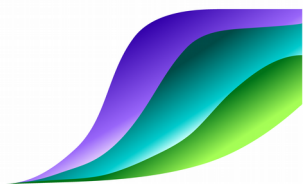


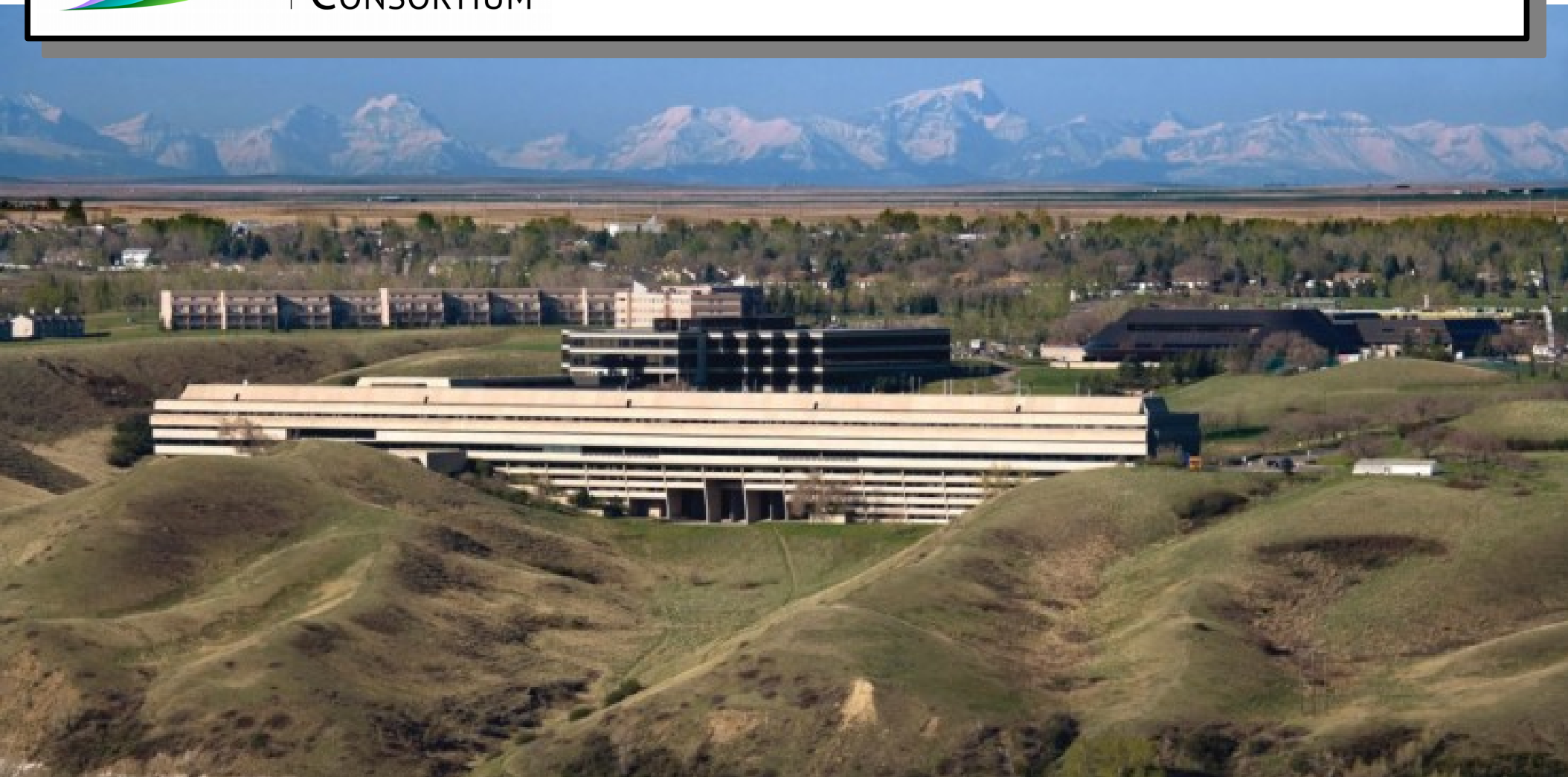


Borries Demeler, Ph.D.  
Dept. of Chemistry and Biochemistry  
Canada 150 Research Chair  
in Biophysics

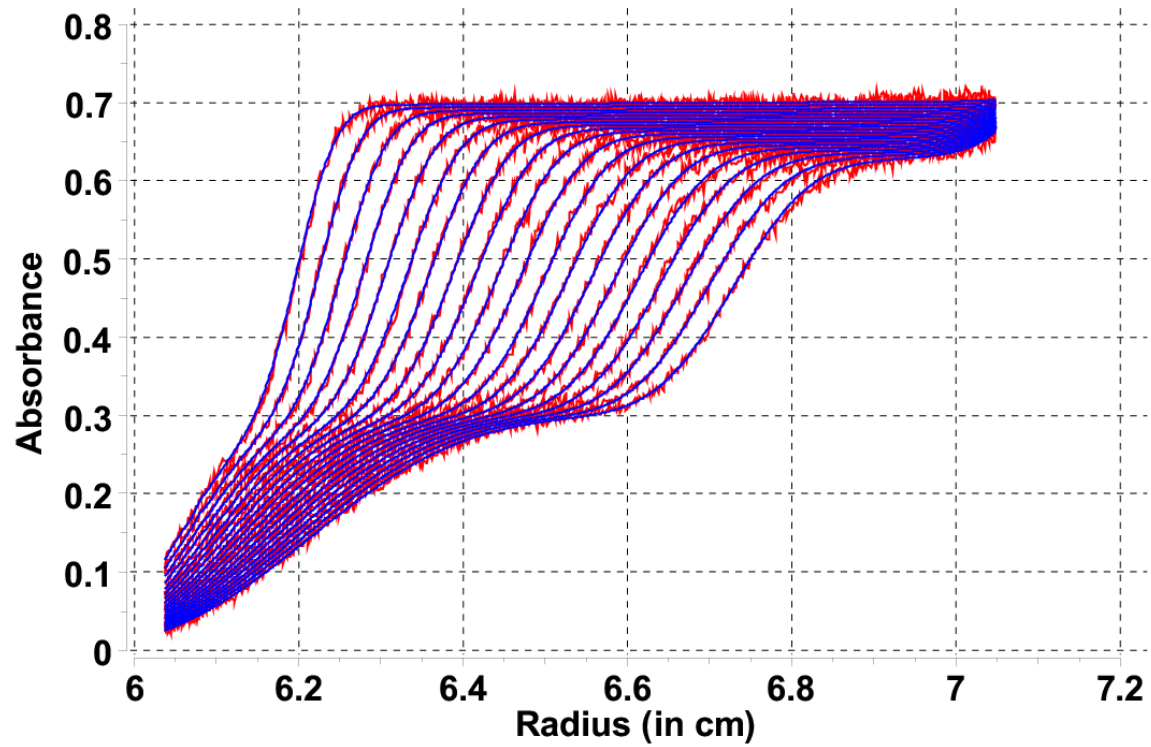


NORTHWEST  
BIOPHYSICS  
CONSORTIUM

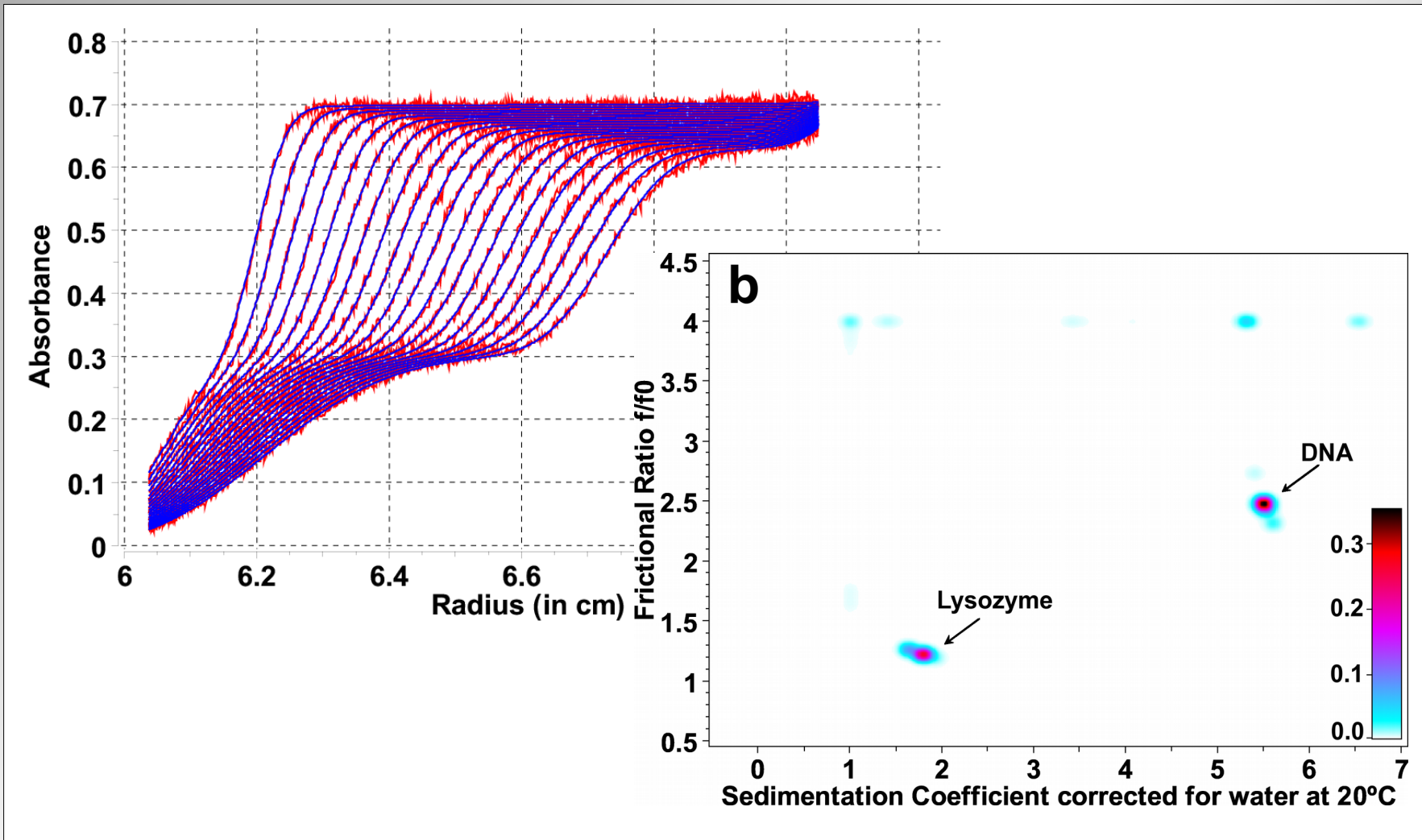
## Reversible Associations and Reactions



## ***Background - Reversible Associations***



## Background - Reversible Associations



# ***Background - Reversible Associations***

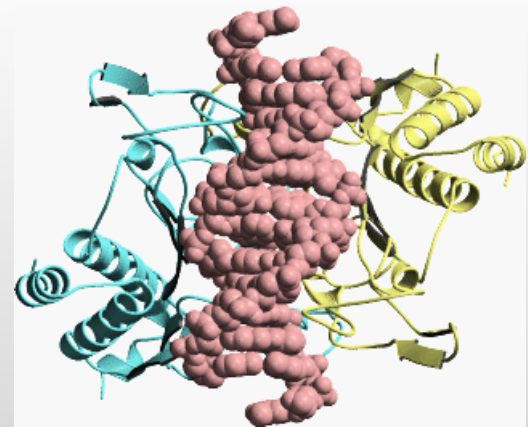
## **Reversible Systems:**

**Self-association, hetero-association, or multiple reactions ( $A + A + B \rightleftharpoons A_2B$ )**

**The concentration in one component affects the concentration of another**

**Reactions can be fast (diffusion controlled) or slow (kinetically limited)**

**...and they observe mass action laws**





## ***Background - Reversible Associations***

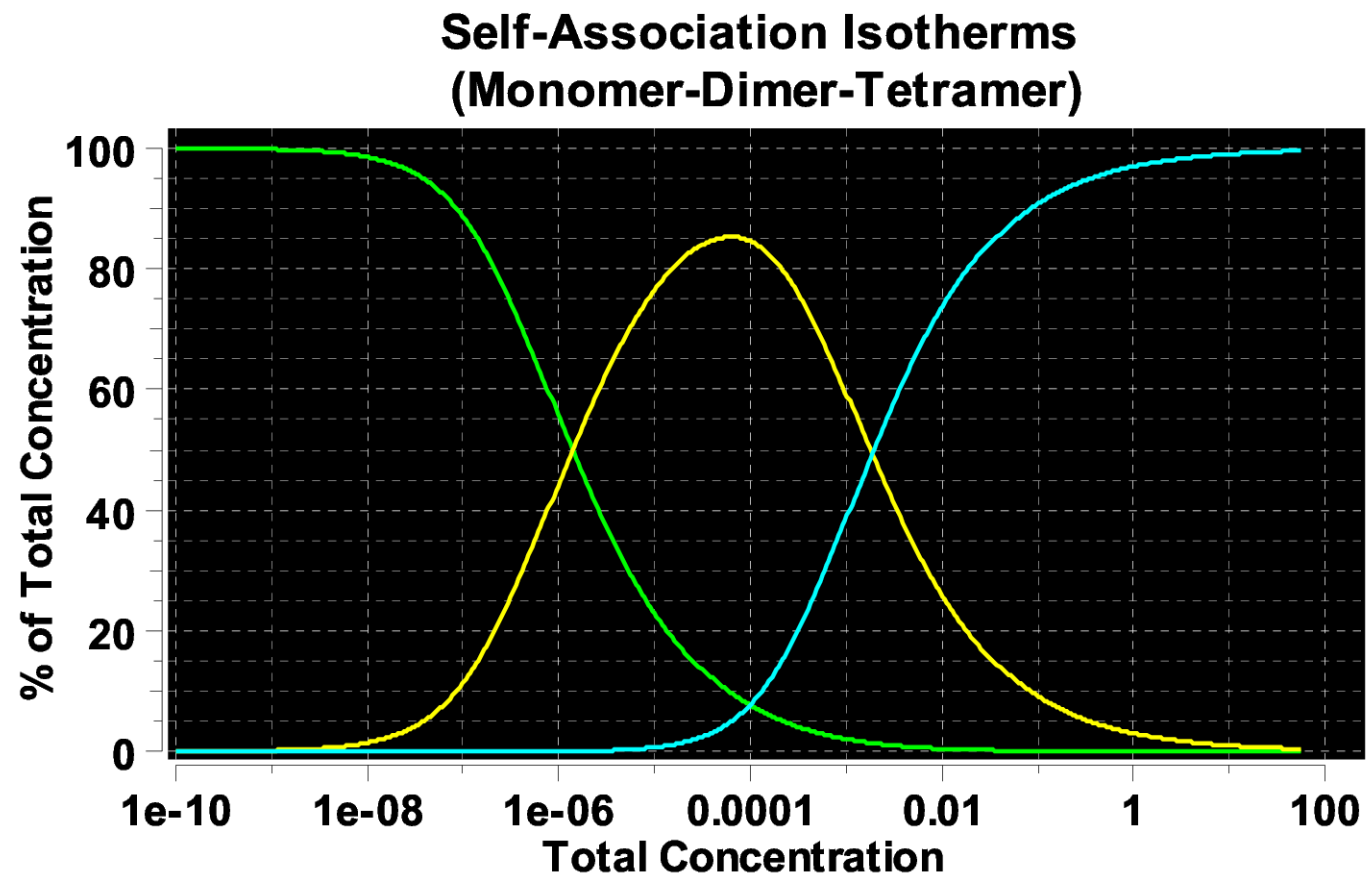
**Equilibrium Constant:**  $\sum_{i=1}^n M = M_n$   $K_A = \frac{[M_n]}{[M]^n}$

