Studying proteins and their interactions in concentrated and crowded environments via tracer sedimentation equilibrium and static light scattering

Cristina Fernández
Centro de Investigaciones Biológicas
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German Rivas

Allen P. Minton

CENTRO DE INVESTIGACIONES BIOLÓGICAS CSIC

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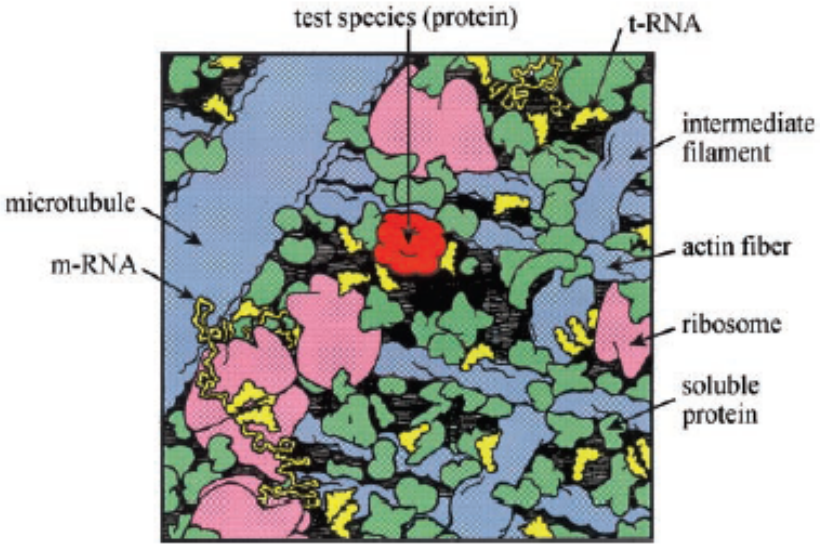
Study of macromolecules at high concentration

The quantitative characterization of proteins in highly concentrated solutions is of interest for at least two reasons:

1. **Biological.**

   To characterize nonspecific interactions that can influence macromolecular reactivity and state of association in biological media.



