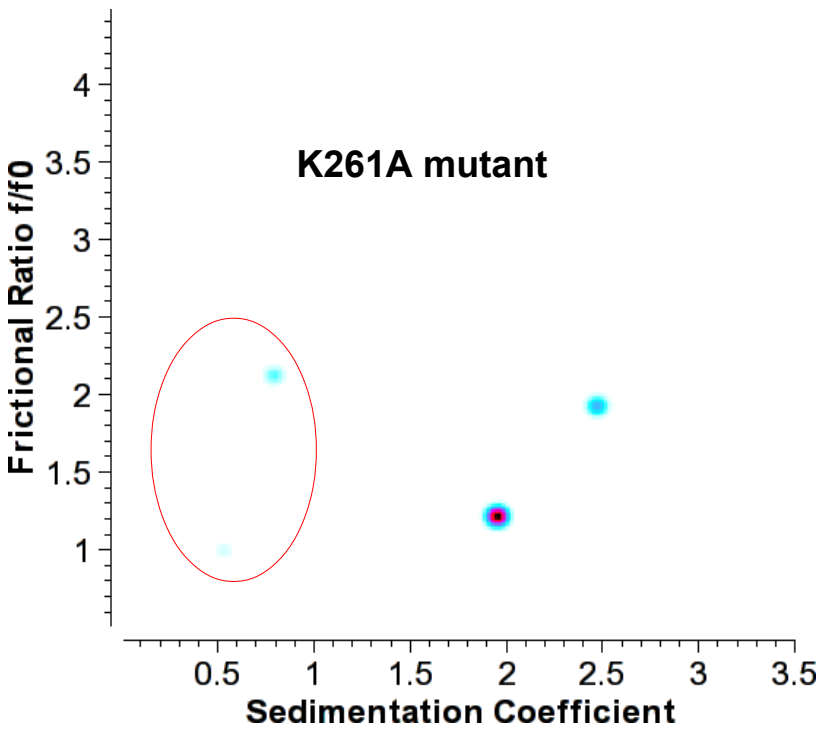
Answer: acidic residues seriously disrupt the dimer interface, while non-polar or basic residues have a slighter effect. But clearly the dimer interface observed in the crystal is present in solution as well.

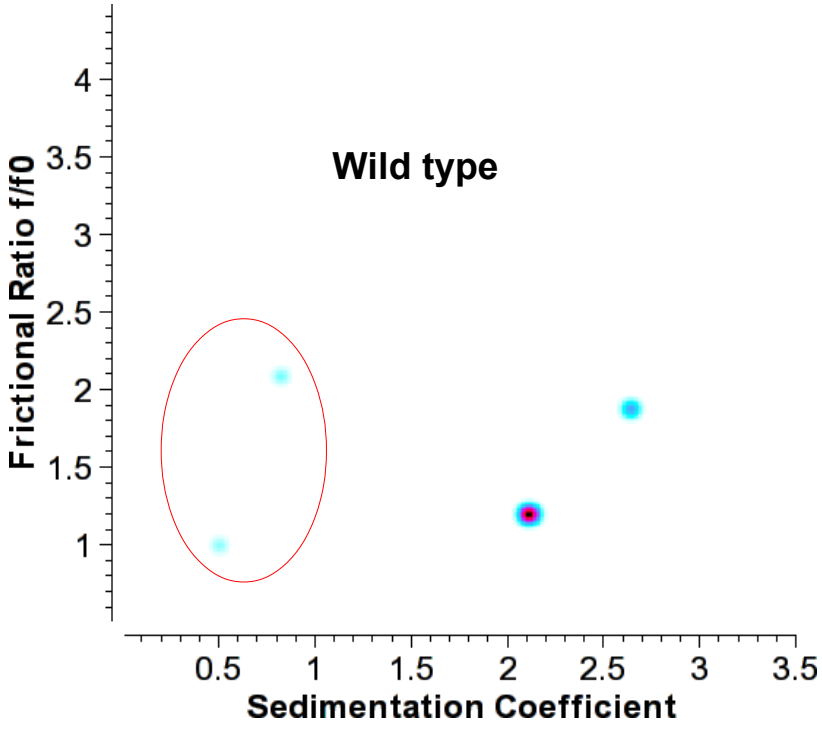
Monomer-Dimer Interface Mutation Analysis



Monomer-Dimer Interface Mutation Analysis

Non-interacting analysis (GA)