**Kinetics:**  $K_A = \frac{k_{on}}{k_{off}}$

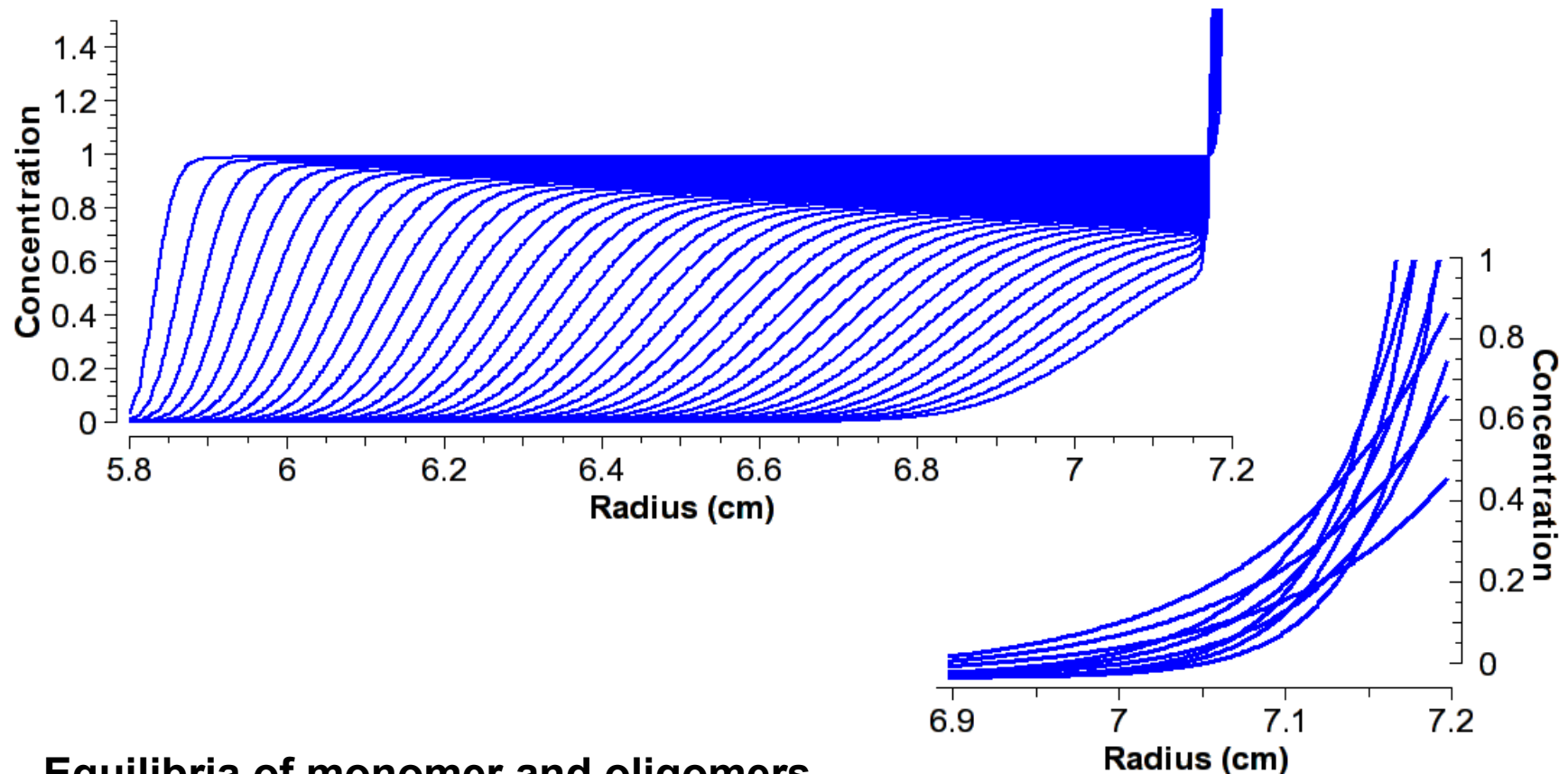
$$[M] + [M_n] = C_{total}$$

**Solve polynomial:**  $[M] + K_A[M]^n - C_{total} = 0$

## ***Background - Reversible Associations***



## ***Background - Reversible Associations***



**Equilibria of monomer and oligomers  
are established according to mass-action  
along the gradients**

## A Model for Reversible Reactions

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

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

Claverie, J.-M., Dreux, H., and R. Cohen (1975). *Sedimentation of Generalized Systems of Interacting Particles. I. Solutions of Systems of Complete Lamm Equations.* Biopolymers 14:1685-1700

Todd GP, Haschemeyer RH. General solution to the inverse problem of the differential equation of the ultracentrifuge. Proc Natl Acad Sci U S A. 1981 78(11):6739-43.

# The ASTFEM-RA Model for Reversible Reactions

$$L(s, D): \left( \frac{\partial C}{\partial t} \right)_r = \frac{-1}{r} \frac{\partial}{\partial r} \left[ s \omega^2 r^2 C - D r \frac{\partial C}{\partial r} \right]_t$$

High accuracy finite element solution for both non-interacting and arbitrary reversible reactions (self- and heteroassociations)

*Cao, W and B. Demeler. Modeling AUC Experiments with an Adaptive Space-Time Finite Element Solution for Multi-Component Reacting Systems. Biophys. J. (2008) 95(1):54-65*

Careful adaptive refinement near meniscus and cell bottom to provide unsurpassed 2<sup>nd</sup> order accuracy and stability

Implicit conservation of mass

Supports multi-speed experiments

Acceleration can be modeled

Band sedimentation experiments

Arbitrary non-interacting and interacting systems, multiple reactions

Determine equilibrium constants and rate constants

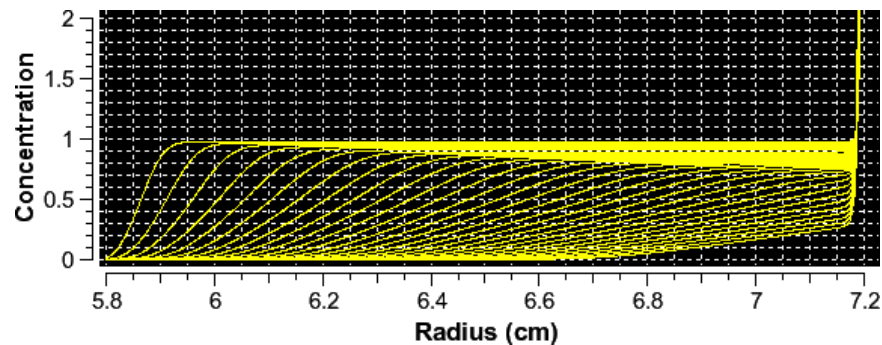
Model flotation experiments



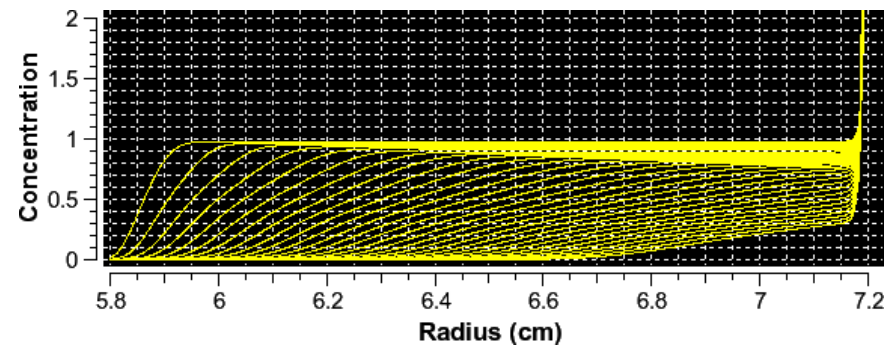
# Models for Reacting Systems:

## Monomer – Trimer Equilibrium, Monomer MW = 50 kDa

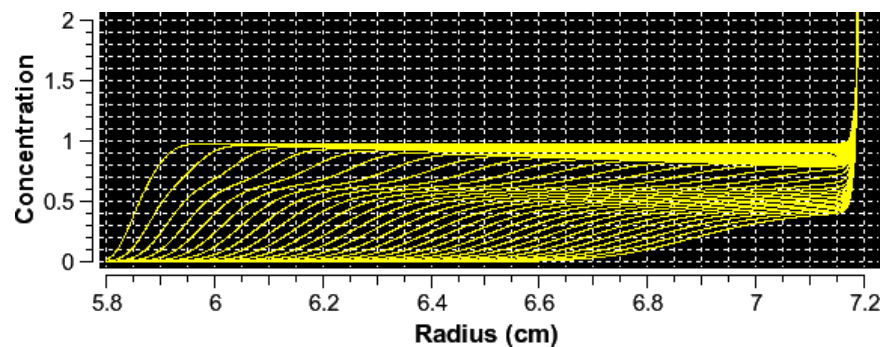
$$K_{\text{off}} = 1.0/\text{sec}$$



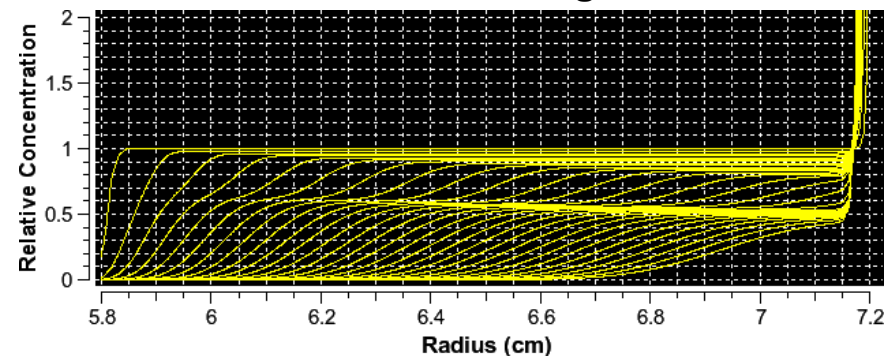
$$K_{\text{off}} = 1.0 \times 10^{-3}/\text{sec}$$



$$K_{\text{off}} = 1.0 \times 10^{-4}/\text{sec}$$



non-interacting



## ***Models for Reacting Systems:***

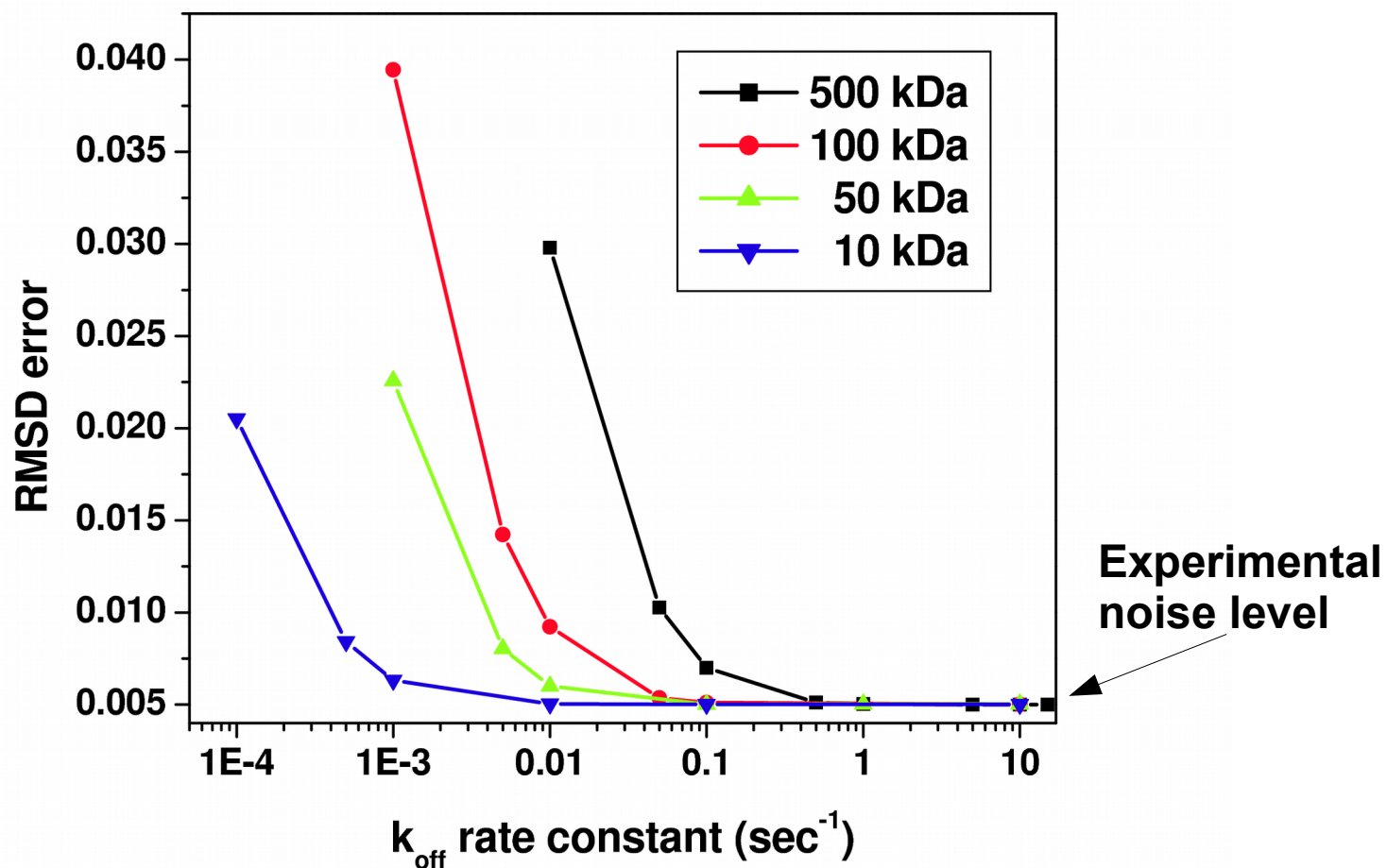
**The magnitude of the measurable off-rate depends on rotor speed and sedimentation coefficient:**

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

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

## *Models for Reacting Systems:*

Range of measurable  $k_{\text{off}}$  rate constants for different MW



# UltraScan Model Builder for Reacting Systems - Monomer:

Discrete Genetic Algorithm Initialization

☒ Database
 ☐ Local Disk

**Model and Constraints**

**General Control**

Discrete Model GA Constraints Editor

**Components**

SKIN2 SAM SAM wt  
SKIN2 SAM SAM wt dimer

Attribute	Value	Low	High	Float?	LogSc?
SKIN2 SAM SAM wt	0.7386	0.66474	0.81246	<input checked="" type="checkbox"/>	
SKIN2 SAM SAM wt dimer	19300	17370	21230	<input type="checkbox"/>	<input type="checkbox"/>
SKIN2 SAM SAM wt	1.45	1.305	1.595	<input checked="" type="checkbox"/>	
SKIN2 SAM SAM wt dimer	1.72607e-13	1.1652e-13	2.37135e-13	<input type="checkbox"/>	
SKIN2 SAM SAM wt	8.29771e-07	7.07898e-07	9.89056e-07	<input type="checkbox"/>	
SKIN2 SAM SAM wt dimer	4.87778e-08	4.09223e-08	5.71755e-08	<input type="checkbox"/>	
SKIN2 SAM SAM wt	0.805	0.79	0.82	<input checked="" type="checkbox"/>	
SKIN2 SAM SAM wt dimer	29280	Wavelength (nm):		280.0	
SKIN2 SAM SAM wt	1	<input checked="" type="checkbox"/> Is Reactant		<input type="checkbox"/> Is Product	
SKIN2 SAM SAM wt dimer	Concentration Dependency of s ( $\sigma$ ):		<input type="checkbox"/> Co-sedimenting Solute		
SKIN2 SAM SAM wt	Concentration Dependency of D ( $\delta$ ):		<input type="button" value="Load C0 from File"/>		
<input type="button" value="Re-compute unselected component attribute values"/>					
<b>Associations (reactions)</b>					
2A1 => 1B2					
Attribute	Value	Low	High	Float?	LogSc?
K_dissociation	5.3e-05	5e-06	0.000101	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
k_off rate	5.75e-05	5e-06	0.00011	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>
<input type="button" value="Help"/>		<input type="button" value="Cancel"/>		<input type="button" value="Accept"/>	

# ***Genetic Algorithm Optimization:***

**Genetic Algorithms (also called evolutionary programming)  
provide a stochastic optimization method**

*Holland J, Adaption in Natural and Artificial Systems, 1975, U. of Michigan Press*