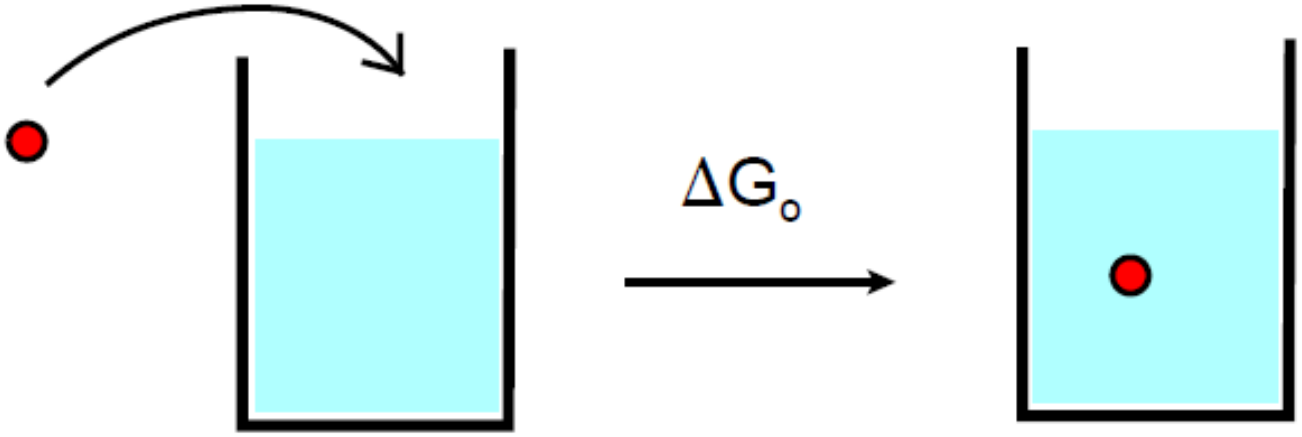
Cartoon of eukaryotic cytoplasm

Modified from Goodsell, 1993

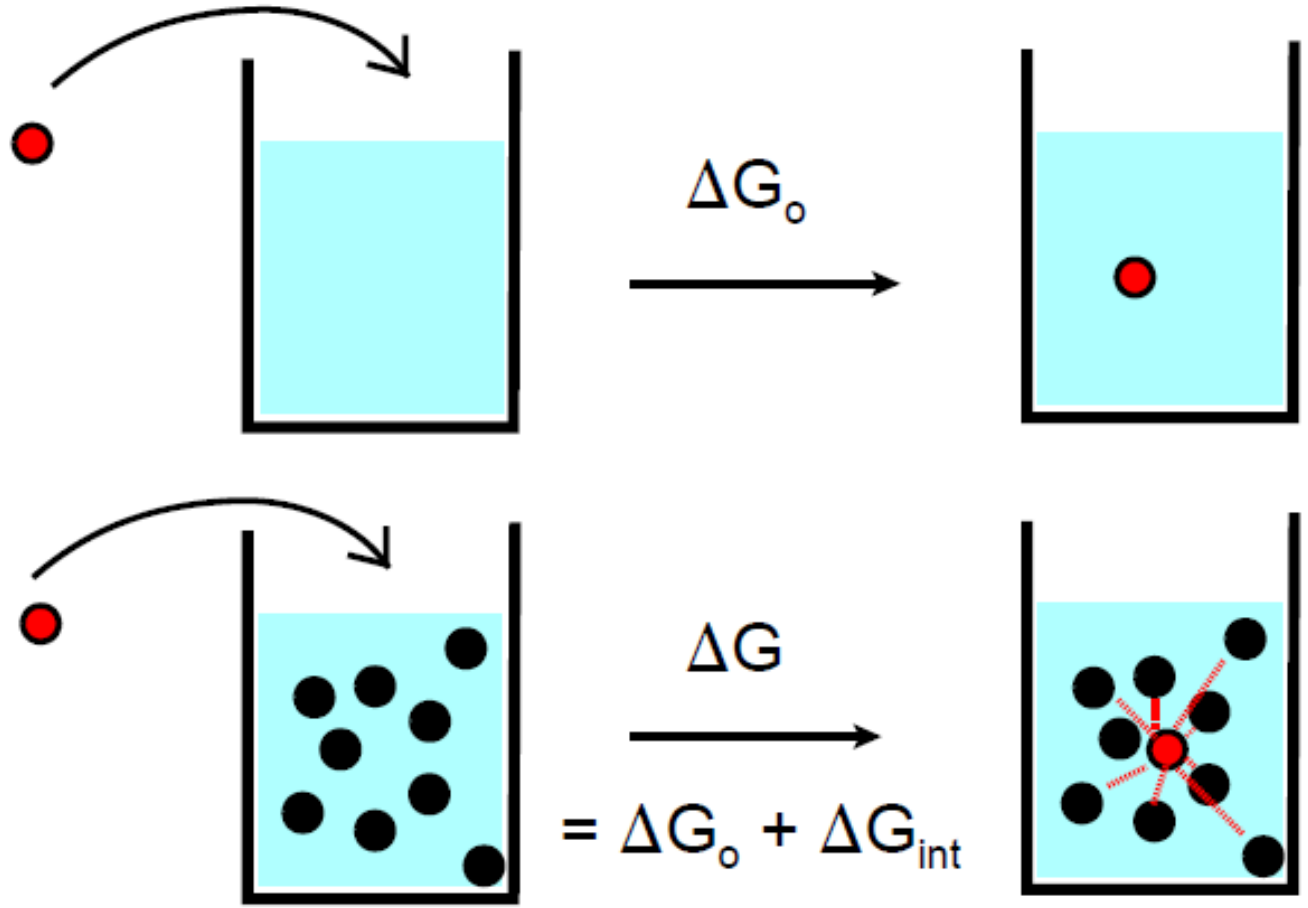
2. **Biotechnological.**

   Biopharmaceutical compounds are stored, administered at high concentration.

A general definition of interactions



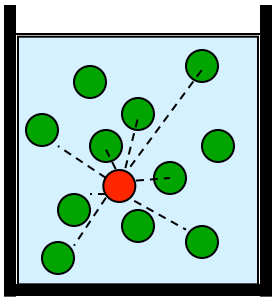
A general definition of interactions



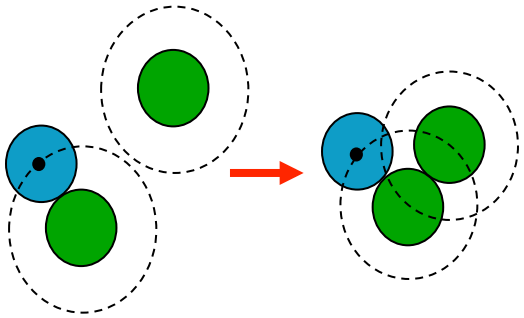
Thermodynamic activity coefficient

$$\left(\frac{\partial \Delta G_{\text{int}}}{\partial c_i} \right) \equiv RT \ln \gamma_i$$

$\ln \gamma_i > 0$ Repulsive solute-solute interaction



$\ln \gamma_i < 0$ Attractive solute-solute interaction or complex formation



Reduction in excluded volume = Reduction in free energy

The challenge is to discriminate between weak associations and nonspecific repulsive interactions, and evaluate the contribution of each to the total experimentally observable behaviour of the solution

Nonspecific interactions do not depend upon detailed structure or function of the macromolecule, but upon gross physical properties:size, shape, surface hydrophobicity/polarity, net charge, dipole moment, etc.

Examples:

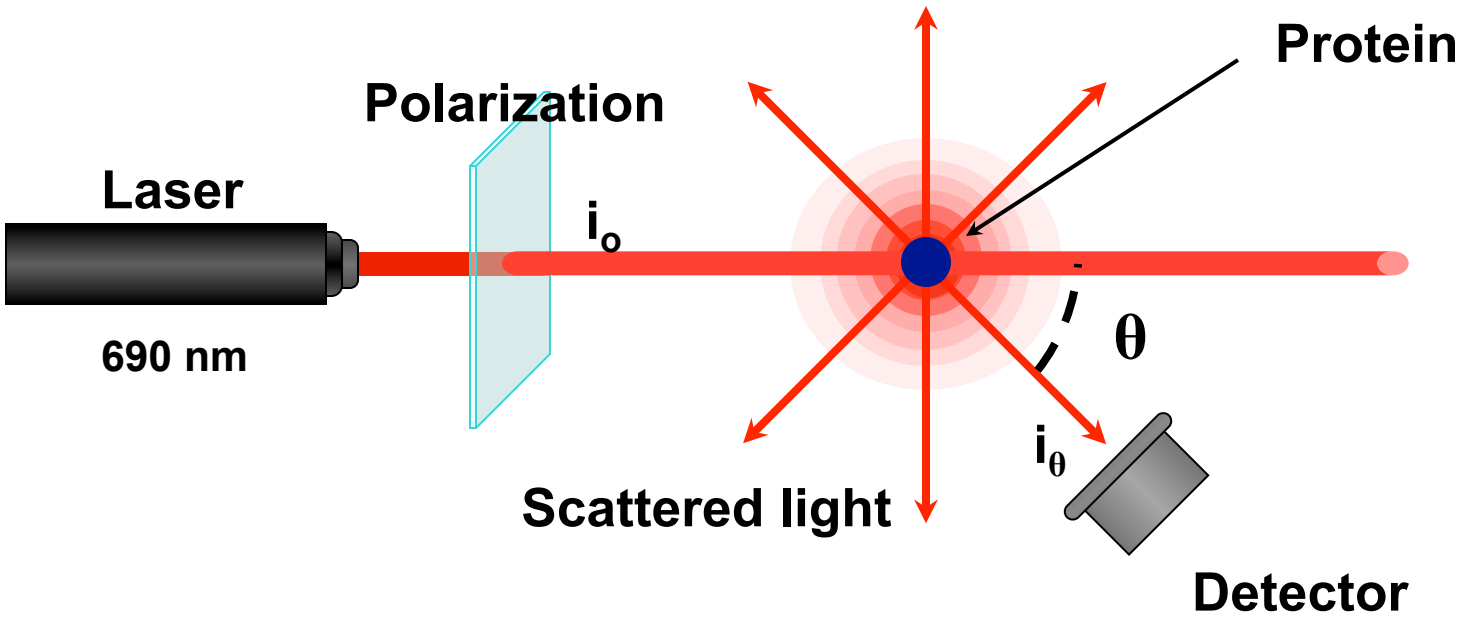
Steric repulsion, monopolar electrostatic repulsion, dipolar electrostatic attraction, short-range attraction between hydrophobic patches

Experimental techniques especially suited for characterization of macromolecular interactions in concentrated solution

1. Concentration gradient – static light scattering

2. Non-ideal tracer sedimentation equilibrium

STATIC LIGHT SCATTERING

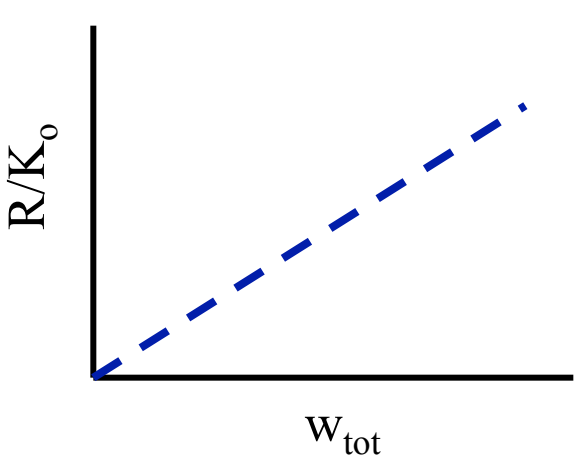


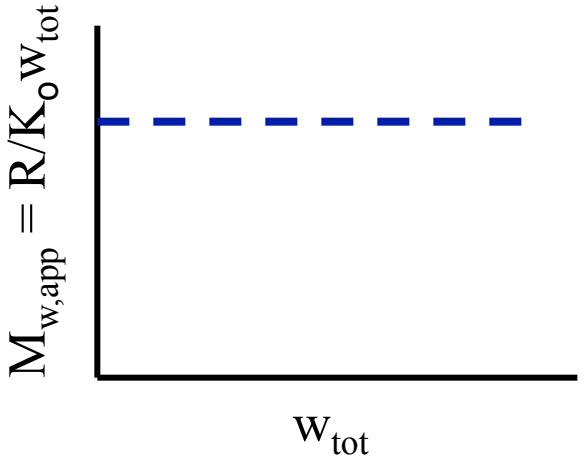
$$R/K_o \propto i/i_o$$

R is the Rayleigh ratio, a quantity used to characterize the scattered intensity.

Ideal behavior, no concentration-dependent association

$$\frac{R}{K_o} = \sum_i M_i w_i = w_{tot} M_w$$





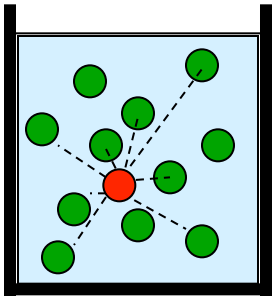
The amount of light scattered is directly proportional to the product of the protein molar mass and concentration.

Effect of nonideality on scattering: single species

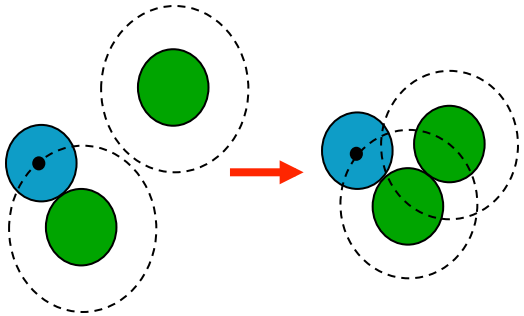
$$\frac{R}{K_o} = \left(\frac{n}{n_o}\right)^2 \frac{Mw}{1 + w\dfrac{d\ln\gamma}{dw}}$$

$$\left(\frac{\partial \Delta G_{\text{int}}}{\partial c_i}\right) \equiv RT \ln \gamma_i$$