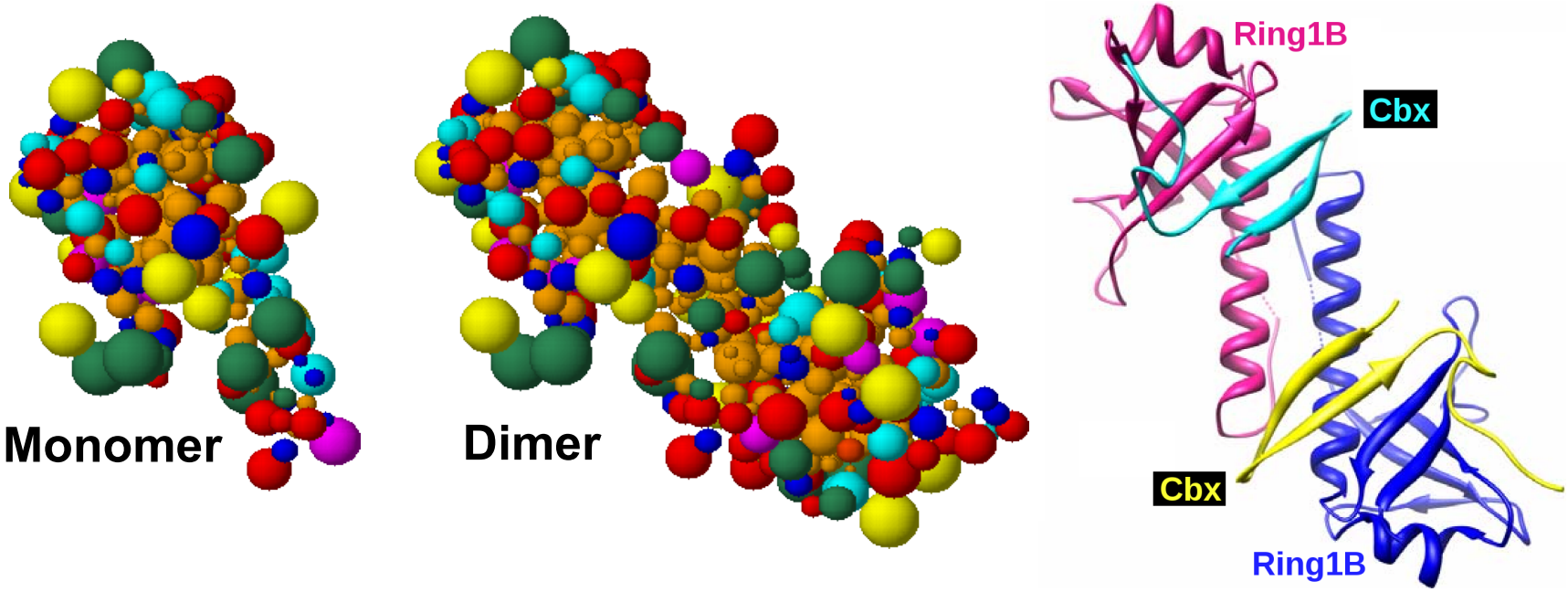


Monomer-Dimer Interface Mutation Analysis Velocity Results

Parameter:	wildtype, 0.9 OD	K261A, 0.9 OD
K_d (μM)	17.6 (14.8, 21.6)	28.5 (25.8, 31.8)
k_{off} (x 10^{-5} sec^{-1})	84.3 (48.6, 120.0)	14.1 (8.1, 20.1)
f/f_o (monomer)	1.14 (1.09, 1.19)	1.19 (1.17, 1.21)
f/f_o (dimer)	1.31 (1.30,1.32)	1.44 (1.43, 1.45)
f/f_o (contaminant)	1.24 (1.18, 1.3)	1.49 (1.47, 1.56)
contam. OD (x0.01)	3.56 (3.37, 3.75)	2.77 (2.58, 2.96)
Co. mol. wt. (x1000)	2.33 (2.09, 2.56)	3.00 (2.93, 3.06)

SV fitting results for C-RING1B wildtype and K261A mutant to a reversible monomer-dimer equilibrium model that allows for the presence of a contaminant. Values in parentheses represent 95% confidence intervals.

Monomer-Dimer Interface Mutation Analysis



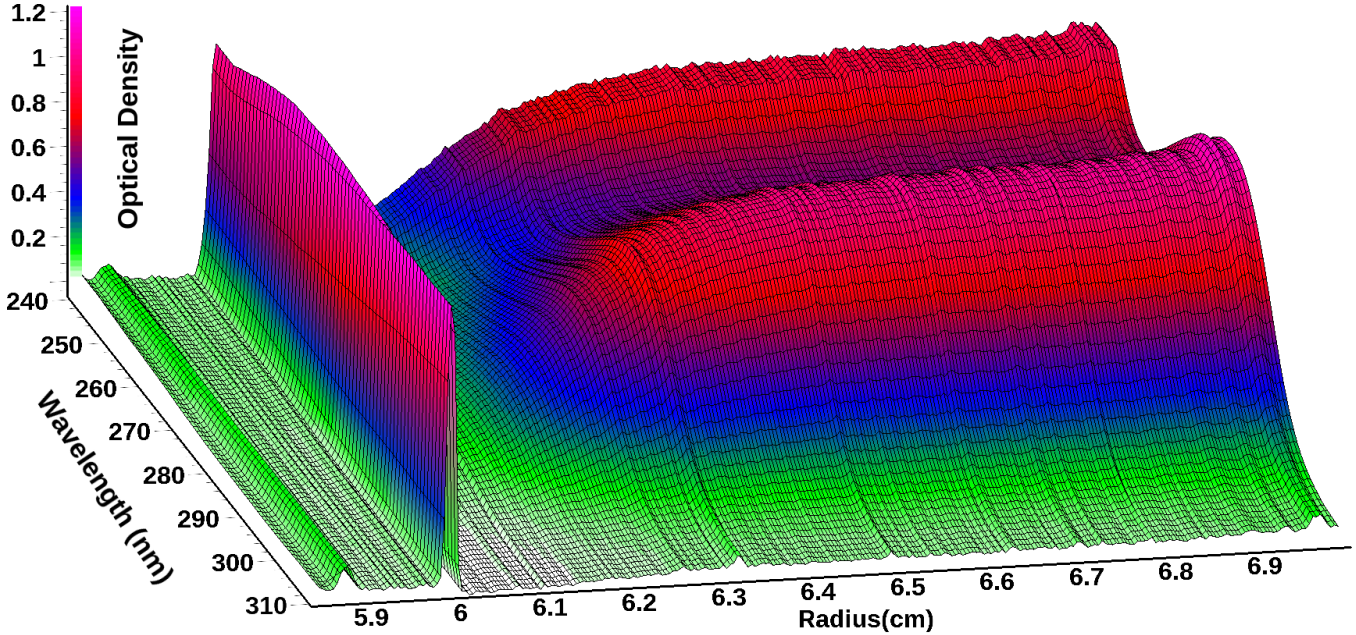
UltraScan SOMO bead model results for Ring1B Wildtype structure:

f/f_0 = 1.26 for the monomer and f/f_0 = 1.32 for the dimer

Measured:

f/f_0 = 1.14 (1.09, 1.19) for monomer, f/f_0 = 1.31 (1.30, 1.32) for dimer

MWL-AUC Analysis of Protein/Nucleic Acid Mixtures

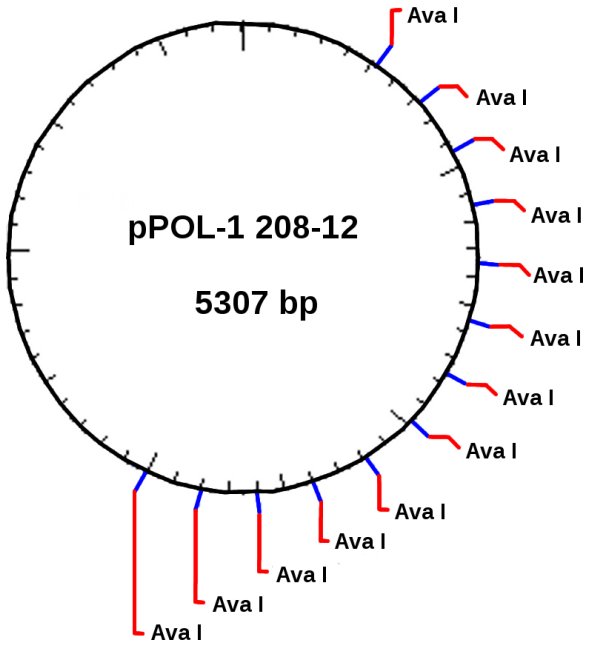


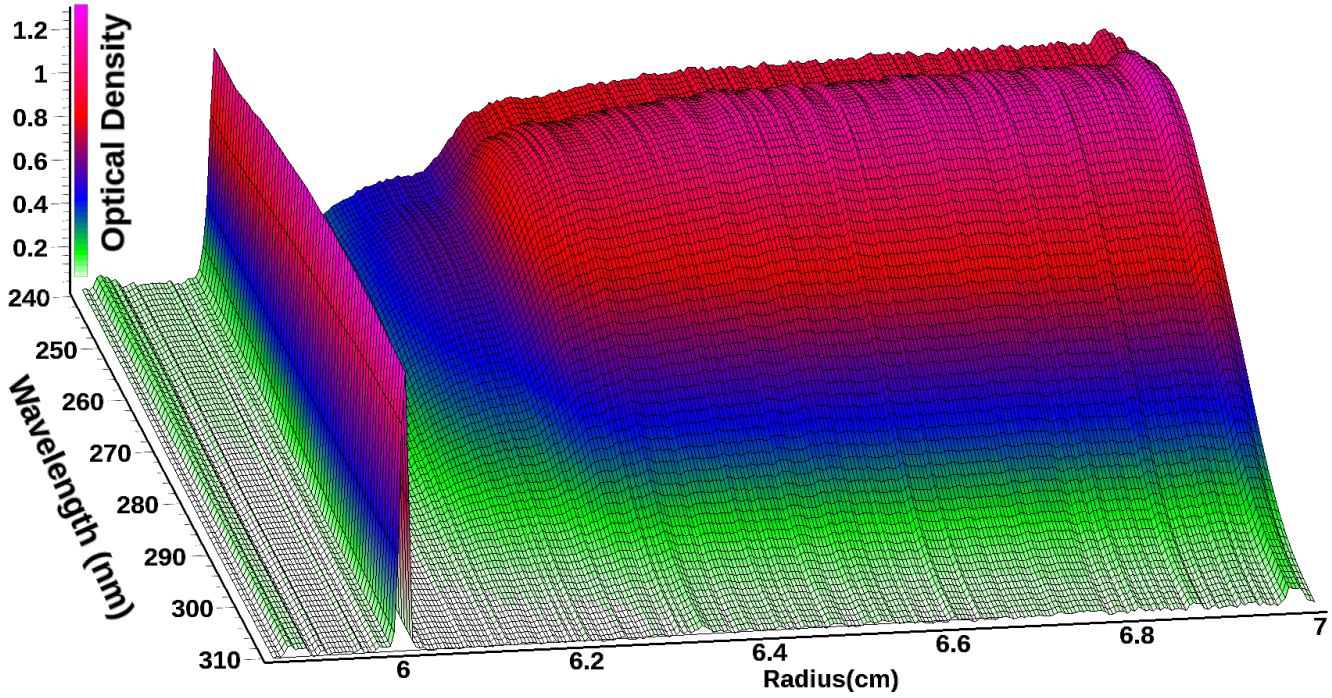
Testing reliability of the method with mixtures of BSA and a DNA plasmid digest:

1 x 2811 bp

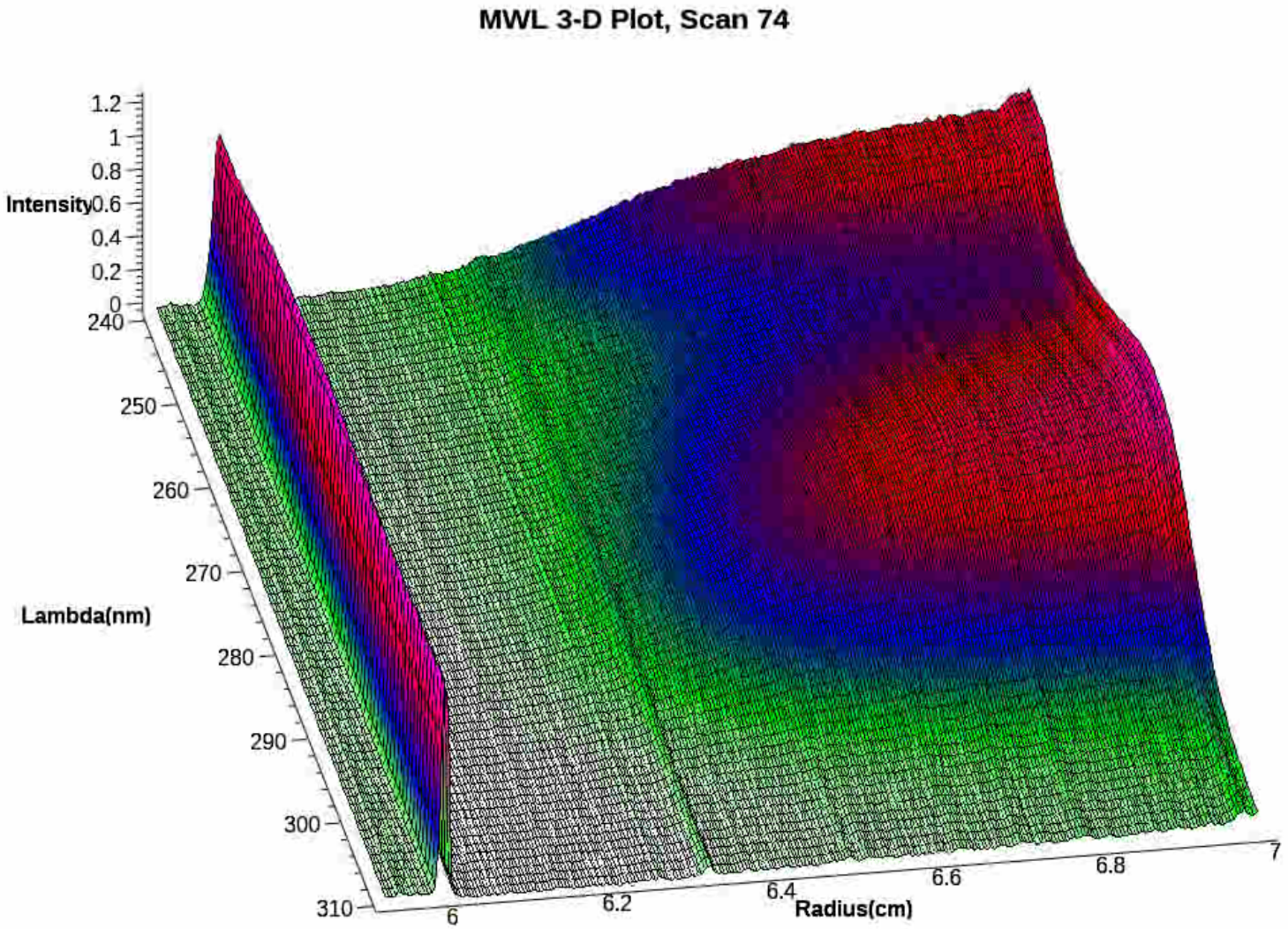
12 x 208 bp

12 x 12 bp

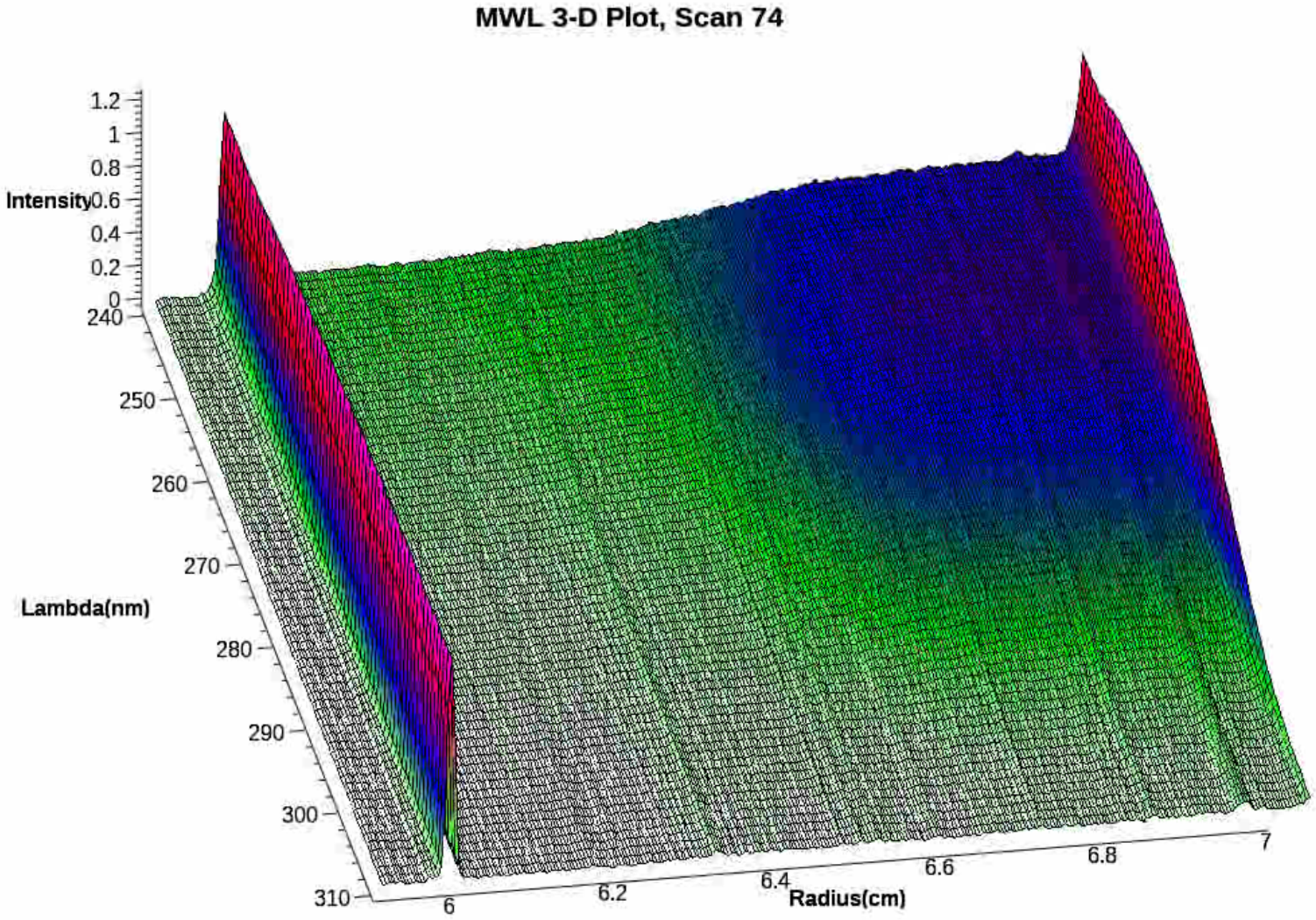




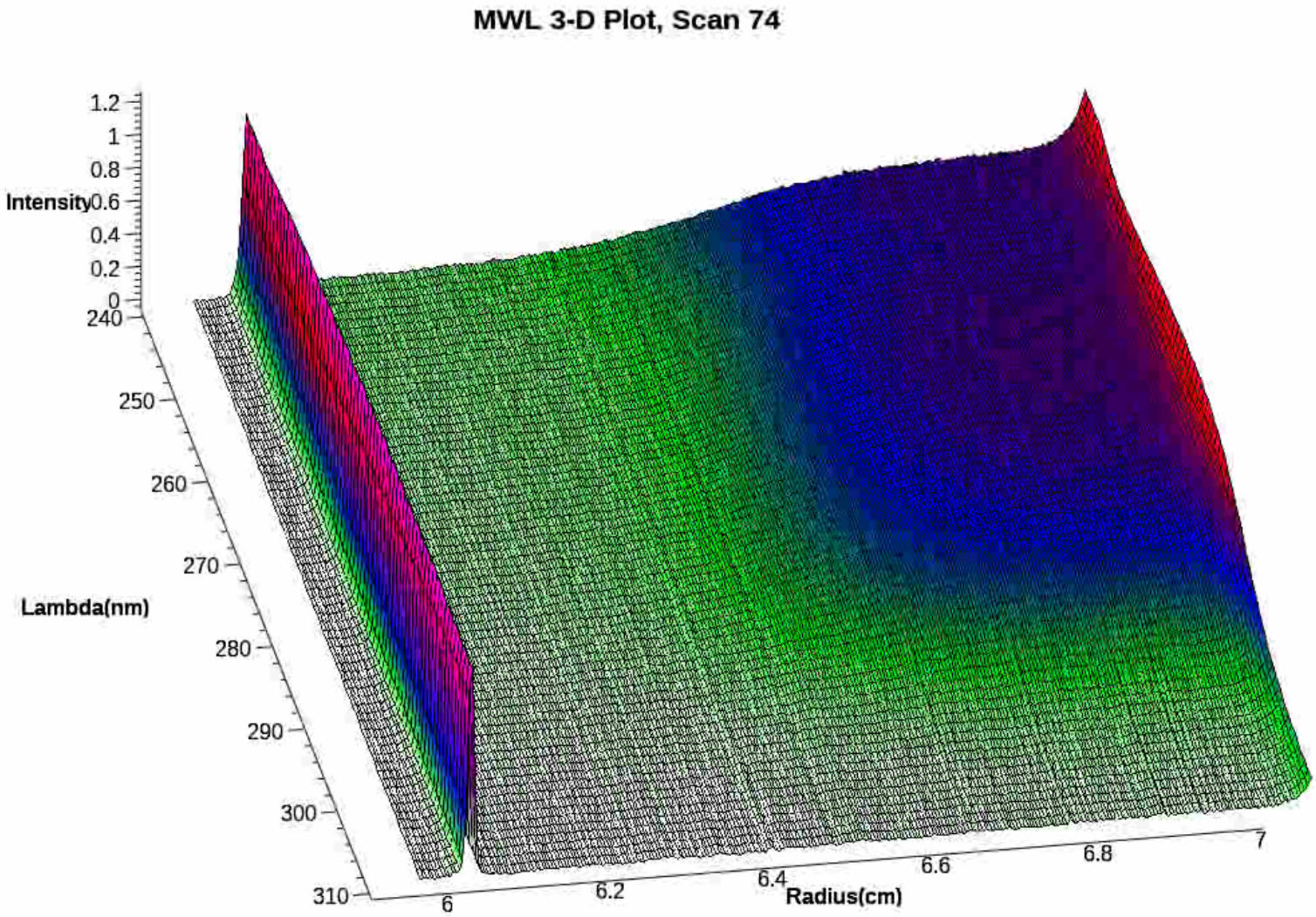
Multi-wavelength Sedimentation Velocity: BSA