**Based on nature – evolutionary paradigm**

**Mutation, recombination, deletion, insertion, crossover operators**

**Random number generators are used to manipulate operators**

**Generational Model – survival of the fittest (...fitting function)**

**Generation → iterations, genes → parameter strings, bases → s, D,  $K_d$ ,  $k_{off}$**

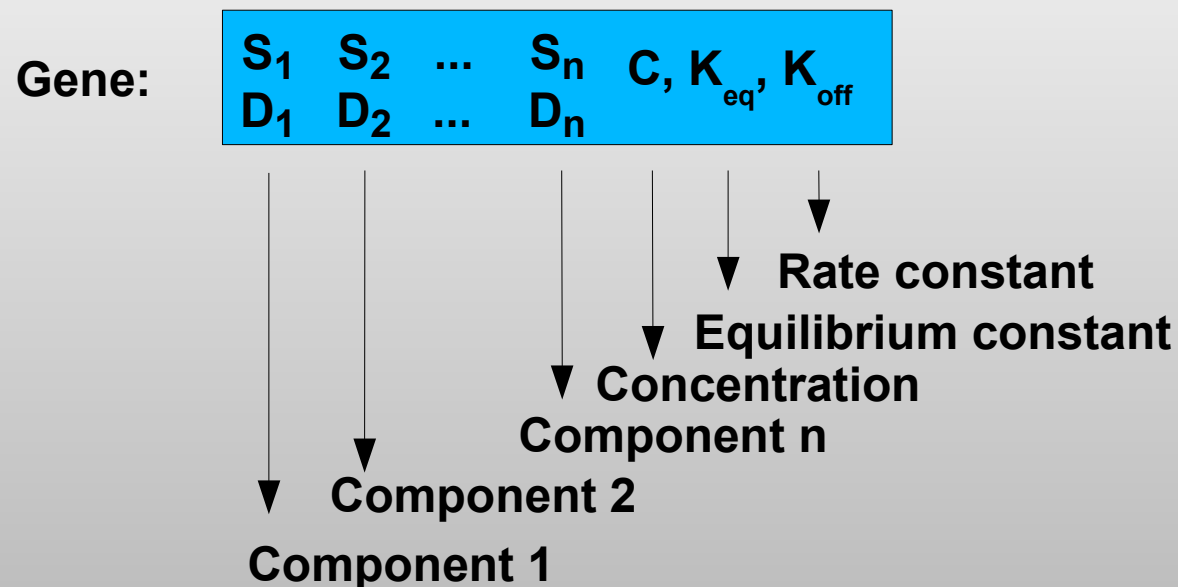
**Use a Monte Carlo analysis to determine confidence level from noisy data**

**Implemented on supercomputer (TeraGrid Science Gateway)**

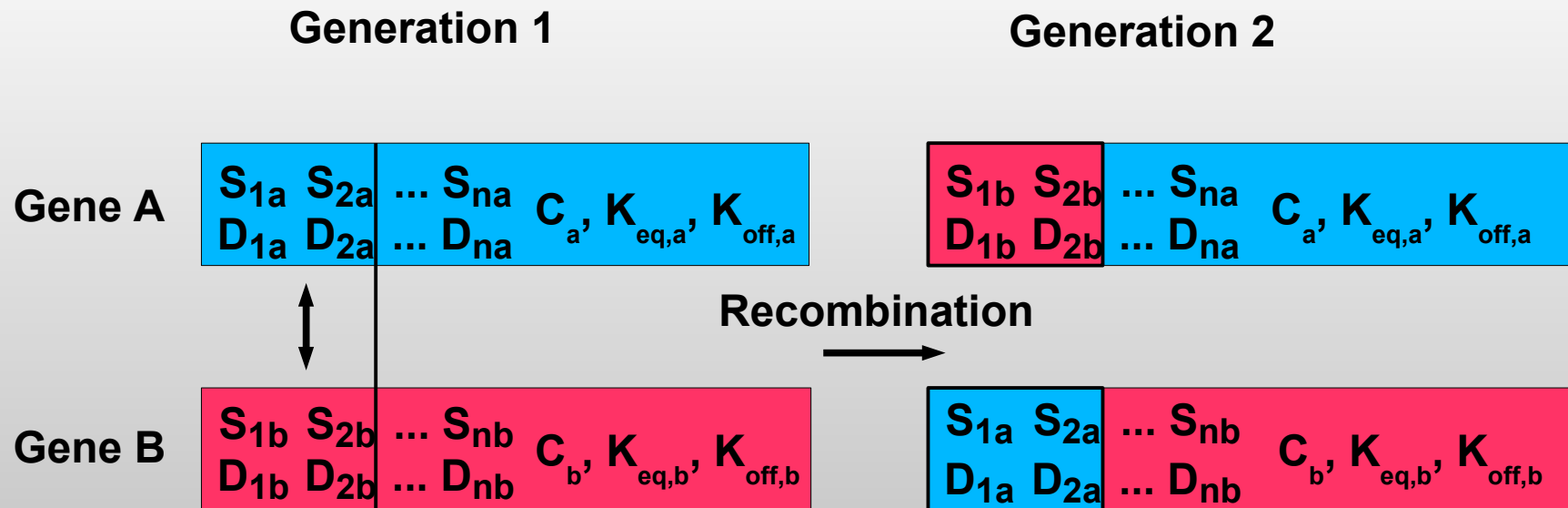


## ***Genetic Algorithm Optimization:***

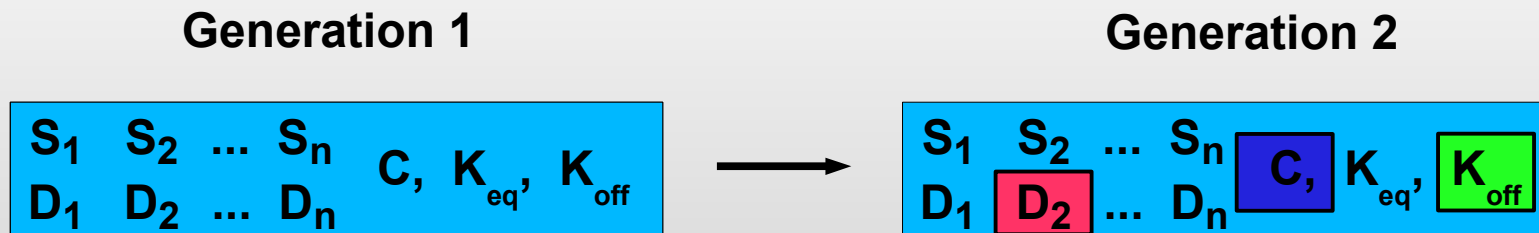
**Genes are strings of parameters, each gene consists of a pair of corresponding sedimentation and diffusion coefficients, loading concentration, equilibrium constants and rate constants.**



# Crossover/Recombination



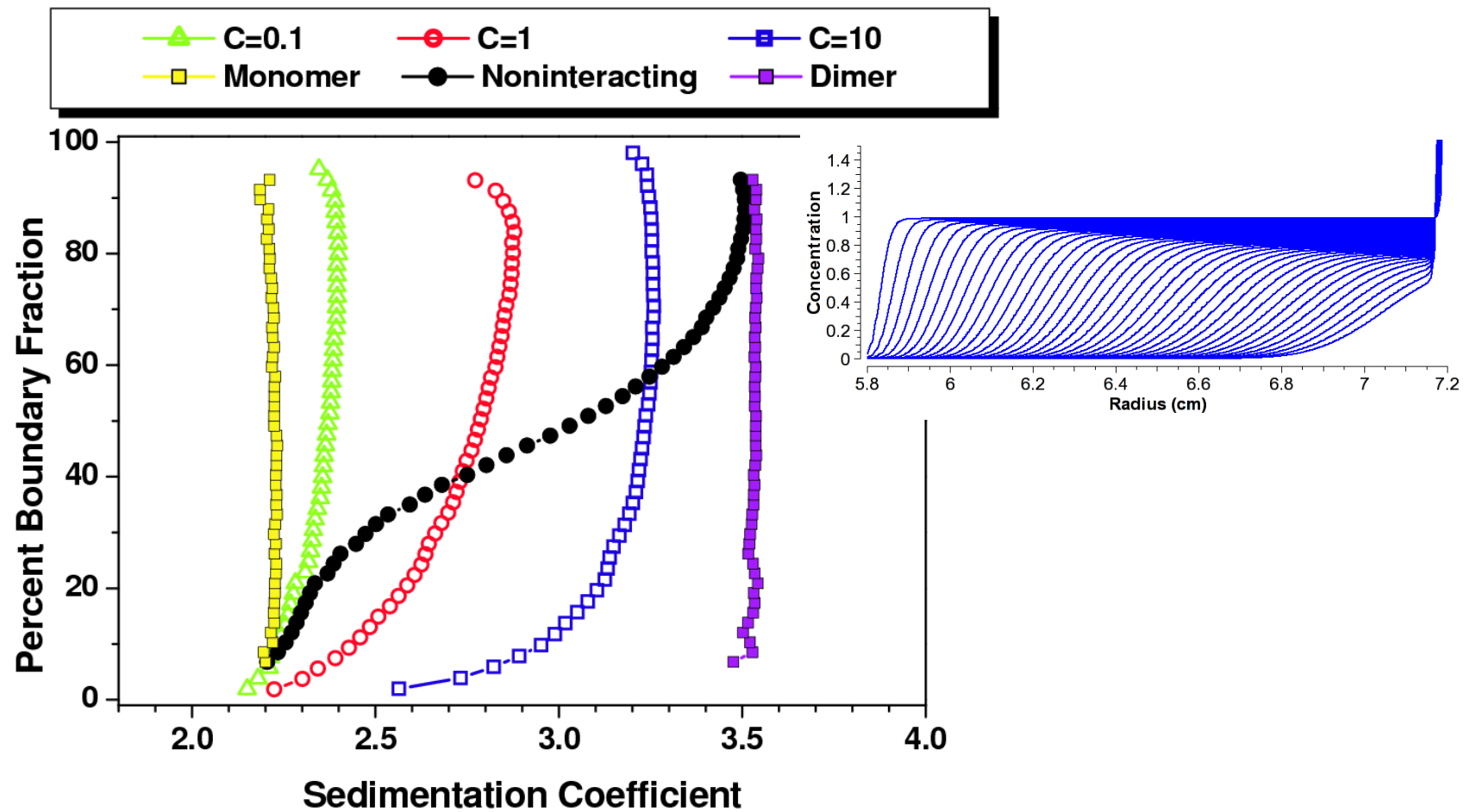
# Mutation



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

# Diagnostics: van Holde – Weischet Analysis

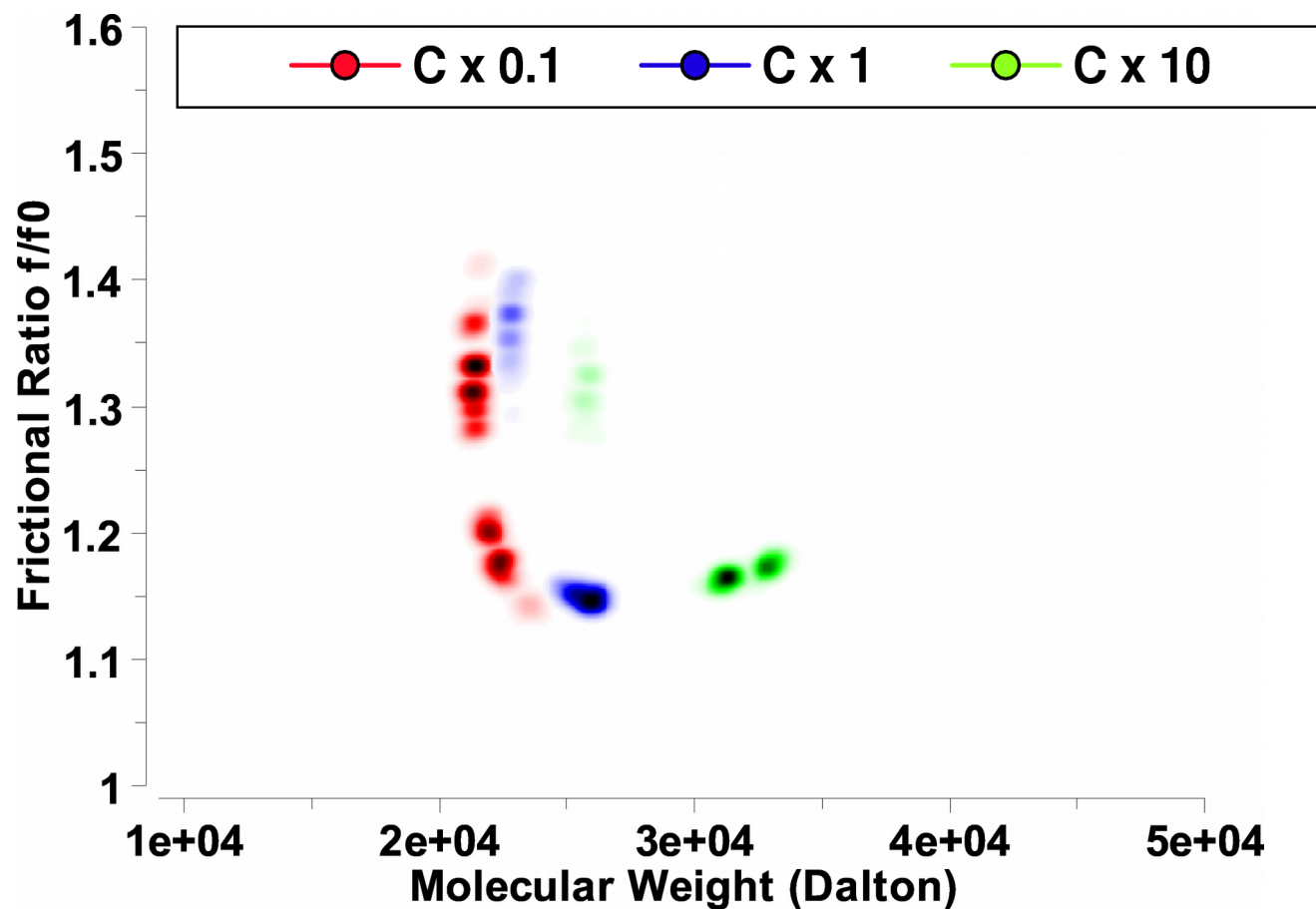
## Example 1: Simulated Monomer – Dimer Equilibrium



Monomer MW = 20 kDa,  $K_d = C \times 1$ ,  $k_{off} = 1 \times 10^{-3}/\text{sec}$ ,  $f/f_0 = 1.25$  (both)

## 2DSA Monte Carlo Analysis

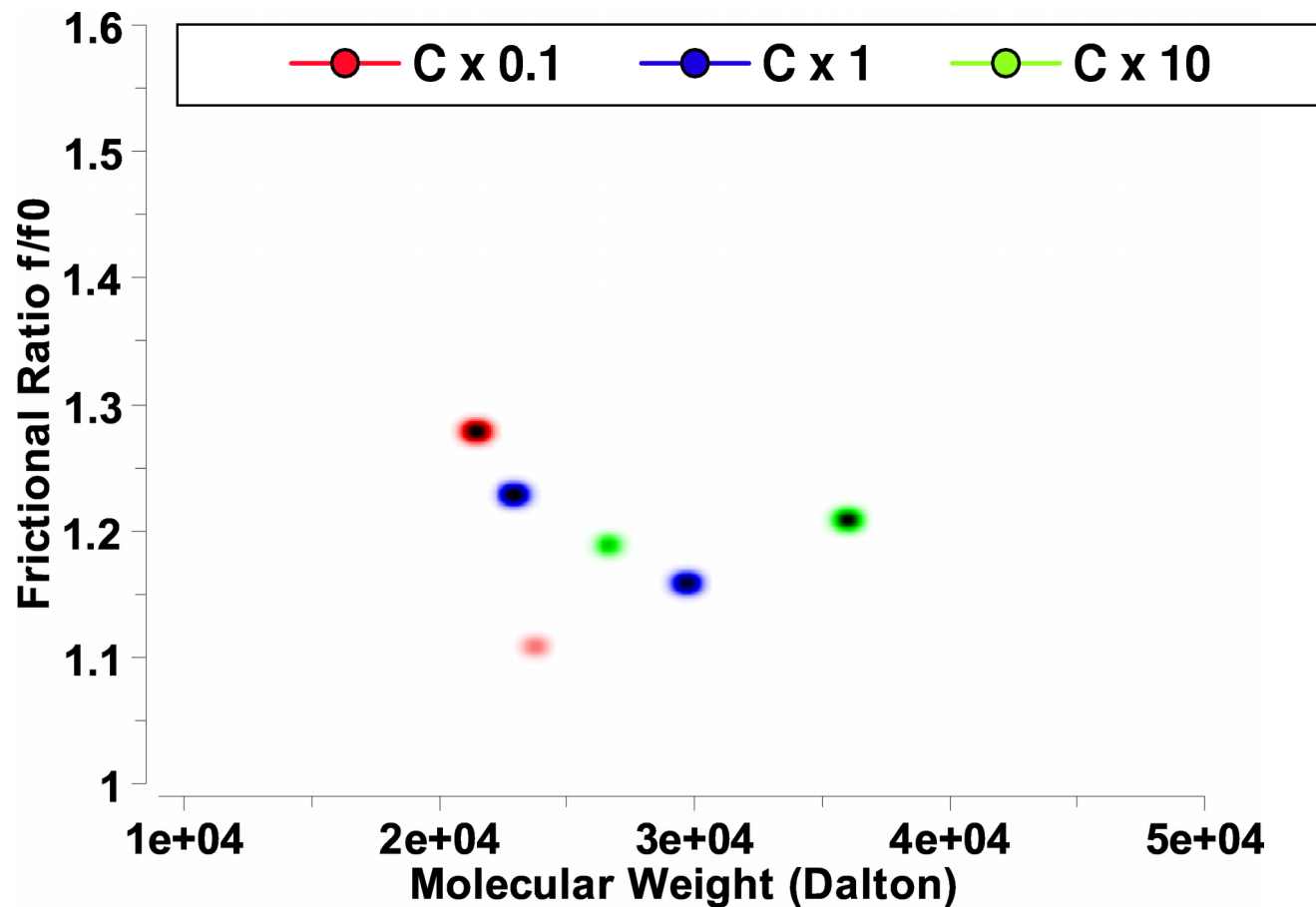
Monomer – Dimer Equilibrium, Monomer MW = 20 kDa