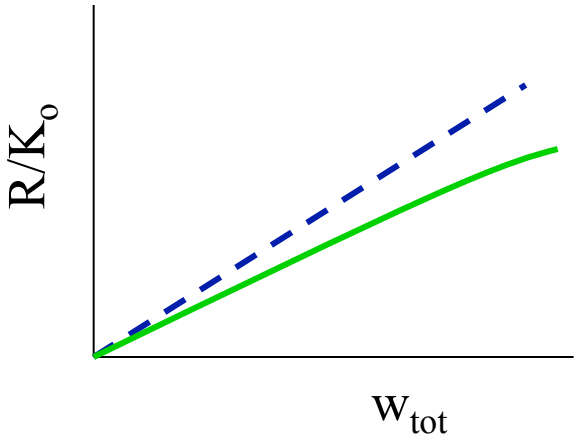
$$w\frac{d\ln\gamma}{dw} > 0$$ Repulsive solute-solute interaction

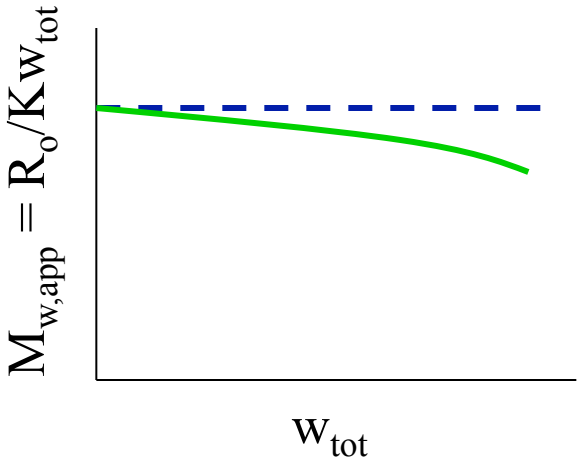


$$w\frac{d\ln\gamma}{dw} < 0$$ Attractive solute-solute interaction



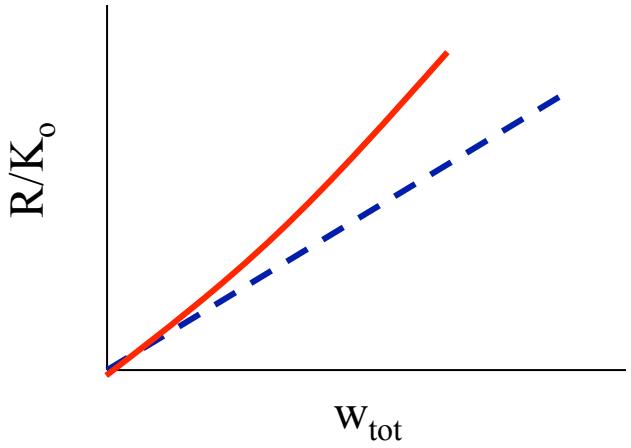
Effect of nonideality on scattering