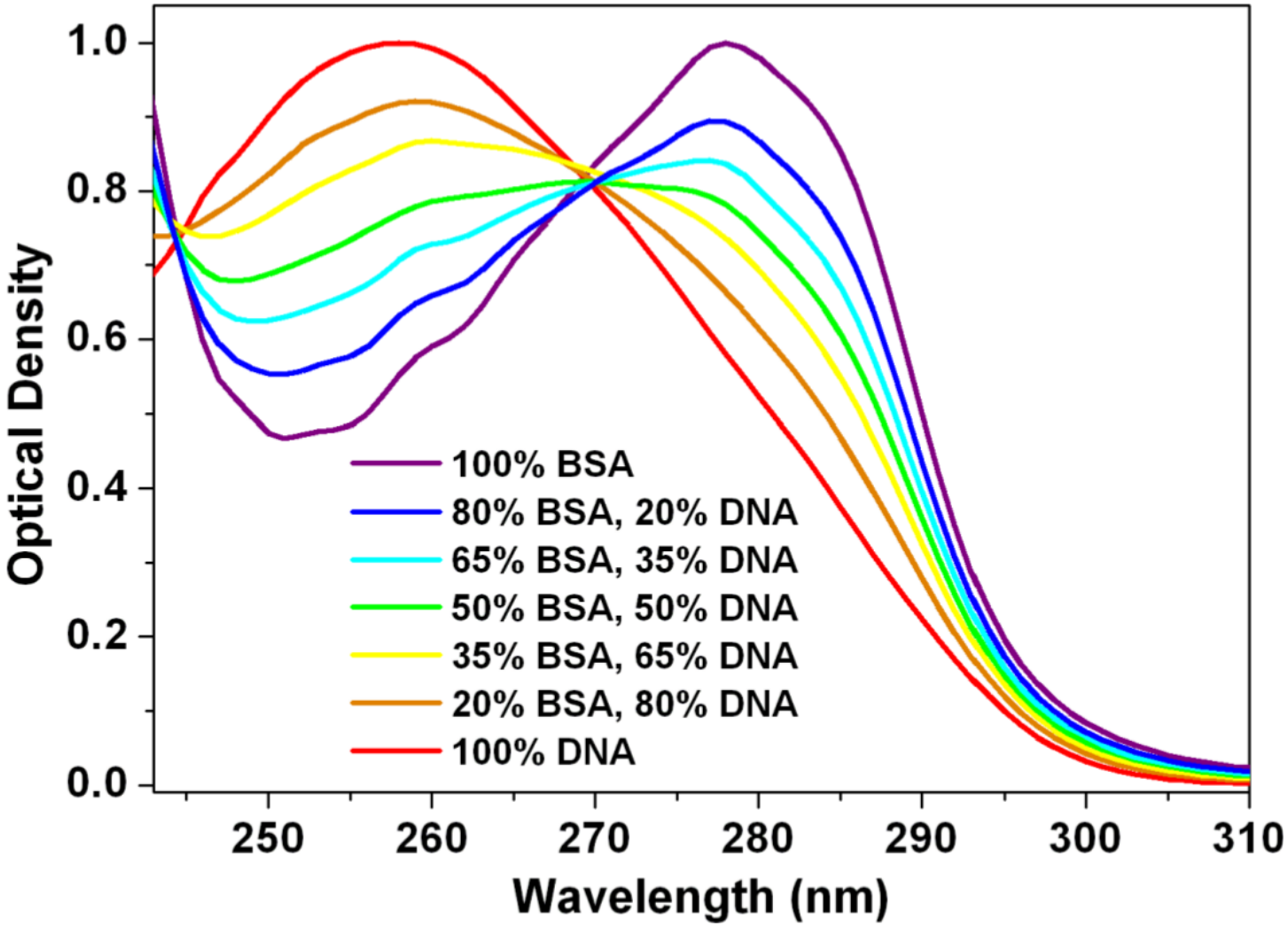
Multi-wavelength Sedimentation Velocity: DNA



Multi-wavelength Sedimentation Velocity: DNA-BSA Mixture

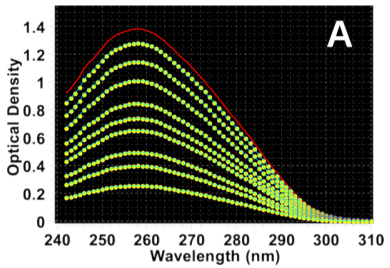


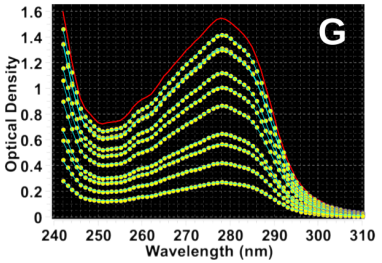
Fitted spectra L_j for different ratios of DNA – BSA Mixture



$$\theta = \cos^{-1}\left[\frac{u \cdot v}{\|u\| \cdot \|v\|}\right]$$

Use L_j Spectra for Spectral Decomposition





Scan 1

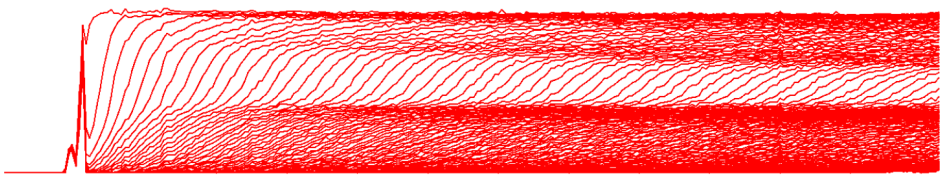
Scan n

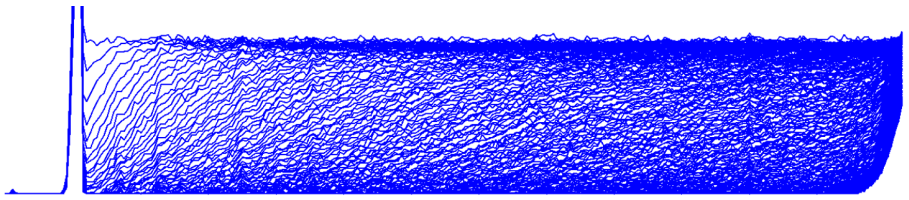
$$C_{MWL} = x_a \begin{bmatrix} \epsilon_{a1} \\ \epsilon_{a2} \\ \dots \\ \epsilon_{ai} \end{bmatrix}_{r,t} + x_b \begin{bmatrix} \epsilon_{b1} \\ \epsilon_{b2} \\ \dots \\ \epsilon_{bi} \end{bmatrix}_{r,t}$$