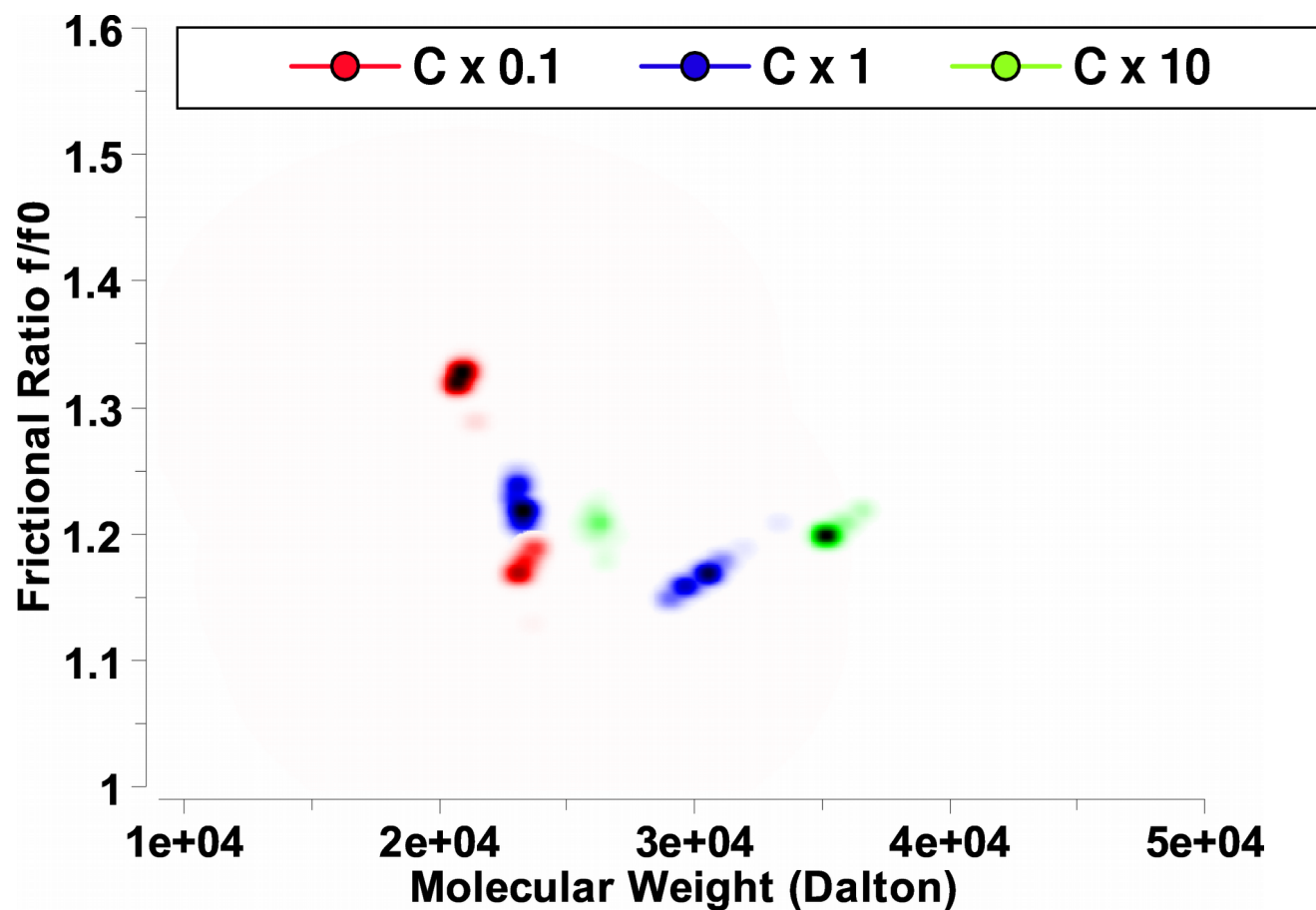
# Genetic Algorithm Analysis

Monomer – Dimer Equilibrium, Monomer MW = 20 kDa



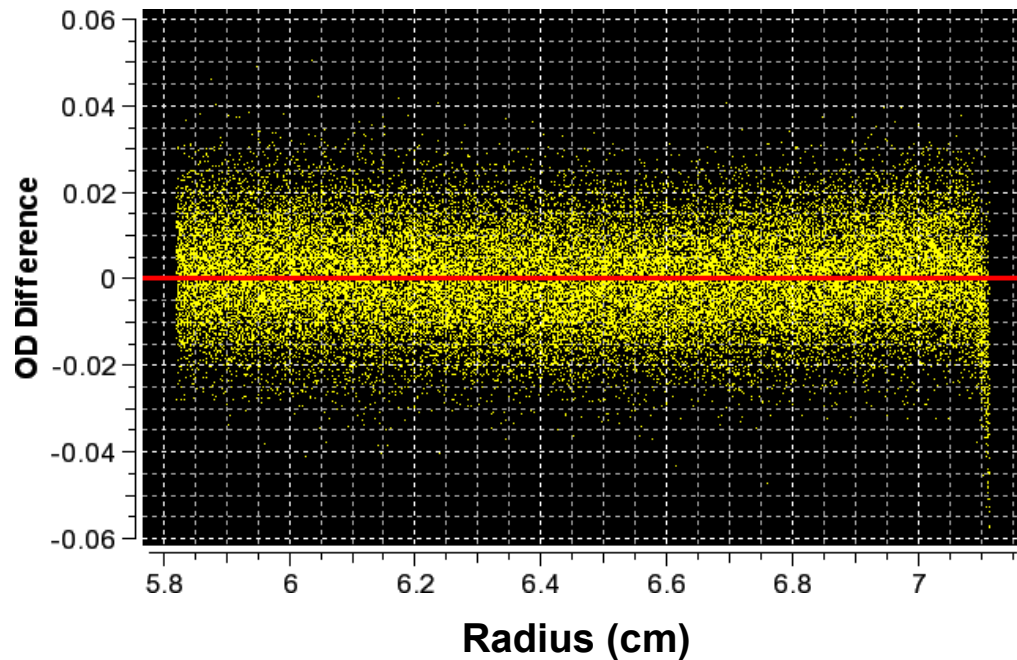
# Genetic Algorithm Monte Carlo Analysis

Monomer – Dimer Equilibrium, Monomer MW = 20 kDa

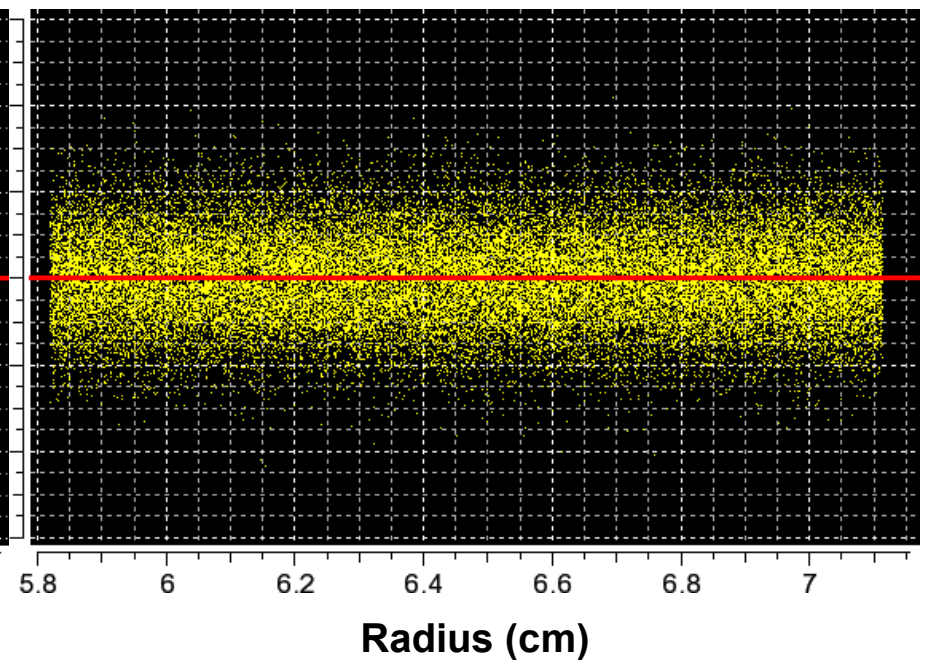


# *UltraScan Model Builder for Reacting Systems - Dimer:*

**GA-MC Non-Interacting Fit**



**GA Reversible Model Fit**



## ***UltraScan Model Builder for Reacting Systems - Dimer:***

**Monte Carlo Statistics for GA fit of C x 1.0 concentration using reacting model (50 iterations):**

<b>Monomer sedimentation coefficient:</b>	<b>2.215e-13</b> (2.166e-13, 2.263e-13)	
<b>Monomer diffusion coefficient:</b>	<b>9.596e-07</b> (8.857e-07, 1.033e-06)	
<b>Monomer molecular weight:</b>	<b>1.996e+04</b> (1.863e+04, 2.129e+04)	<b>20,000</b>
<b>Monomer frictional ratio:</b>	<b>1.250e+00</b> (1.179e+00, 1.321e+00)	<b>1.25</b>
<b>Dimer sedimentation coefficient:</b>	<b>3.578e-13</b> (3.461e-13, 3.696e-13)	
<b>Dimer diffusion coefficient:</b>	<b>7.754e-07</b> (7.363e-07, 8.144e-07)	
<b>Dimer molecular weight:</b>	<b>3.992e+04</b> (3.725e+04, 4.259e+04)	<b>40,000</b>
<b>Dimer frictional ratio:</b>	<b>1.221e+00</b> (1.190e+00, 1.252e+00)	<b>1.25</b>
<b>Reaction 1: equilibrium constant:</b>	<b>9.055e-01</b> (6.962e-01, 1.115e+00)	<b>1.0</b>
<b>Reaction 1: k_off rate:</b>	<b>1.466e-03</b> (8.591e-04, 2.0723e-03)	<b>0.001</b>

# Monomer-Dimer Interface Mutation Analysis

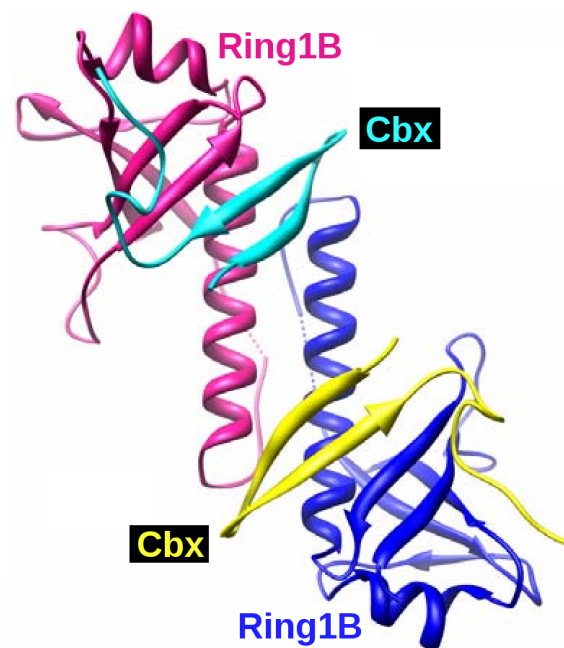
## Example 2: Ring1B mutation analysis (Dr. Chong Kim, UTHSCSA)

**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:**

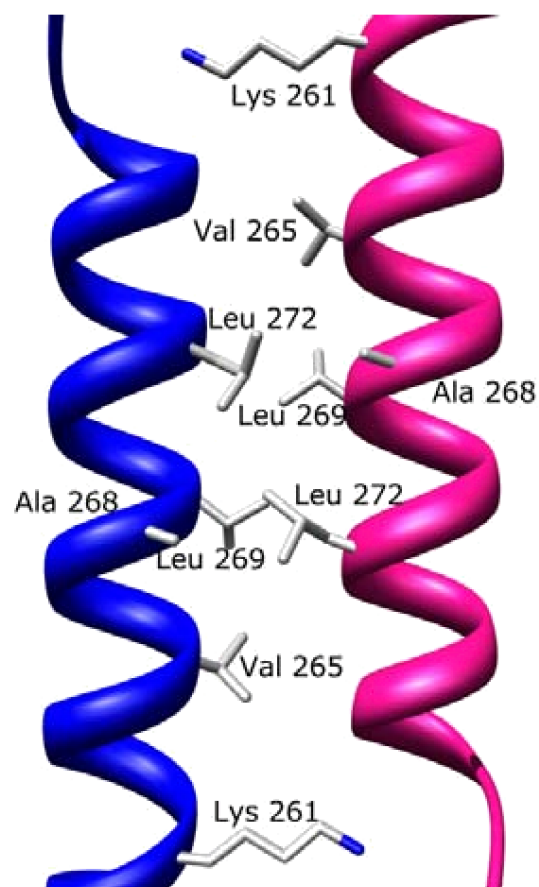
Ring1B binds the C-terminal domain of Polycomb, but crystallizes as a hetero-dimer. In solution without c-polycomb, Ring1B is a dimer. Is the crystal dimer interface the same observed in solution?