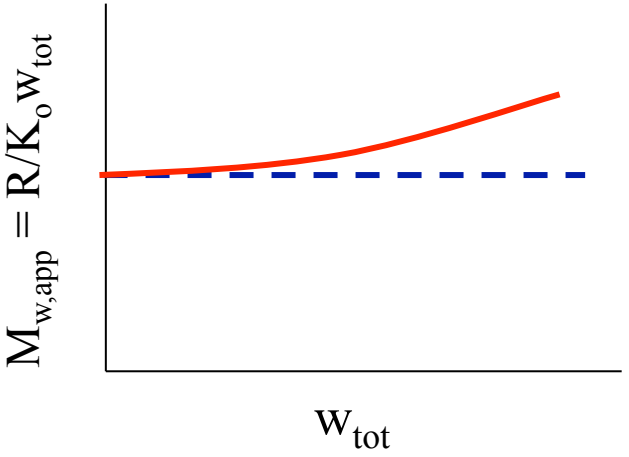




$$w\frac{d\ln\gamma}{dw} > 0$$

Repulsive solute-solute interaction



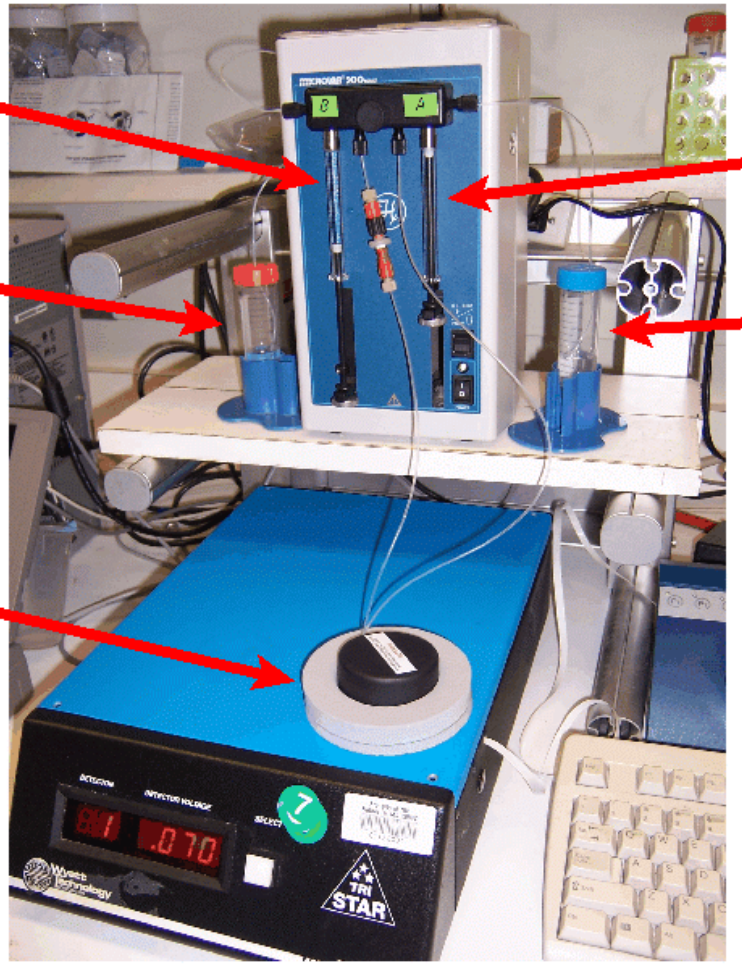


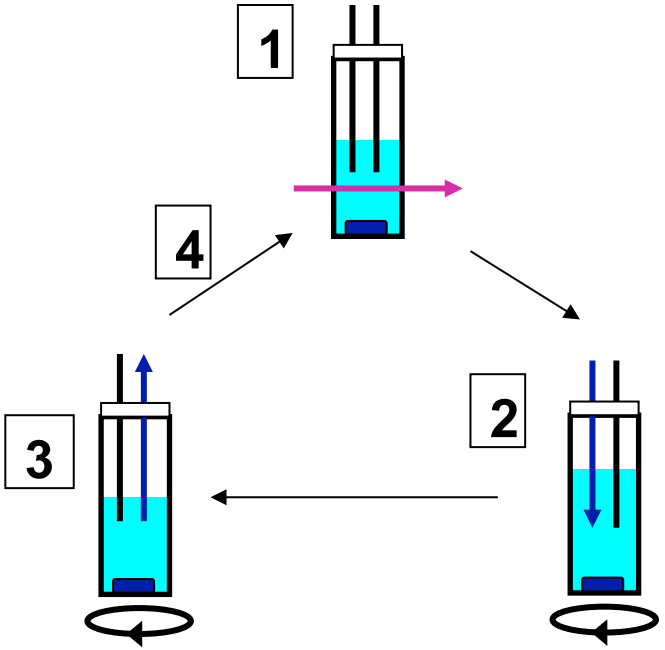
$$w\frac{d\ln\gamma}{dw} < 0$$

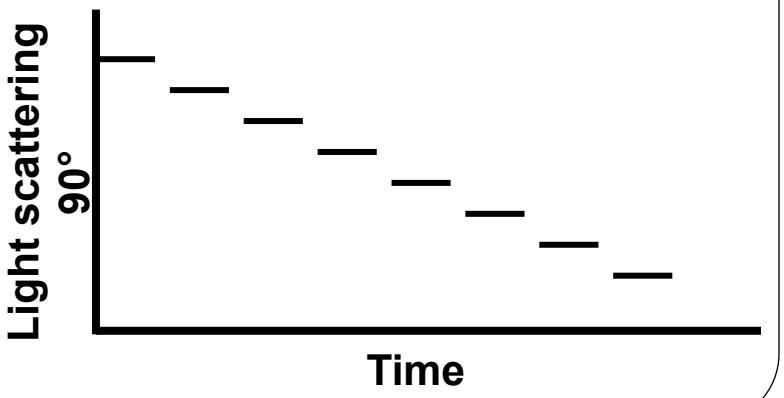
Attractive solute-solute interaction

Experimental set-up