NNLS

L_{DNA}

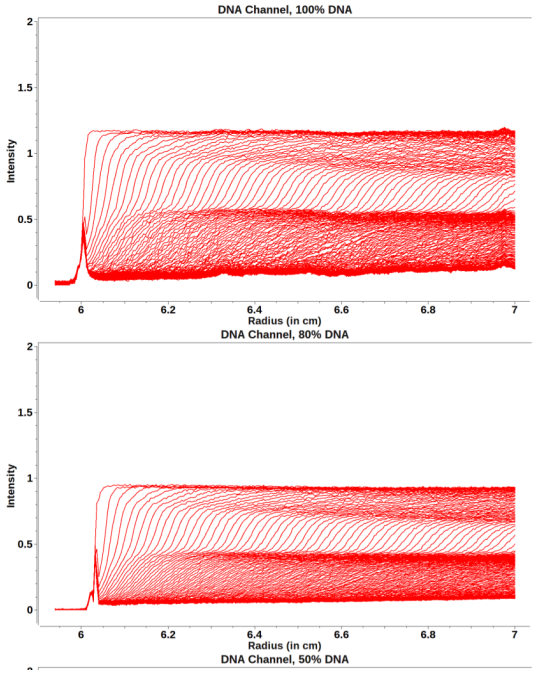
L_{BSA}

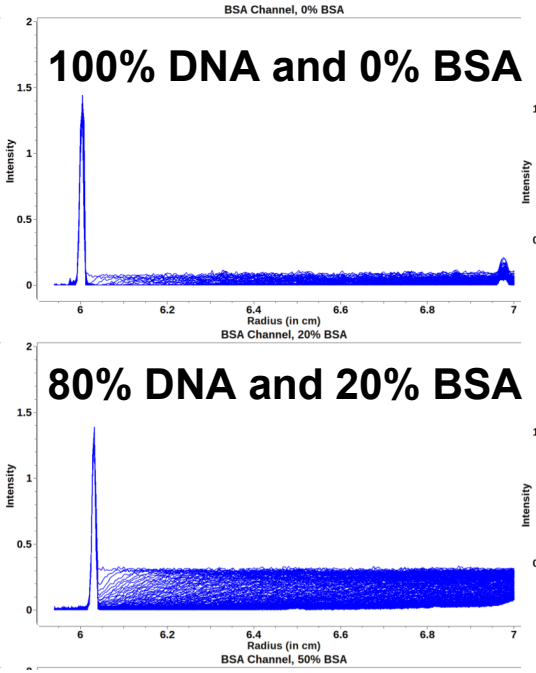


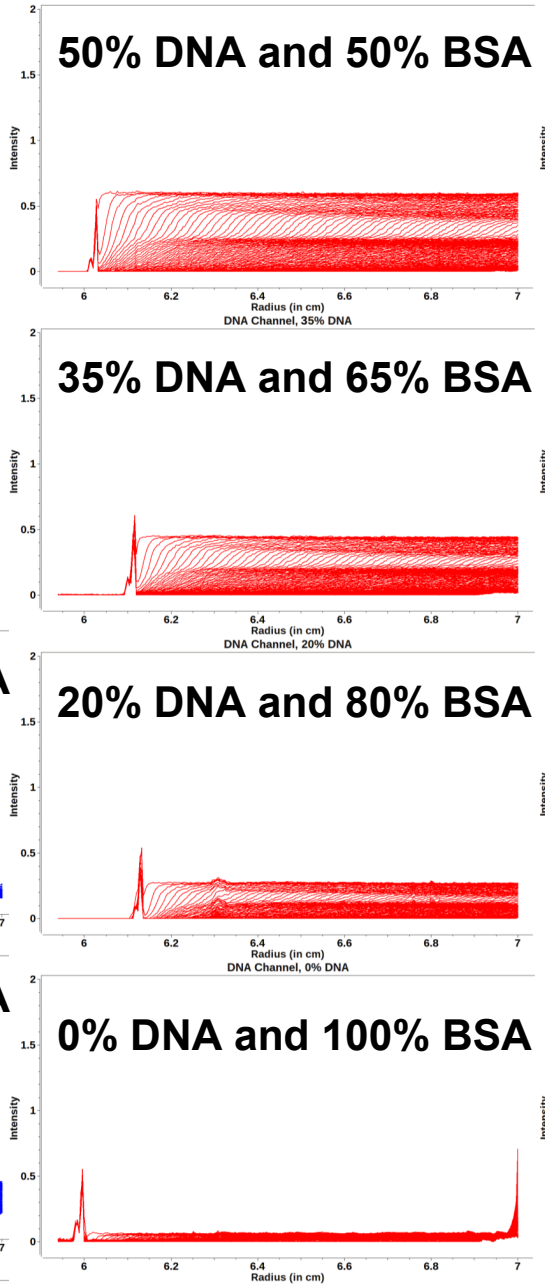


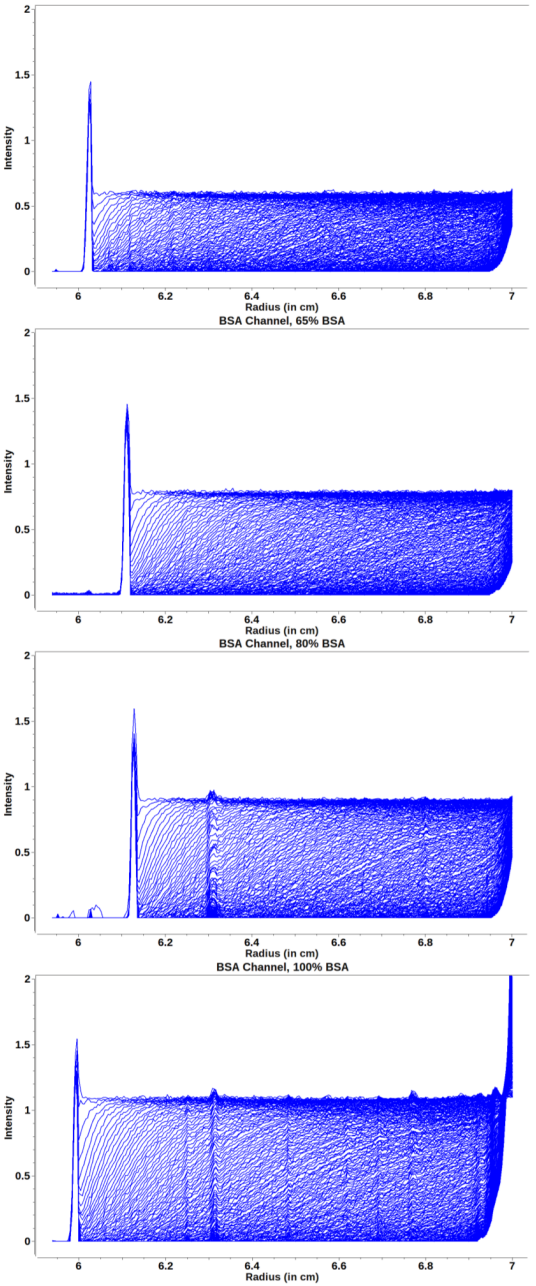
Multi-Wavelength Decomposition of a range of DNA/BSA mixtures using pure 100% DNA and 100% BSA spectra:

$$C_{MWL} = x_a \underbrace{\begin{bmatrix} \epsilon_{a1} \\ \epsilon_{a2} \\ \dots \\ \epsilon_{ai} \end{bmatrix}_{r,t}}_{L_{DNA}} + x_b \underbrace{\begin{bmatrix} \epsilon_{b1} \\ \epsilon_{b2} \\ \dots \\ \epsilon_{bi} \end{bmatrix}_{r,t}}_{L_{BSA}}$$