# ***Monomer-Dimer Interface Mutation Analysis***

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

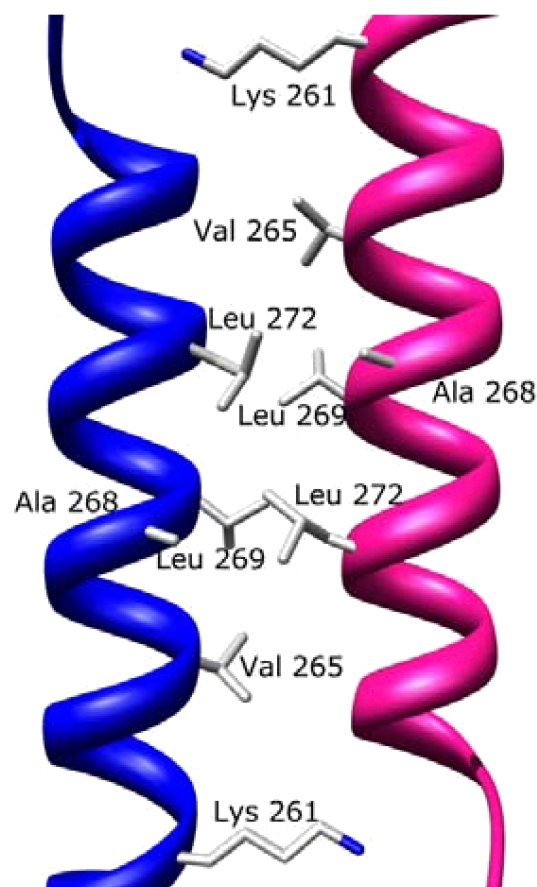


**Question: Is the dimerization interface observed in crystal structure responsible for dimerization in solution?**

**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)



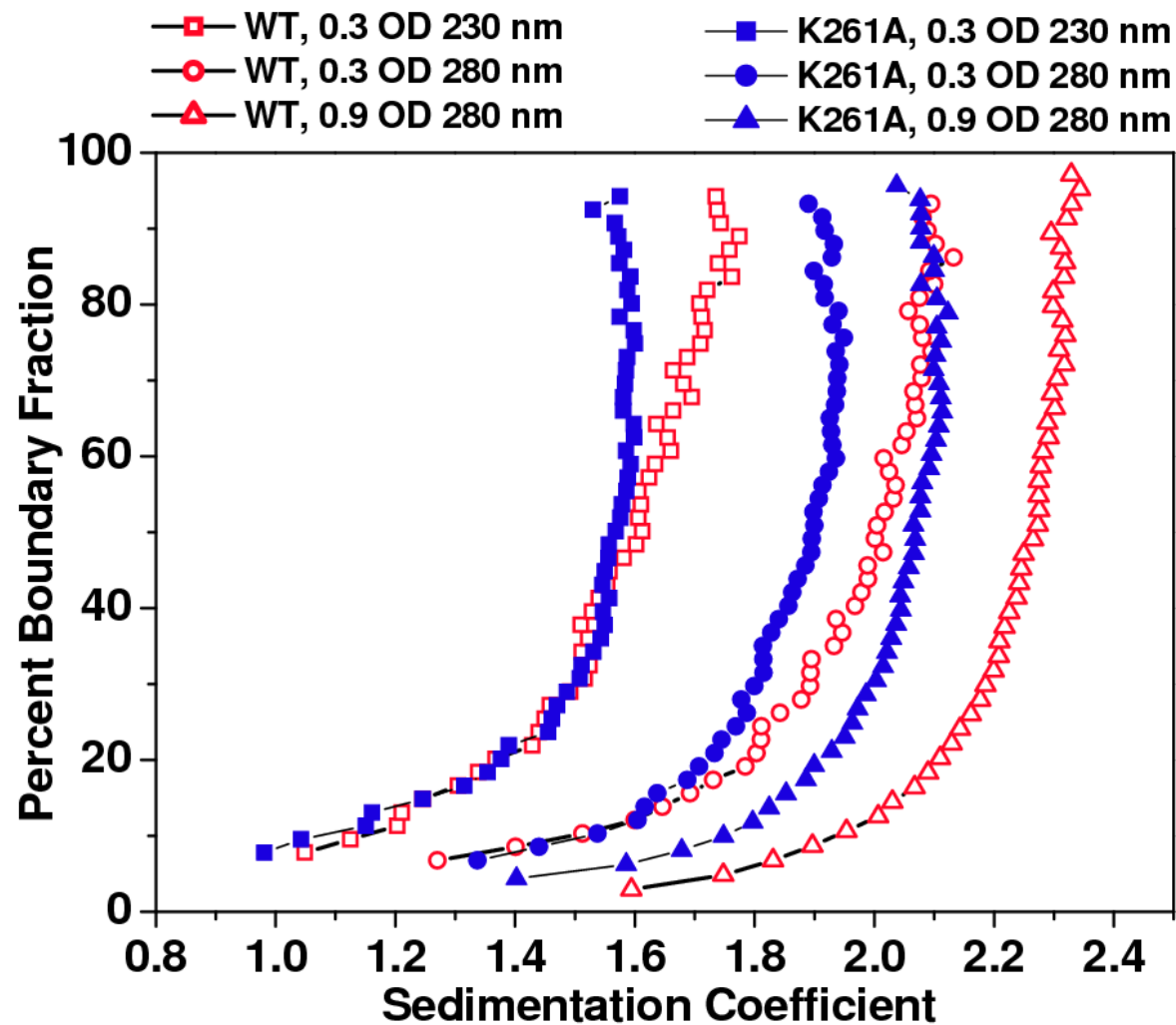
Hydrophobic residues were replaced by polar residues in dimerization study:

Dimerizes?

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

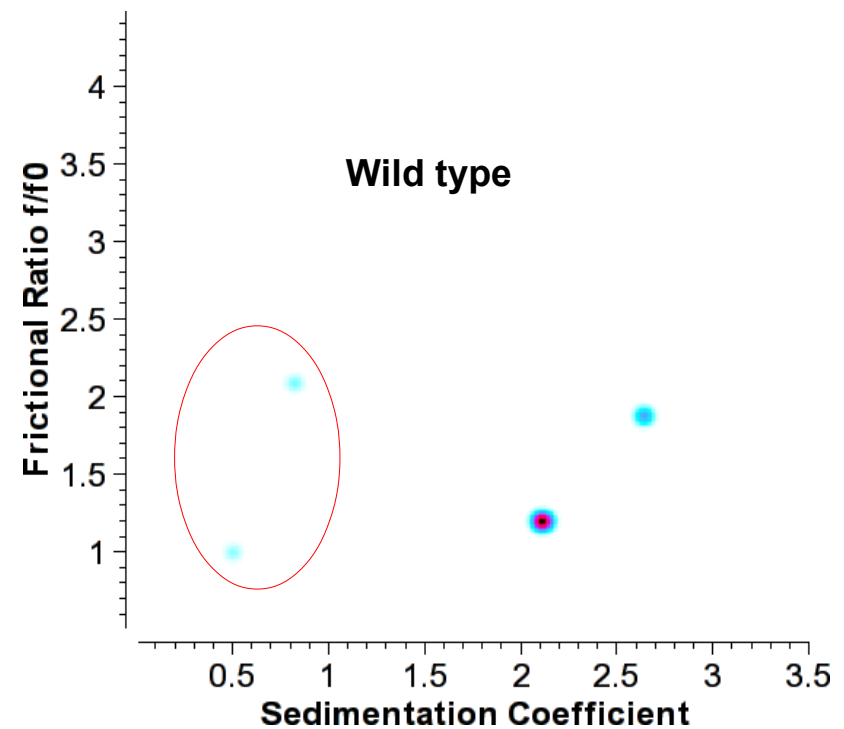
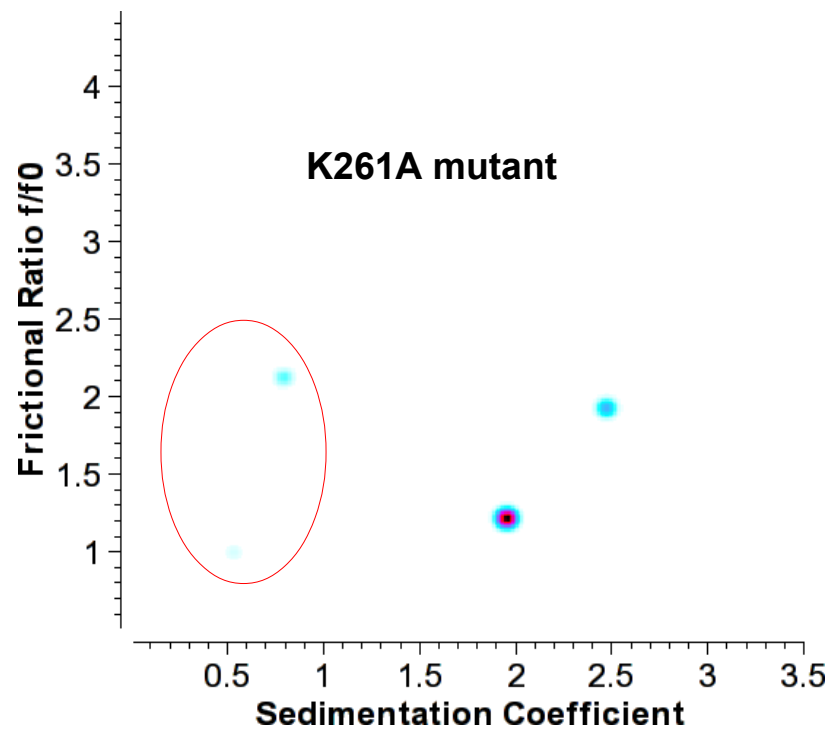
**Answer:** acidic residues seriously disrupt the dimer interface, while non-polar or basic residues have a slighter effect. But clearly the dimer interface observed in the crystal is present in solution as well.

# Monomer-Dimer Interface Mutation Analysis



# Monomer-Dimer Interface Mutation Analysis

## Non-interacting analysis (GA)



## ***Monomer-Dimer Interface Mutation Analysis Equilibrium Results***

<b>Fitting Model:</b>	<b>K261A:</b>	<b>Wildtype:</b>
1-species MW	1.898 x 10 <sup>4</sup> Da	1.909 x 10 <sup>4</sup> Da
1-species RMSD	7.05 x 10 <sup>-3</sup>	9.48 x 10 <sup>-3</sup>
Fixed MW dist. RMSD	4.46 x 10 <sup>-3</sup>	4.26 x 10 <sup>-3</sup>
1-2 reversible RMSD	5.56 x 10 <sup>-3</sup>	5.41 x 10 <sup>-3</sup>
1-2 reversible Kd	54.0 μM (29.1, 166.1)	22.7 μM (8.64, 63.0)
SV 0.3 OD <sub>280</sub>	17.1 μM (15.9, 18.4)	10.4 μM (9.62, 11.4)
SV 0.9 OD <sub>280</sub>	28.5 μM (25.8, 31.8)	17.6 μM (14.8, 21.6)

Sedimentation Equilibrium fitting results for single species and reversible monomer-dimer models, as well as a fixed molecular weight distribution model with 100 species ranging between 1-50 kDa. Values in parentheses represent 95% confidence intervals.

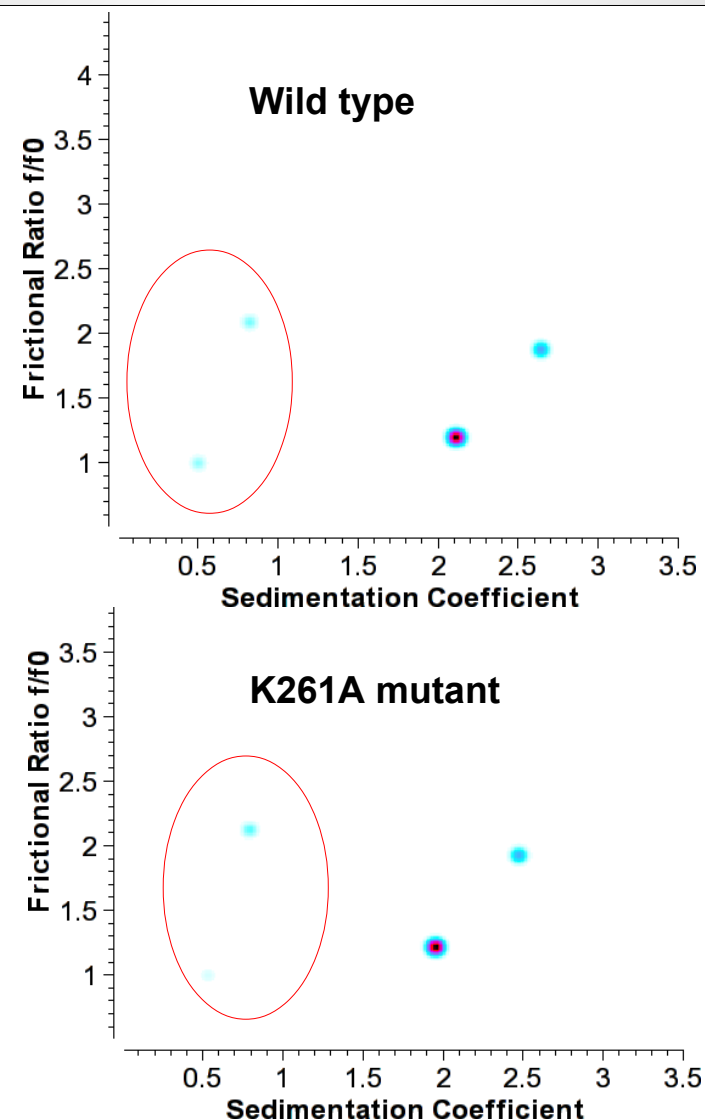
## ***Monomer-Dimer Interface Mutation Analysis Velocity Results***

<b>Parameter:</b>	<b>wildtype, 0.3 OD</b>	<b>wildtype, 0.9 OD</b>	<b>K261A, 0.3 OD</b>	<b>K261A, 0.9 OD</b>
$K_d$ ( $\mu\text{M}$ )	10.4 (9.62, 11.4)	17.6 (14.8, 21.6)	17.1 (15.9, 18.4)	28.5 (25.8, 31.8)
$k_{\text{off}}$ ( $\times 10^{-5} \text{ sec}^{-1}$ )	72.7 (26.5, 118.9)	84.3 (48.6, 120.0)	84.0 (46.4, 121.6)	14.1 (8.1, 20.1)
$f/f_o$ (monomer)	1.31 (1.28, 1.34)	1.14 (1.09, 1.19)	1.33 (1.32, 1.35)	1.19 (1.17, 1.21)
$f/f_o$ (dimer)	1.35 (1.33, 1.37)	1.31 (1.30, 1.32)	1.43 (1.42, 1.45)	1.44 (1.43, 1.45)
$f/f_o$ (contaminant)	1.23 (1.18, 1.27)	1.24 (1.18, 1.3)	1.17 (1.12, 1.21)	1.49 (1.47, 1.56)
contam. OD ( $\times 0.01$ )	3.49 (3.41, 3.57)	3.56 (3.37, 3.75)	3.35 (3.27, 3.44)	2.77 (2.58, 2.96)
Co. mol. wt. ( $\times 1000$ )	1.84 (1.71, 1.97)	2.33 (2.09, 2.56)	1.71 (1.59, 1.82)	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.

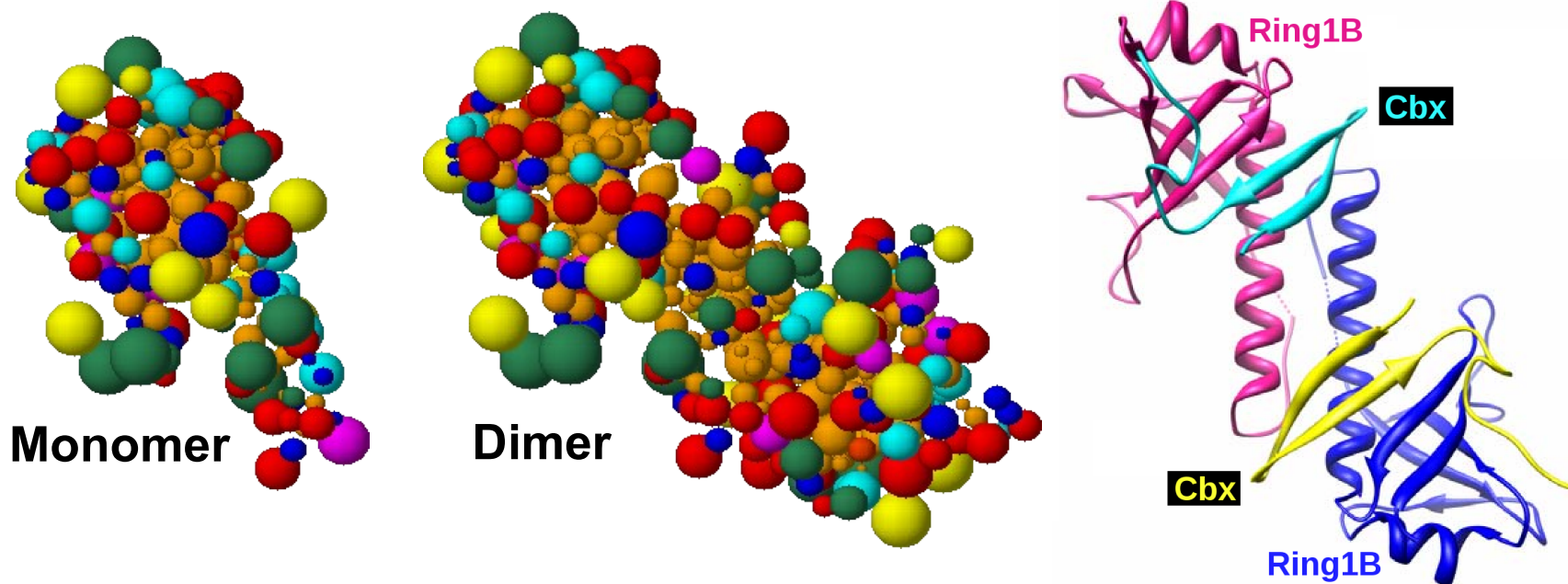
# Monomer-Dimer Interface Mutation Analysis