Automated dilution system

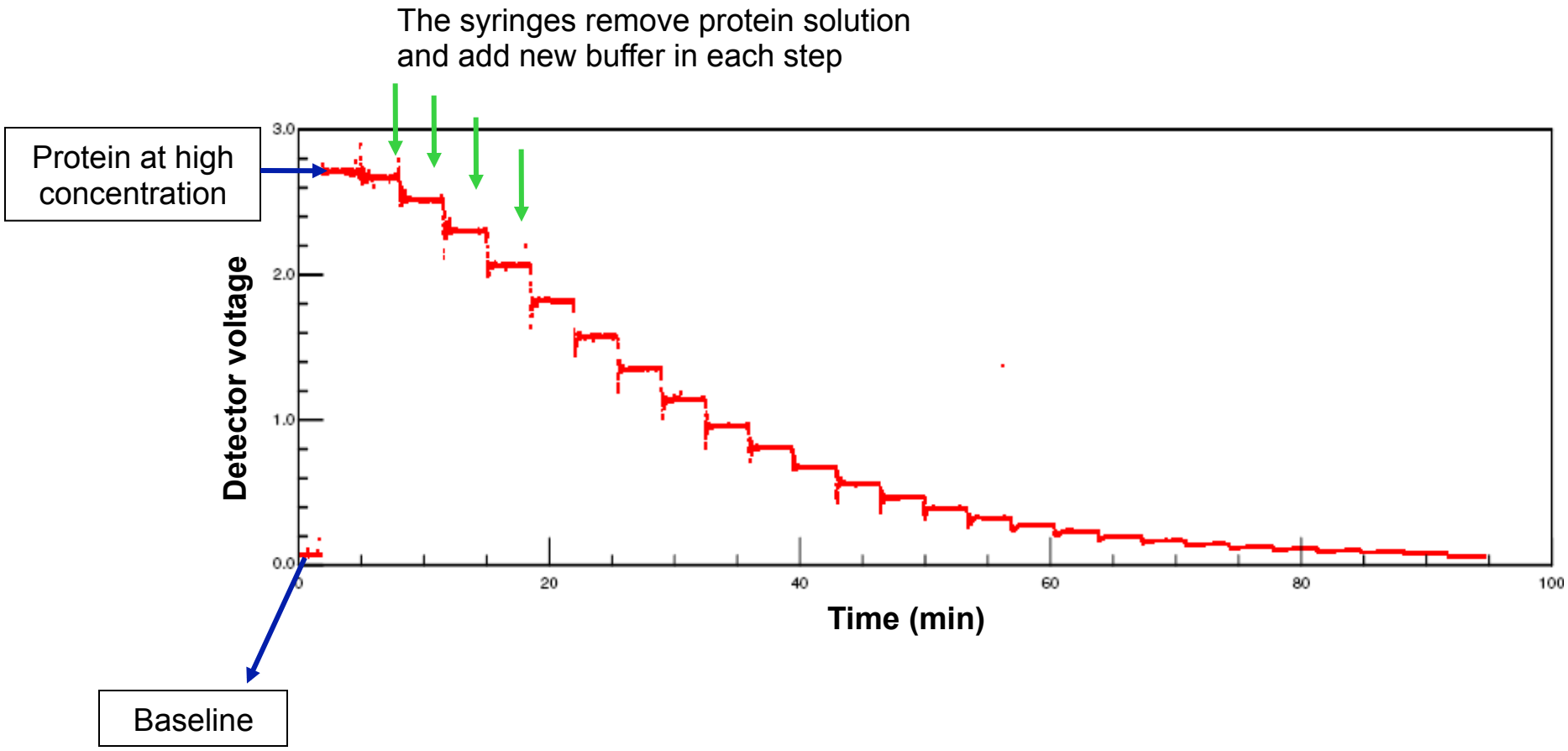






Experimental data - BSA

BSA (80 g/l), PBS pH 7.4, 150 mM NaCl

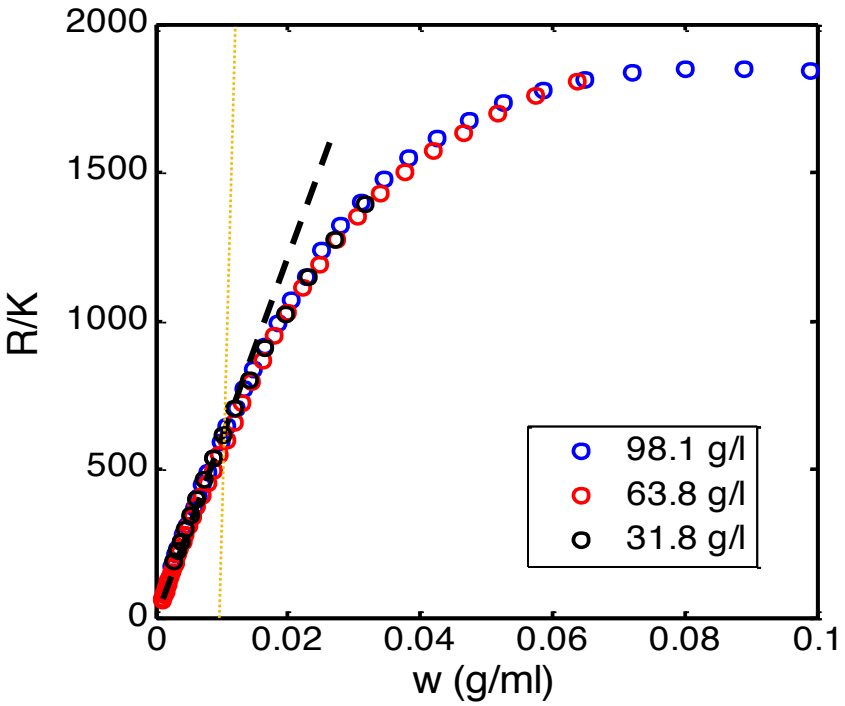


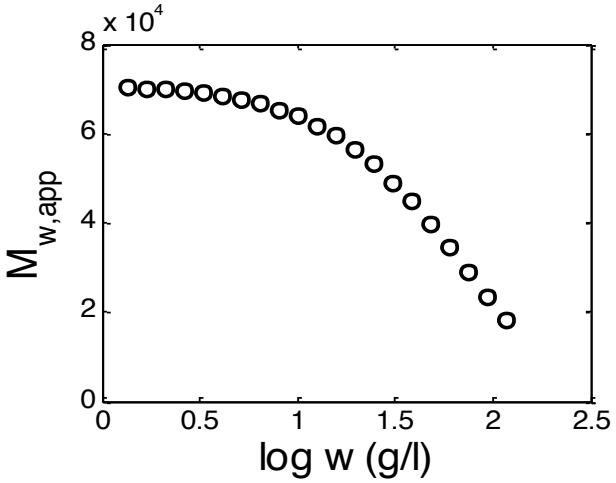
We measured the light scattering as a function of concentration over the range 1-100 g/l.