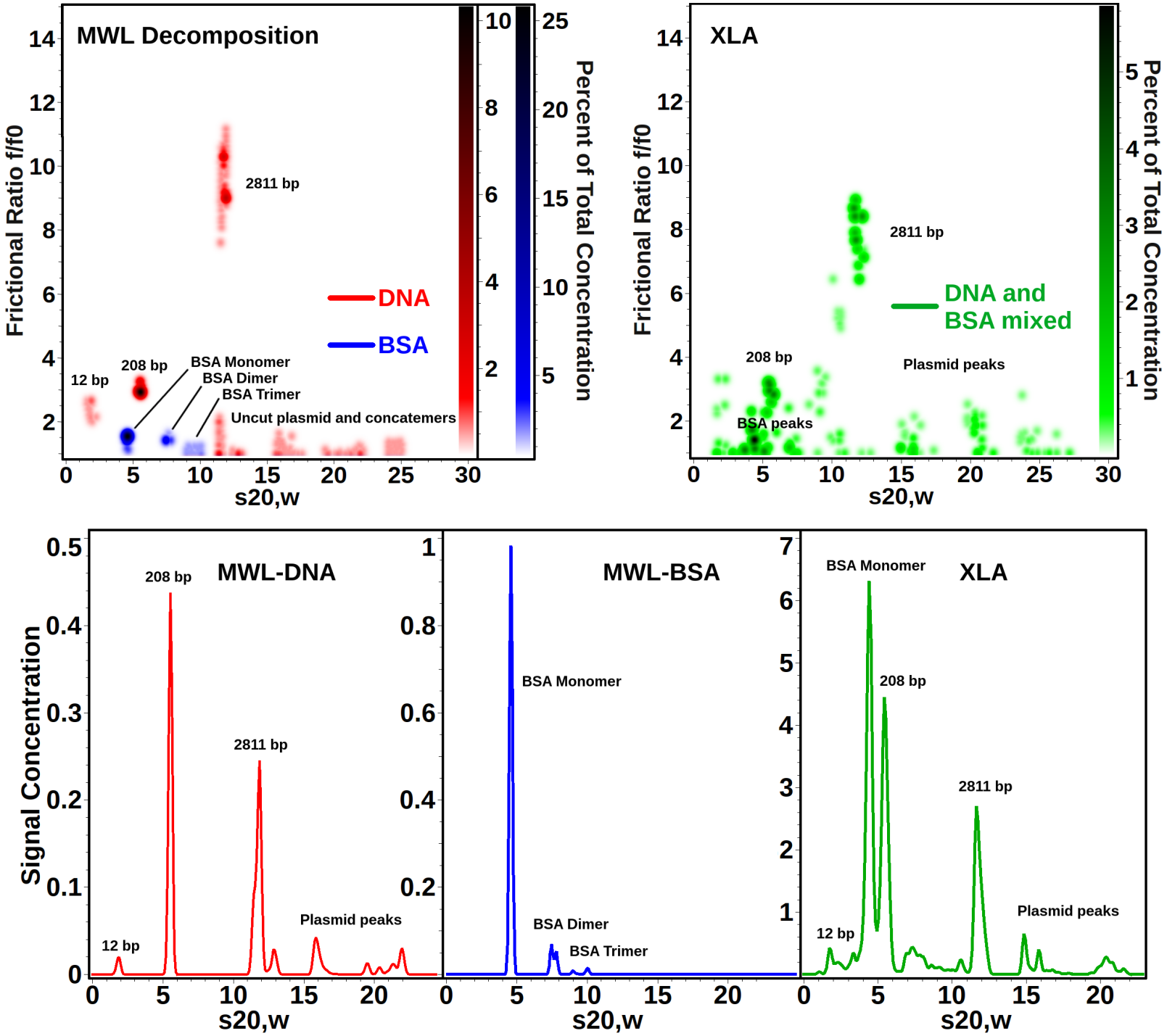








Global Genetic Algorithm Analysis



Homework (p1 of 3)

TABLE 4.3 *Diffusion coefficients*[a]

Substance	*Molecular Weight*	$D_{20,w} \times 10^6$ [b]	*Method*[c]
Glycine[d]	75	9.335	G
Sucrose[d]	342	4.586	G
Ribonuclease	13,683	1.068	R
Serum albumin[d] (bovine)	66,500	0.603	R
Tropomyosin	93,000	0.224	S
Fibrinogen[d] (human)	330,000	0.197	R
Myosin[d]	440,000	0.105	S
Tobacco mosaic virus	About 40,000,000	0.053	S
Robbit papilloma virus	About 47,000,000	0.059	S

[a]The diffusion coefficients have been corrected to water at 20°C (see Chapter 5 for the procedure).

[b]Note that D generally decreases with molecular weight but that elongated molecules such as tropomyosin, fibrinogen, myosin, and TMV have unusually low values. The dimensions of D are cm^2/sec.

[c]G, Gouy; R, Rayleigh; S, Schlieren.

[d]Extrapolated to zero concentration.

Homework (p2 of 3)

(A) Given the molecular weights and diffusion coefficients in the table, calculate the following values for Bovine Serum Albumin (vbar = 0.732 ml/g), Ribonuclease (vbar = 0.708 ml/g), Myosin (vbar = 0.731 ml/g), and for a 208 bp double-stranded DNA fragment (vbar = 0.55 ml/g, $D=1.9 \times 10^{-7}$ cm^2/sec, MW=131,000 Da):

1. sedimentation coefficient (10%), 2. frictional ratio (10%), 3. Stokes radius (10%), 4. minimal radius (10%)

5. Which of these molecules is most non-globular? (10%)

6. Using your answer from (3), calculate the amount of water for each protein that would have to be bound to account for the Stokes radius of the molecule in terms of the ratio of grams of water : grams of protein. Is that reasonable? (10%)

Homework (p3 of 3)

(B) Indicate if *s* and *D* increase, decrease or stay the same when the following events occur, and justify your answer:

1. an anisotropic (asymmetric) monomeric protein aggregates into a large globular blob (8 %)

2. a monomeric protein unit elongates through head-to-tail association, forming a fibril shape (8 %)

3. a DNA molecule is dialyzed from a 500 mM NaCl solution into a 5 mM NaCl solution (8 %)

4. a globular, well-folded protein unfolds into a denatured state (8 %)

5. a monomeric, globular protein associates to form a globular hexamer (8 %)

Show ALL work, print legibly!

Homework

a) The anhydrous density of a protein is 1.43 g/ml. When measured in the ultracentrifuge, the partial specific volume was determined to be 0.742 ml/g. How many microliters of water are bound to 1 ml of solvated protein? Assume a water density of 1 g/ml.

b) A researcher studies a 50 kDa protein by sedimentation velocity. At high salt, Genetic algorithm Monte Carlo analysis shows a single species with a mean s-value of 3.5 S and a frictional ratio of about 1.45. As the salt concentration.is decreased, a second species appears with an s-value of about 6.6 S and a frictional ratio of about 1.23. Assume the partial specific volume is constant at all salt concentrations at 0.72 ml/g. Explain a possible model for this observation. What can you conclude about the function of salt with respect to any oligomerization? What can you conclude about the oligomerization state? Draw a model of a molecule that would match these observations (Hint: keep in mind the frictional ratios!)