Parameter:	wildtype, 0.9 OD	K261A, 0.9 OD
Sed. veloc. $K_d$ ( $\mu\text{M}$ )	17.6 (14.8, 21.6)	28.5 (25.8, 31.8)
Sed. equil. $K_d$ ( $\mu\text{M}$ )	22.7 (8.64, 63.0)	54.0 (29.1, 166.1)
$k_{\text{off}}$ ( $\times 10^{-5} \text{ sec}^{-1}$ )	84.3 (48.6, 120.0)	14.1 (8.1, 20.1)
$f/f_0$ (monomer)	1.14 (1.09, 1.19)	1.19 (1.17, 1.21)
$f/f_0$ (dimer)	1.31 (1.30, 1.32)	1.44 (1.43, 1.45)
$f/f_0$ (contaminant)	1.24 (1.18, 1.3)	1.49 (1.47, 1.56)
contam. OD ( $\times 0.01$ )	3.56 (3.37, 3.75)	2.77 (2.58, 2.96)
mol. wt. ( $\times 1000$ )	2.33 (2.09, 2.56)	3.00 (2.93, 3.06)





# 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

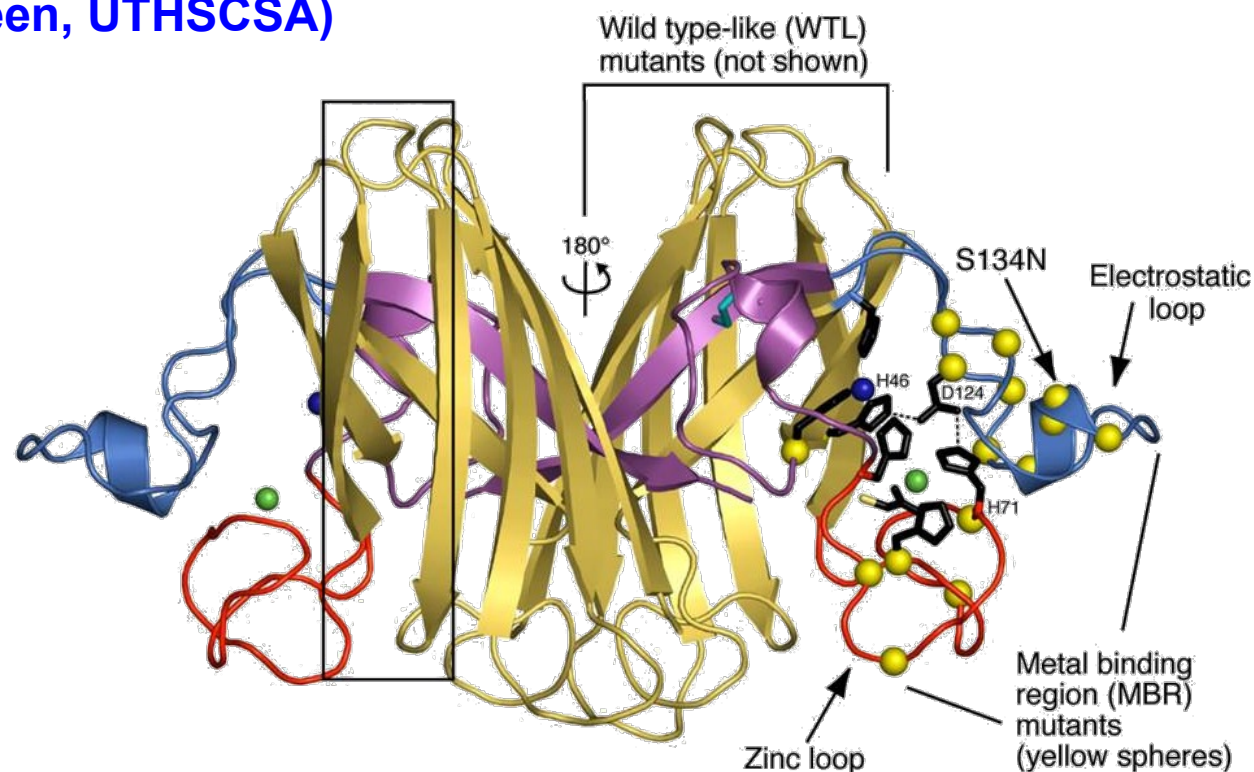
Measured:

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



# Protein Stability Analysis

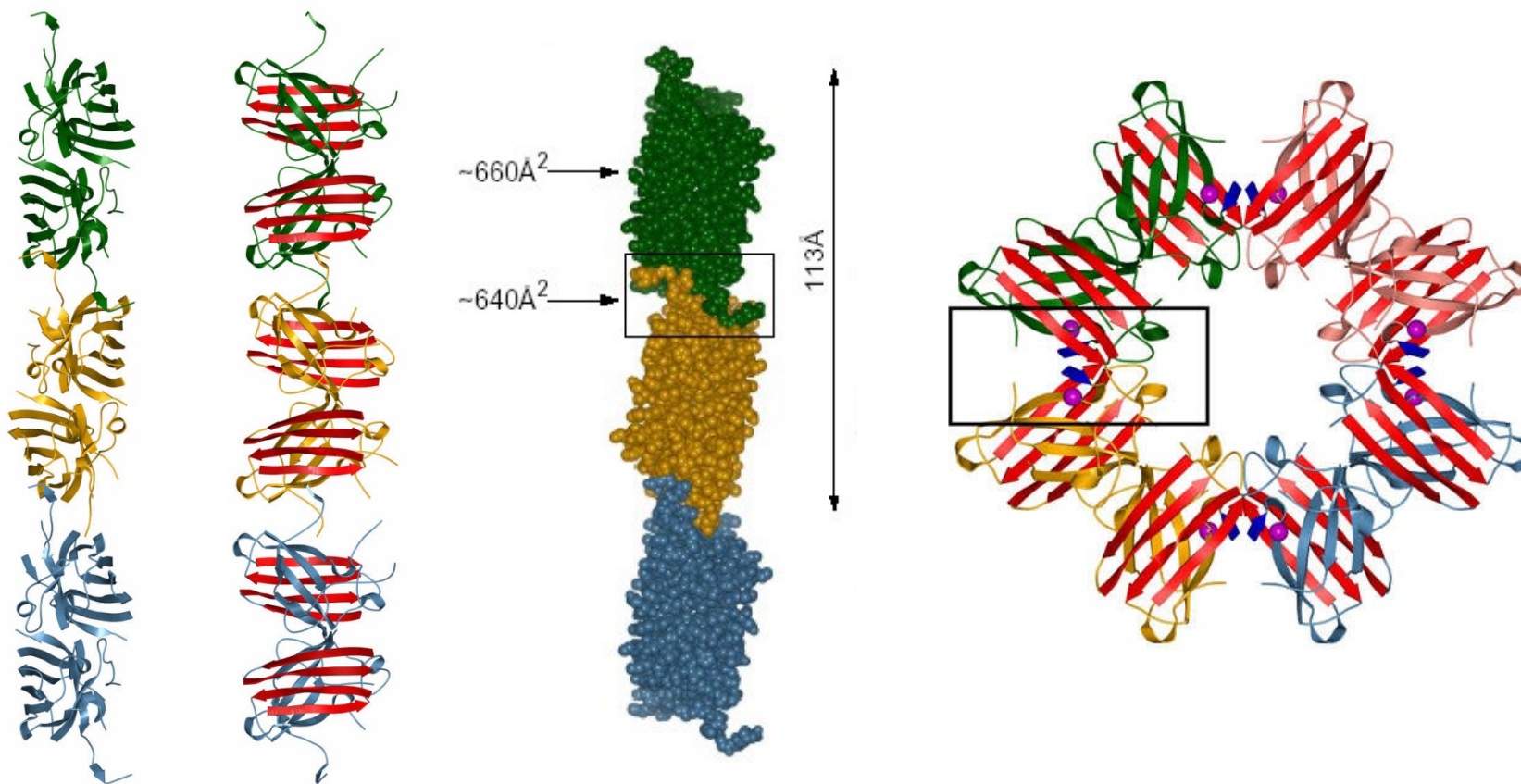
## Example 3: Cu/Zn Superoxide Dismutase (Dr. John Hart, Dr. Ahmad Galaleldeen, UTHSCSA)



**Questions: Loss of function or gain of toxic function? Various experiments suggest aggregation. Do structural changes cause a gain of toxic (aggregation) function? Are metal-binding mutants destabilized? Do they dissociate more easily? Is that a pathway to aggregation?**

# Protein Stability Analysis

## MBR mutants S134N and H46R

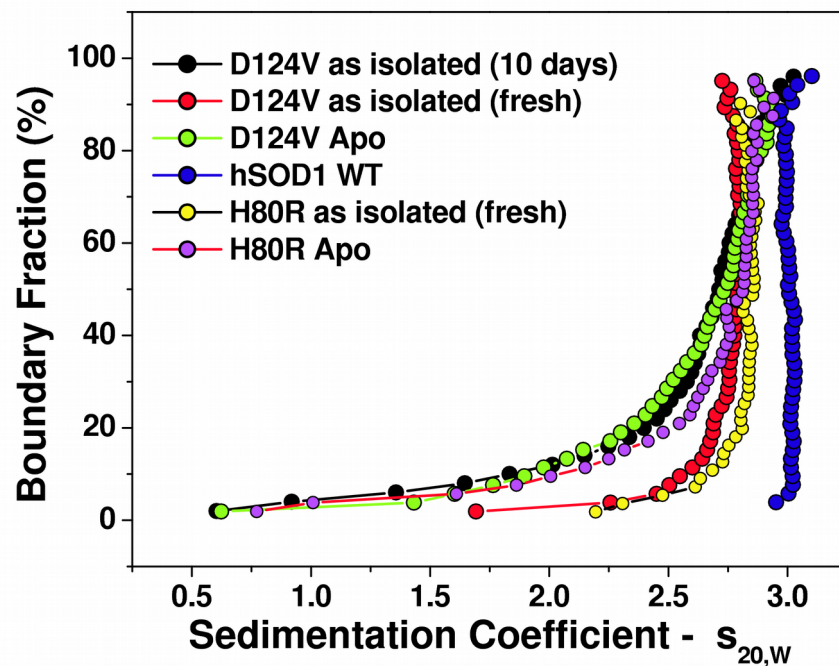


Elam, J. S., Taylor, A. B., Strange, R., Antonyuk, A., Doucette, P. A., Rodriguez, J. A., Hasnain, S. S., Hayward, L. J., Valentine, J. S., Yeates, T. O., and Hart, P. J. (2003) *Nat Struct Biol* **10**, 461-467