- Non self-associating proteins
- Self-associating proteins

Non-associating protein: BSA

Experimentally obtained values of normalized scattering intensity (R/K_o) plotted as a function of concentration





$$\frac{R}{K_o} = \left(\frac{n}{n_o}\right)^2 \frac{Mw_{tot}}{1 + w\dfrac{d\ln\gamma}{dw}}$$

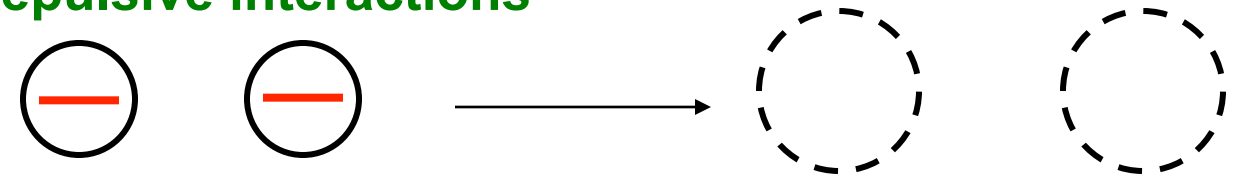
Repulsive solute-solute interaction

Hard sphere model

- In the effective hard particle theory, nonspecific interactions are modeled as hard core interactions between hard convex particles of specified shape and size.

- Additional “soft” interactions (e. g. electrostatic repulsion or attraction, or hydrophobic attraction) are incorporated into the model by modifying the size of the effective hard particle.

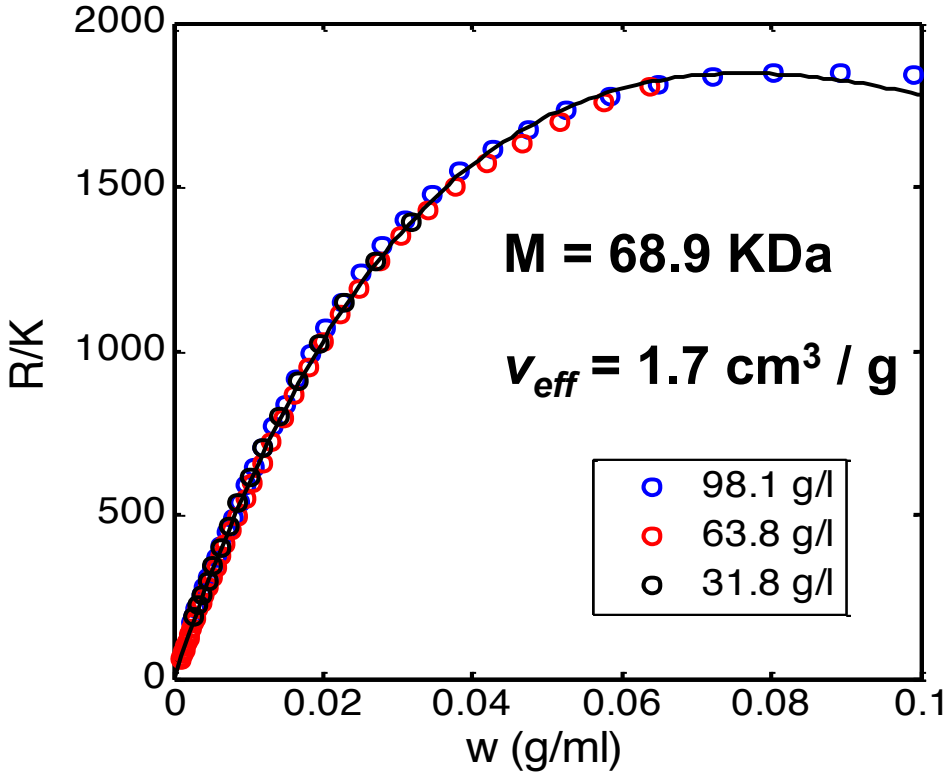
Repulsive interactions



Hall & Minton. (2003) *Biochim. Biophys. Acta.* 1649:127-39.

Best fit of hard sphere scattering model to BSA data

We model the dependence of the apparent weight-average molecular weight ($M_{w,app}$) upon concentration (w) in non-ideal solutions.