Homework

c) A researcher has created a 2,500 basepair DNA molecule with 12 nucleosome binding sites and reconstitutes the DNA with histone proteins to create an artificial chromatin molecule. He wants to find out what the effect of adding magnesium is on the reconstituted molecule, and performs velocity experiments in 0, 0.5 and 2 mM $MgCl_2$. He observes a heterogeneous s-value distribution (see next page). To his surprise, an equilibrium experiment when analyzed with an lnC vs. r^2-r0^2 plot revealed a good fit to a straight line. The slope of the linear fit for all salt concentrations was identical and gave a value of 2.092. (assume 20C, 3000 rpm, a viscosity of 0.01poise, a density of 1.0 g/ccm for all salt concentrations, vbar of 0.65 g/ccm, The gas constant (R) is 8.314 x 10^7 erg. Temperature should be expressed in Kelvin).

c1) What do you conclude from the **velocity** results? Explain the different distributions observed and what they indicate. Suggest at least two sample characteristics that could account for the observed **velocity** distributions.

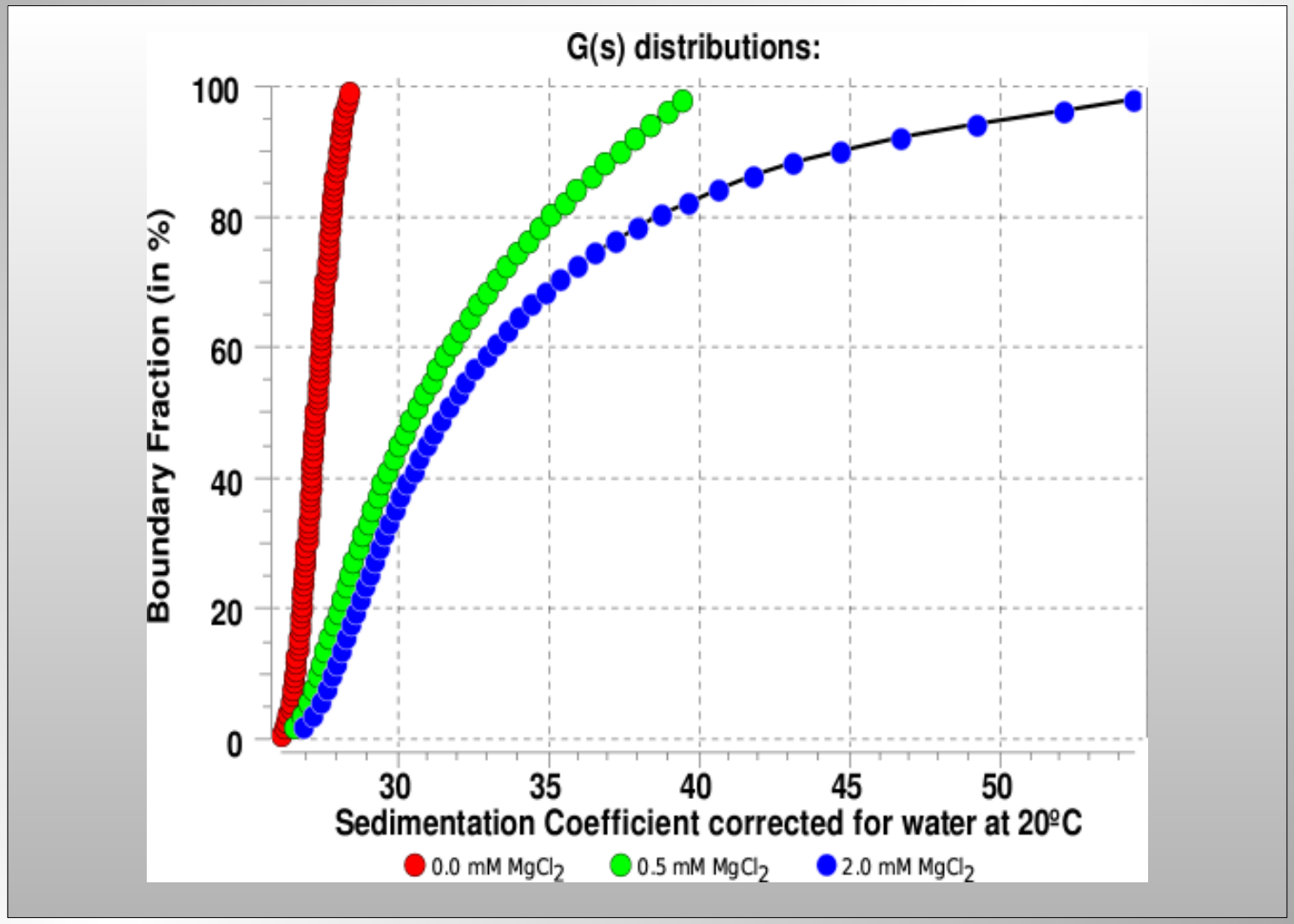
c2) What do you conclude from the **equilibrium** results? Explain.

c3) How can you reconcile the equilibrium results with the velocity results? Explain. Calculate the molecular weight suggested by the slope. What does it say about the chromatin molecule (hint: a histone octamer is about 111.5 kDa, the average DNA molecular weight is ~660 Da/bp.)

c4) Suggest what may have happen to the chromatin molecule as magnesium chloride is added to the buffer. Suggest a model that will account for these results.

c5) calculate the frictional ratio for the 27S and the fastest moving species in each salt conc.

Homework


G(s) distributions:
Boundary Fraction (in %)
100
80
60
40
20
0
30
35
40
45
50
Sedimentation Coefficient corrected for water at 20ºC
0.0 mM MgCl2
0.5 mM MgCl2
2.0 mM MgCl2

Useful Equations:

$$f = \frac{RT}{N D}$$

$$M = \frac{s N f}{1 - \bar{\nu}\rho}$$

$$V = \frac{M \nu}{N}$$

$$r_0 = \left(\frac{3V}{4\pi}\right)^{1/3}$$

$$f_0 = 6\pi\eta r_0$$

$$\phi = \frac{f}{f_0}$$

Equilibrium Equation:

$$C = C_0 \exp^{\frac{M\omega^2(1-\bar{\nu}\rho)}{2RT}\left(r^2 - r_0^2\right)}$$

This function can be linearized by taking the log on both sides:

$$\log C = C_0 + \frac{M\omega^2(1-\bar{\nu}\rho)}{2RT}\left(r^2 - r_0^2\right)$$

Plotting $\log C$ **vs.** $r^2 - r_0^2$ **gives a straight line with slope**

$$\frac{M\omega^2(1-\bar{\nu}\rho)}{2RT}$$

$$\omega = \pi/30 * rpm$$