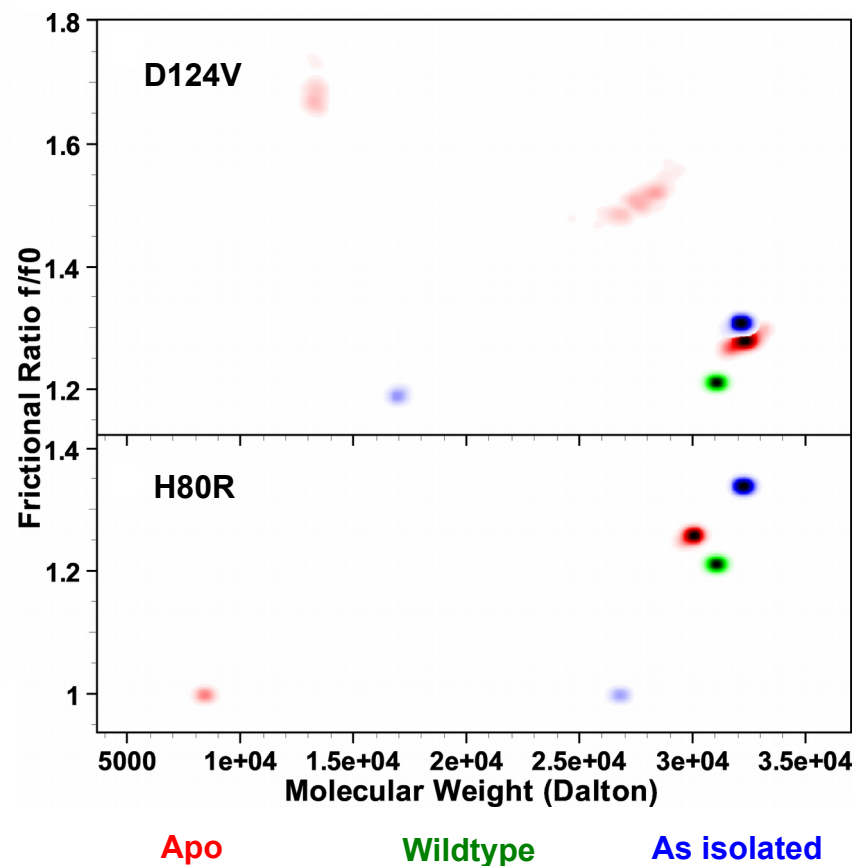
# Protein Stability Analysis

## MBR mutants D124V and H80R

### van Holde – Weischet Analysis



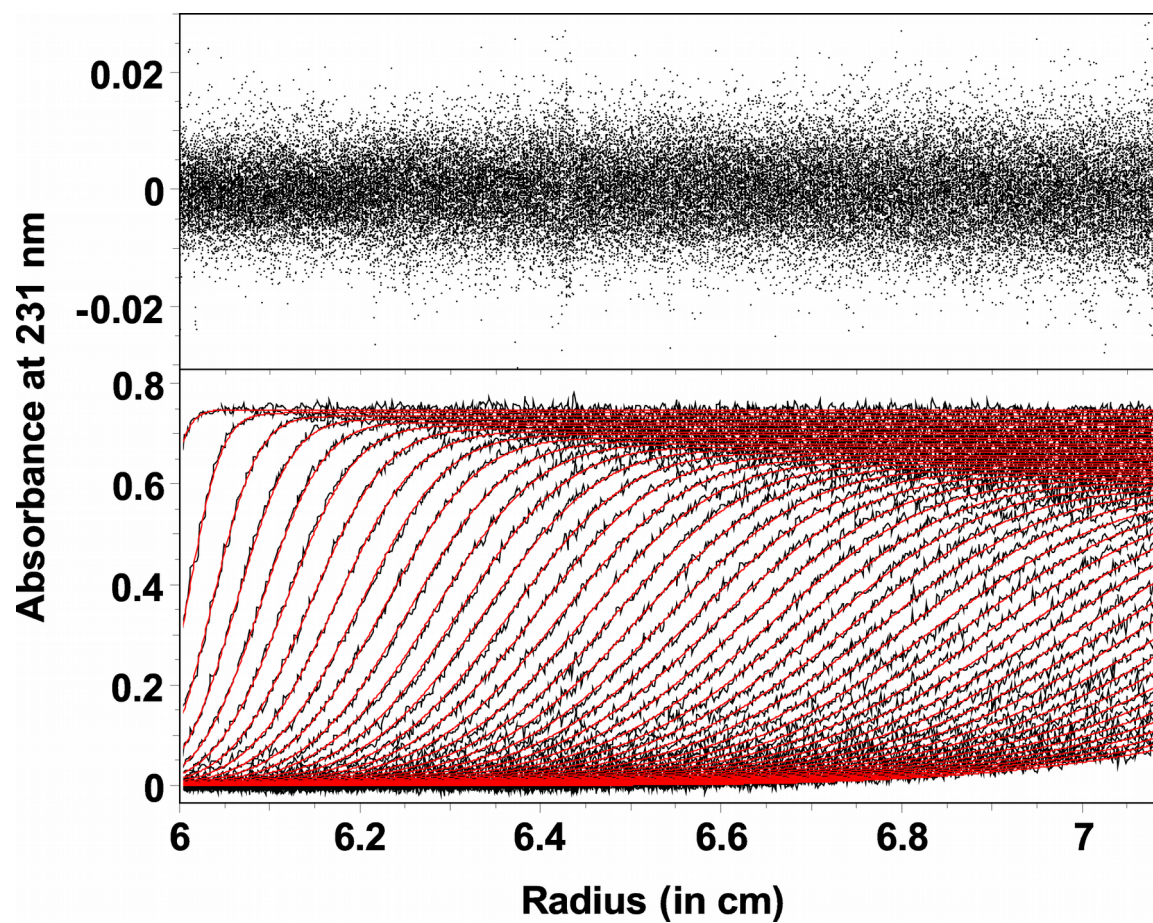
### Non-interacting GA-MC fit





# ***Protein Stability Analysis***

**D124V, SVE fitted with a reversible monomer-dimer equilibrium model**



# ***Protein Stability Analysis***

## **D124V, as isolated: Monomer-dimer model**

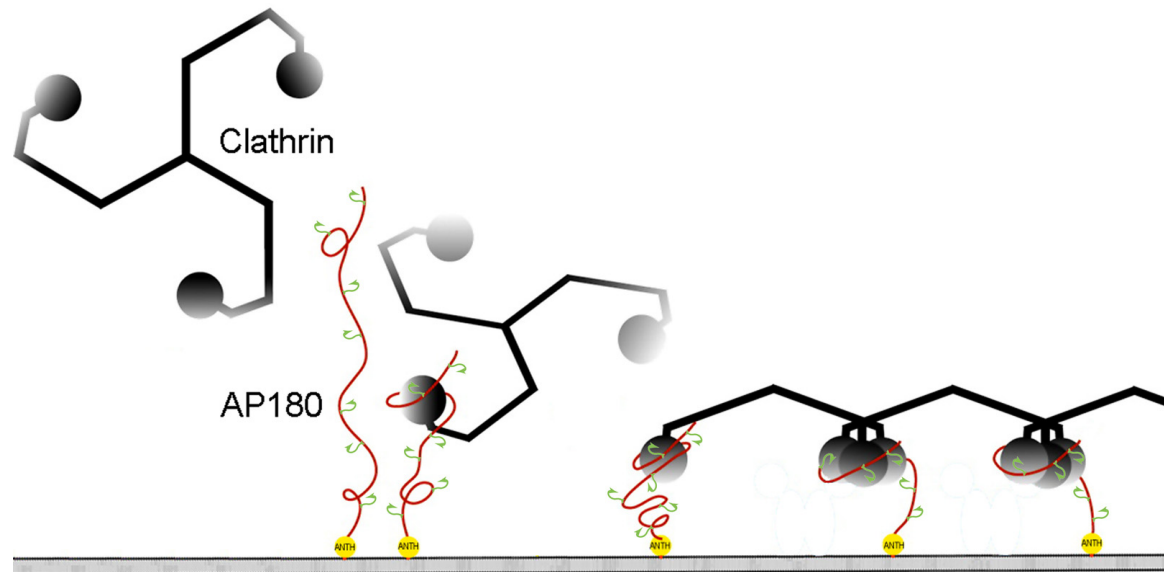
	<b>Reversible Monomer-dimer self-associating model</b>	<b>Non-interacting model</b>
<b>Monomer MW</b>	<b>16.1 (15.8, 16.4) kDa</b>	<b>16.8 (16.6, 17.1) kDa</b>
<b>Dimer MW</b>	<b>Constrained to 2 x Monomer MW</b>	<b>31.8 (31.6, 3.23) kDa (fitted)</b>
<b>Monomer <math>f/f_0</math></b>	<b>1.12 (1.09, 1.14)</b>	<b>1.19 (1.18, 1.20)</b>
<b>Dimer <math>f/f_0</math></b>	<b>1.29 (1.28, 1.31)</b>	<b>1.31 (1.30, 1.31)</b>
<b>Equilibrium const.</b>	<b>0.65 <math>\mu</math>M (0.49 <math>\mu</math>M, 0.96 <math>\mu</math>M)</b>	<b>0.66 <math>\mu</math>M (based on partial C.)</b>
<b><math>K_{\text{off}}</math> rate const.</b>	<b><math>1.03 \times 10^{-4}</math> (<math>0.96 \times 10^{-4}</math>, <math>1.11 \times 10^{-4}</math>) <math>\text{s}^{-1}</math></b>	<b>n/a</b>
<b><math>K_{\text{on}}</math> rate const.</b>	<b><math>6.69 \times 10^{-5} \text{ M s}^{-1}</math></b>	<b>n/a</b>

### **Conclusion:**

**Metal binding region mutants destabilize the homodimer and cause monomerization. The monomer is thought to aid aggregation, and the destabilized metal binding region contributes to aggregation. Further studies need to be performed to evaluate the aggregation behavior.**

# Hetero-associations:

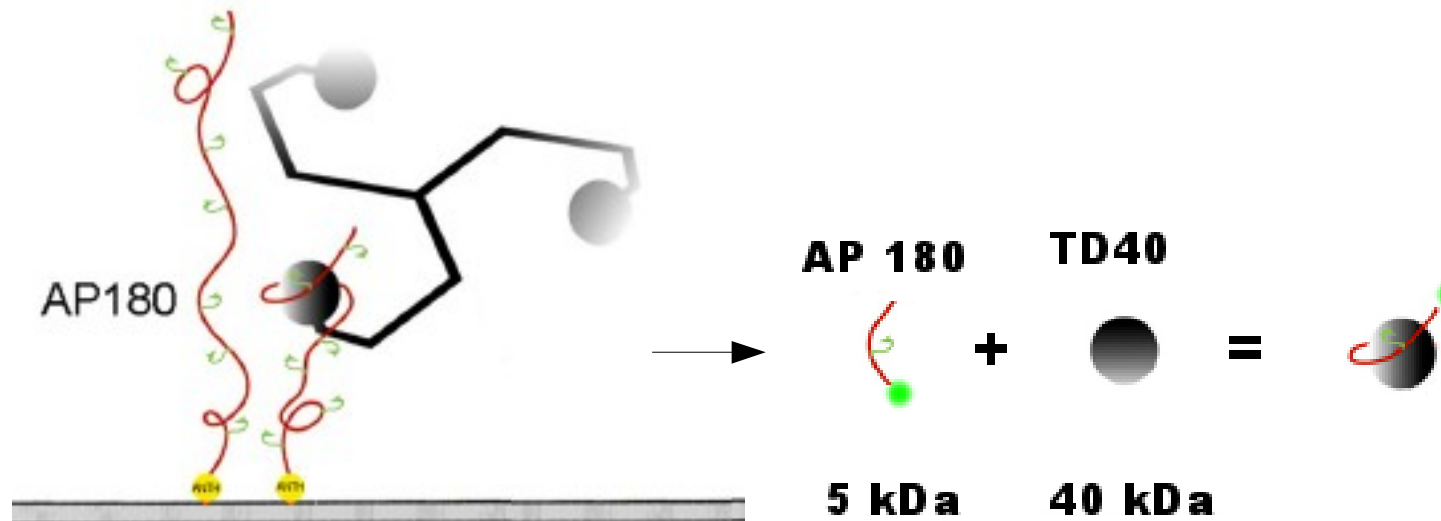
## Example Application: Clathrin assembly/Ligand Binding



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  $\text{PIP}_2$ , 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

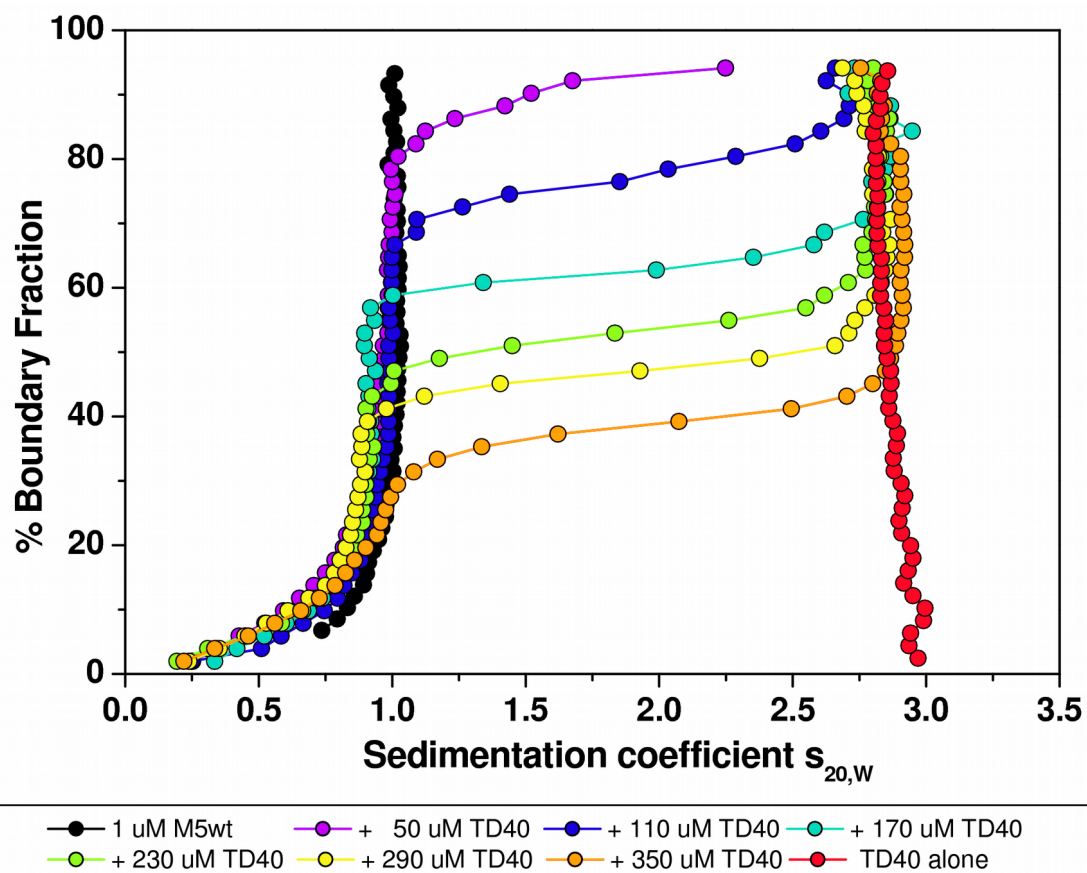


### Strategy:

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

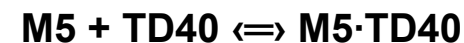
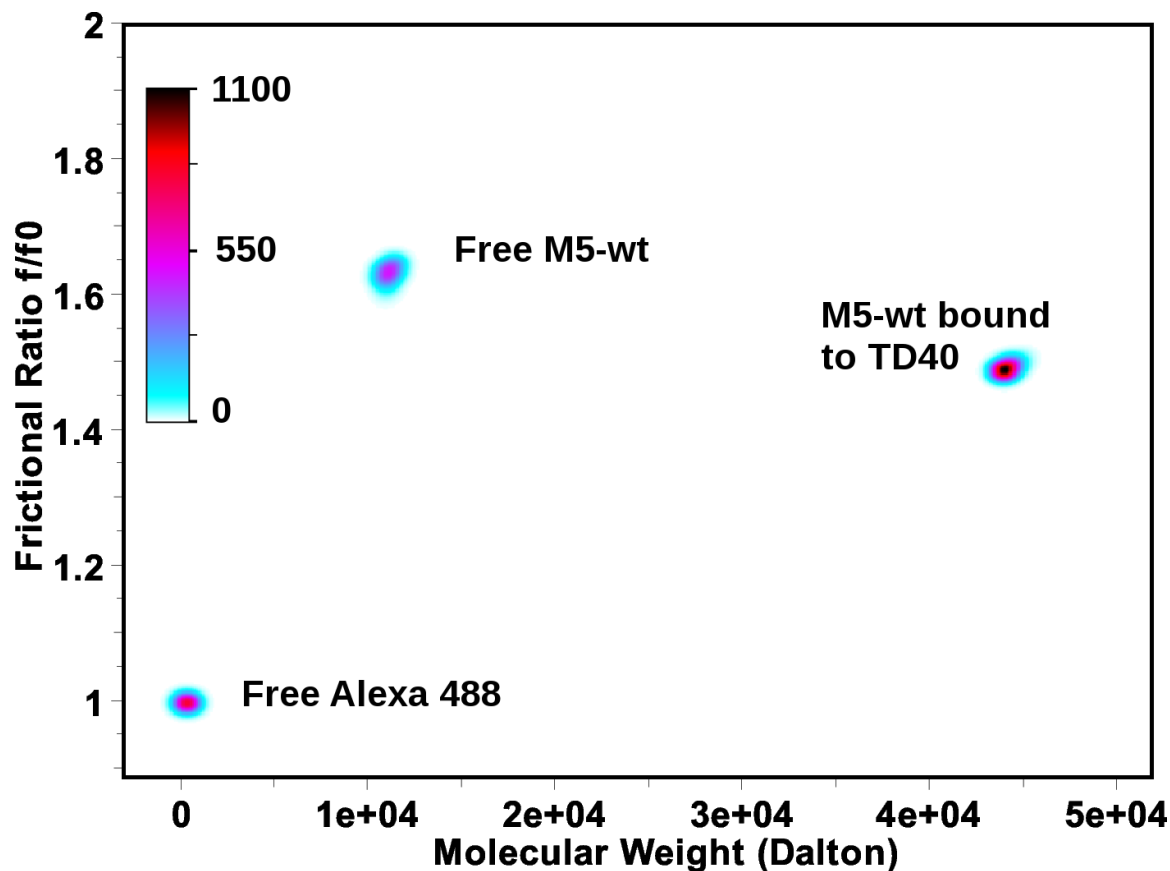
Titrate free TD40 against fixed amount of free M5 ligand and watch complex TD40\*M5 appear. Measure amounts of free M5 and complex TD40\*M5 and use that to calculate the  $K_d$





## AUC Applications

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



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

Measure:

fraction  $f$  of free M5

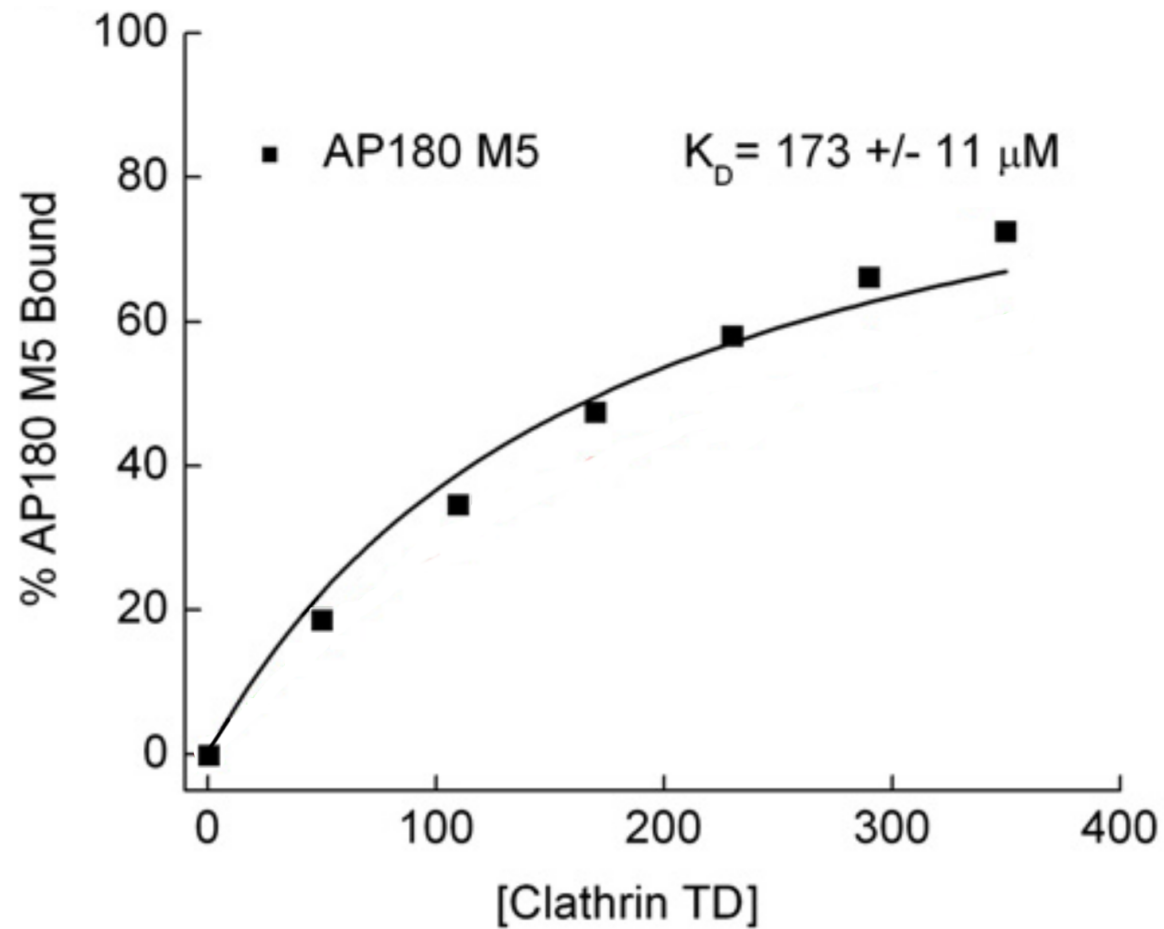
$$[M5] = f * [M5]_{\text{loading}}$$

$$[M5 \cdot TD40] = (1 - f) [M5]_{\text{loading}}$$

$$[TD40] = [TD40]_{\text{loading}} - [M5 \cdot TD40]$$

## AUC Applications

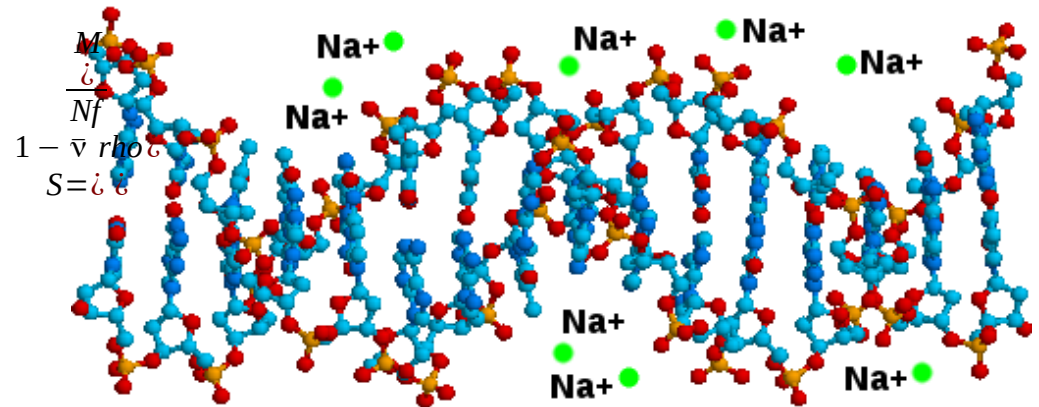
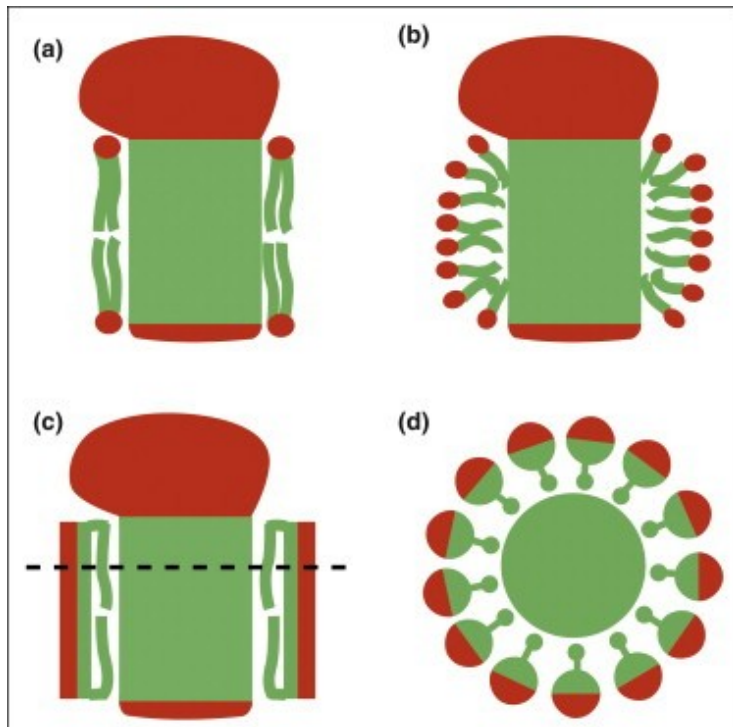
Binding Curve for M5 and TD40:



## Partial specific volume in hetero-associating Systems

Whenever there is binding, the  $\bar{v}$  will correspond to the  $\bar{v}$  of the sedimenting particle, and must include all components transiently bound to the molecule of interest during sedimentation, including salt ions and water.

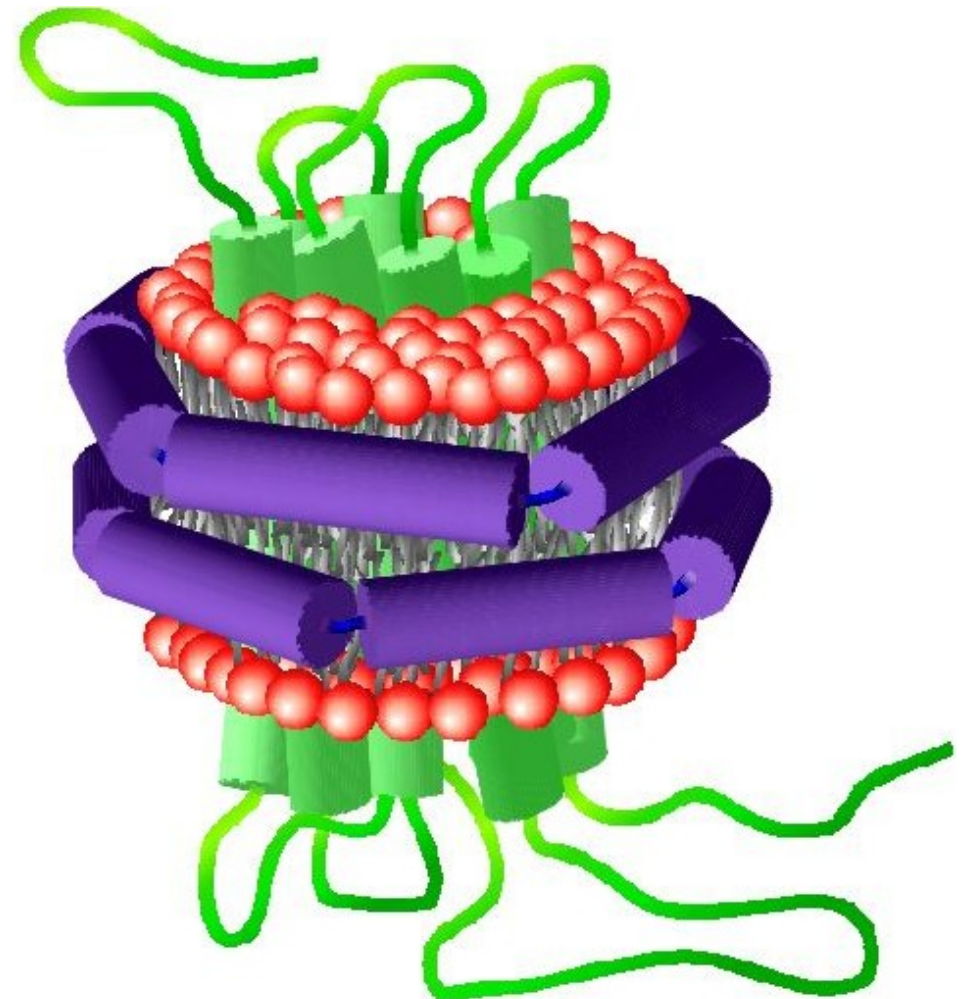
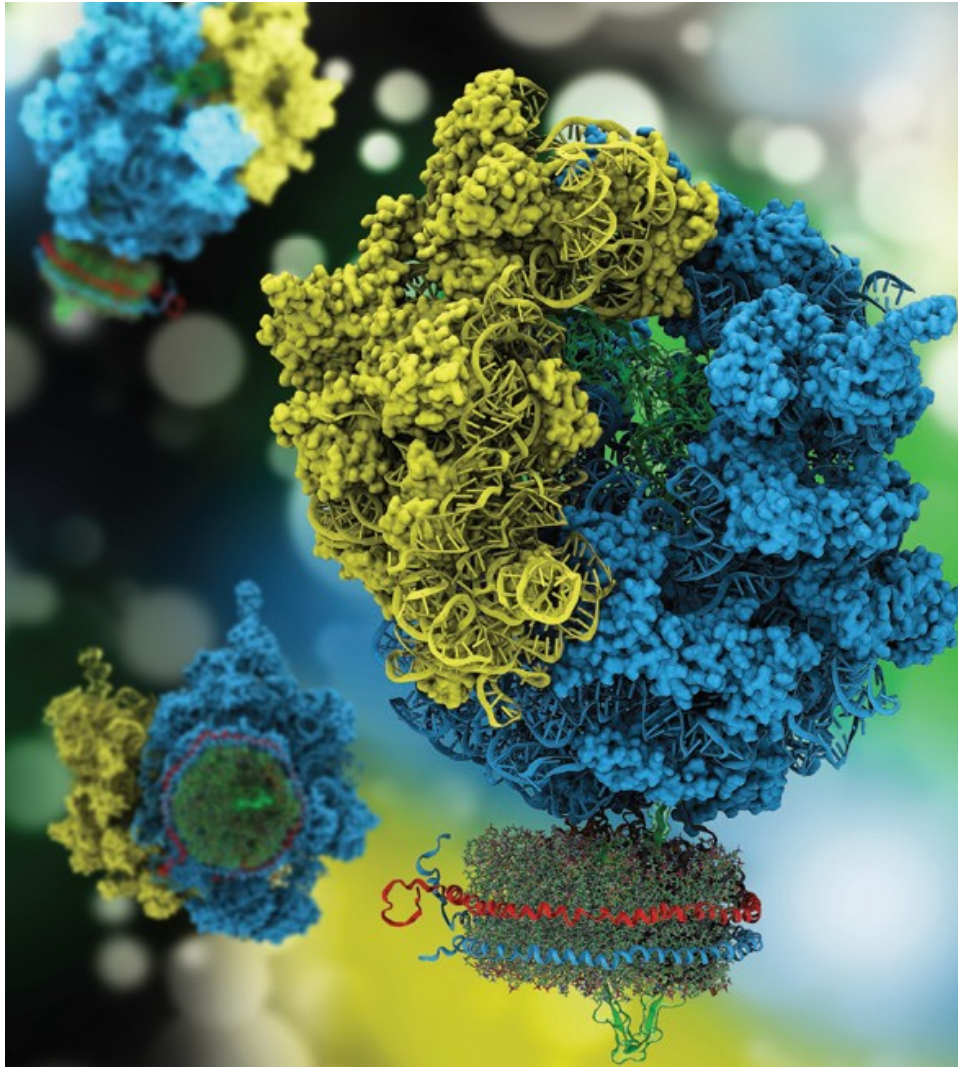
However, transiently bound molecules are *NOT* part of the molecular weight.



$$(1 - \Phi \rho)_{sp} = (1 - \bar{v}_{prot} \rho) + \delta_{det} (1 - \bar{v}_{det} \rho) + \dots$$

$$s = \frac{M(1 - \bar{v} \rho)}{Nf}$$

## *Partial specific volume in hetero-associating Systems*





## ***Partial specific volume in hetero-associating Systems***

### **Strategies for measuring MW of proteins when detergent is bound:**

- 1. Change the solvent density so that the solvent density is equivalent to the detergent density. Both detergent density and solvent density need to be independently validated. If both are the same, the bound detergent will not contribute to the buoyancy of the molecule, and:**

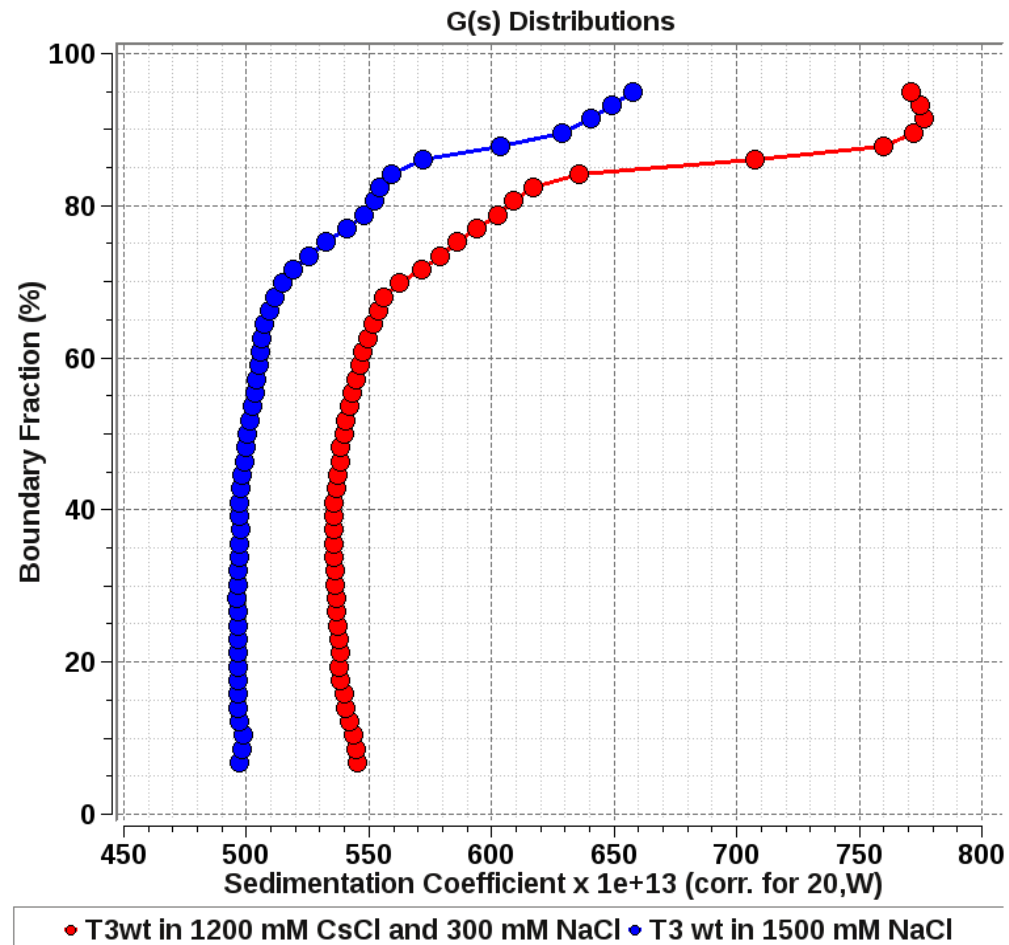
$$\rho = 1/\bar{v}_{det} \text{ where: } \delta_{det}(1 - \bar{v}_{det} \rho) = 0 \text{ and}$$
$$M(1 - \Phi\rho) = M(1 - \bar{v}_{prot} \rho)$$

**Then the detergent will not contribute.**

- 2. If the density of the detergent cannot be matched by any heavy water concentration, the same principle can be used by making multiple measurements in different concentrations of D<sub>2</sub>O or H<sub>2</sub>O<sub>18</sub> and evaluating the buoyancy term. Plot the buoyancy term as a function of solvent density and extrapolate this function to the density of the detergent. At this point the buoyancy term only reflects the protein, and the molecular weight reflects the entire sedimenting particle.**

## Partial specific volume in hetero-associating Systems

Example application: virus particle in two different salts:



## ***Reversible Systems - Summary:***

**Reversible systems can be analyzed by sedimentation velocity, and equilibrium constants can be determined more reliably.**

**Reversible systems are actually better fitted by constrained reversible models than by degenerate non-interacting models**

**Kinetic parameters can be measured**

**Reliable kinetics can be observed only if they fall in the regime of the timescale of the sedimentation experiment, and are best analyzed at high speed:**

**Faster kinetics can best be determined for large/fast sedimenting systems  
Range of detection is between  $10^{-2}$ /sec –  $10^{-6}$ /sec**

**Sedimentation velocity measurements outperform sedimentation equilibrium measurement due to increased composition resolution and due to much greater data density (outperform = increased resolution, more information, enhanced accuracy and precision)**

**Sedimentation velocity experiments also provide shape information**

**Sedimentation velocity experiments are more sensitive and can resolve low concentration contaminants, and can account for their presence separately in the fit, yielding more accurate parameters for the remaining solutes.**