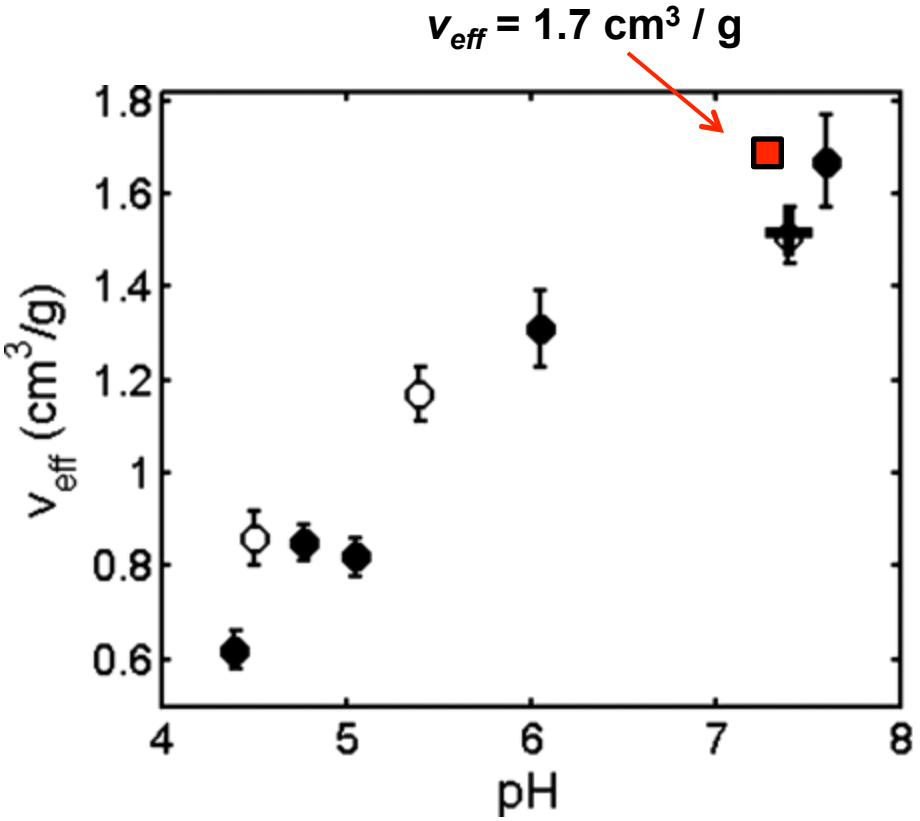


$$\frac{R}{K_o} = \left(\frac{n}{n_o}\right)^2 \frac{Mw_{tot}}{1 + w\frac{d\ln\gamma}{dw}}$$

$$\frac{\nu_{eff}\,(cm^3/g)}{M}$$

Non-associating protein: BSA

BSA data are described by the Effective Hard Particle Model



Specific volume of equivalent hard particle best accounting for concentration dependence of BSA in 0.15 M NaCl, plotted as a function of pH.

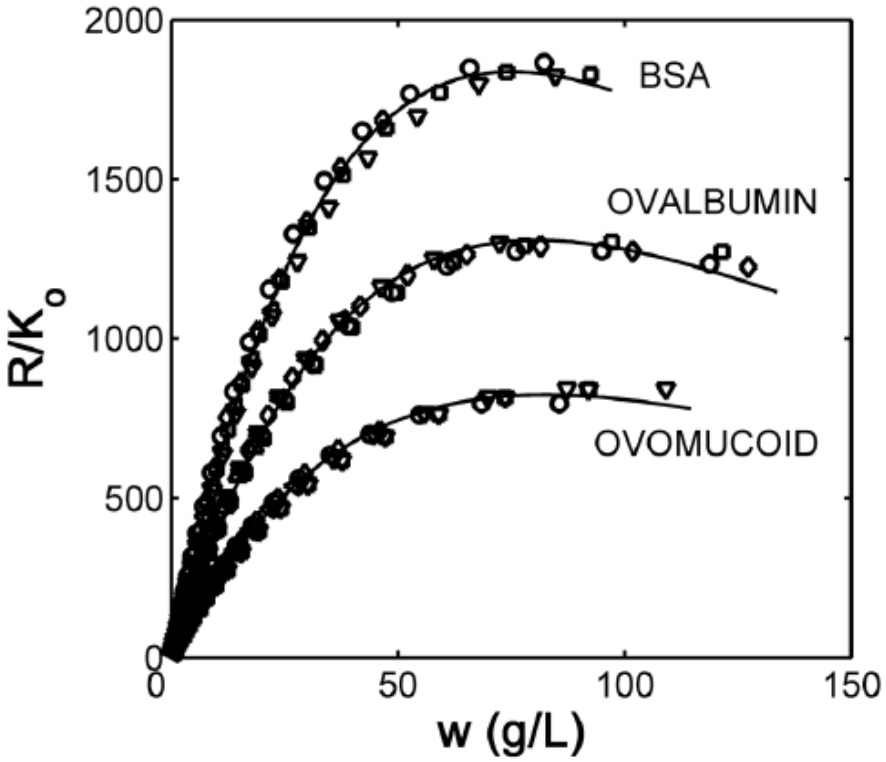
Light scattering: closed circles
Edsall et al (1950)

Sedimentation equilibrium: cross
Millar (1983)

Osmotic pressure: open circles
Vilker et al. (1974)

Minton A.P. (2007) *J Pharm Sci.* 96:3466-9.

Experimentally obtained values of normalized scattering intensity (R/K_o) plotted as a function of concentration (w)



Data for all three proteins may be quantitatively accounted for by an effective hard sphere model with a single nonideality parameter for each protein.

Best fit parameters	**BSA**	**Ovalbumin**	**Ovomucoid**
M/1000	68.7± 1.6	45.5 ± 1.0	28.0 ± 0.8
v_{eff} **(cm^3/g)**	1.77 ± 0.06	1.64 ± 0.07	1.61 ± 0.05

Fernandez & Minton (2008) *Anal. Biochem.* 381:254-257

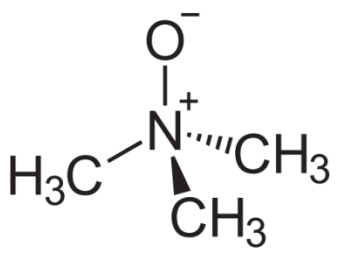
Non-associating mixtures of protein

- Bovine serum albumin (BSA) M ~ 67000 Da
- Albumin (from chicken egg white) M ~ 45000 Da
- Ovomucoid (Trypsin inhibitor) M ~ 28000 Da

Fernandez & Minton (2009) *Biophys. J.* 96:1992-98

Mixtures of proteins and other compounds

- Proteins + Trimethylamine N-oxide (TMAO) at pH 7.2



pK_a TMAO = 4.7

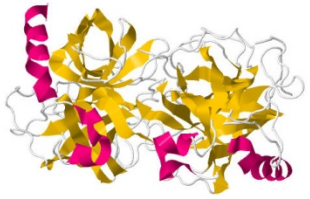
M = 75.11 Da

r_{TMAO} = 3 Å

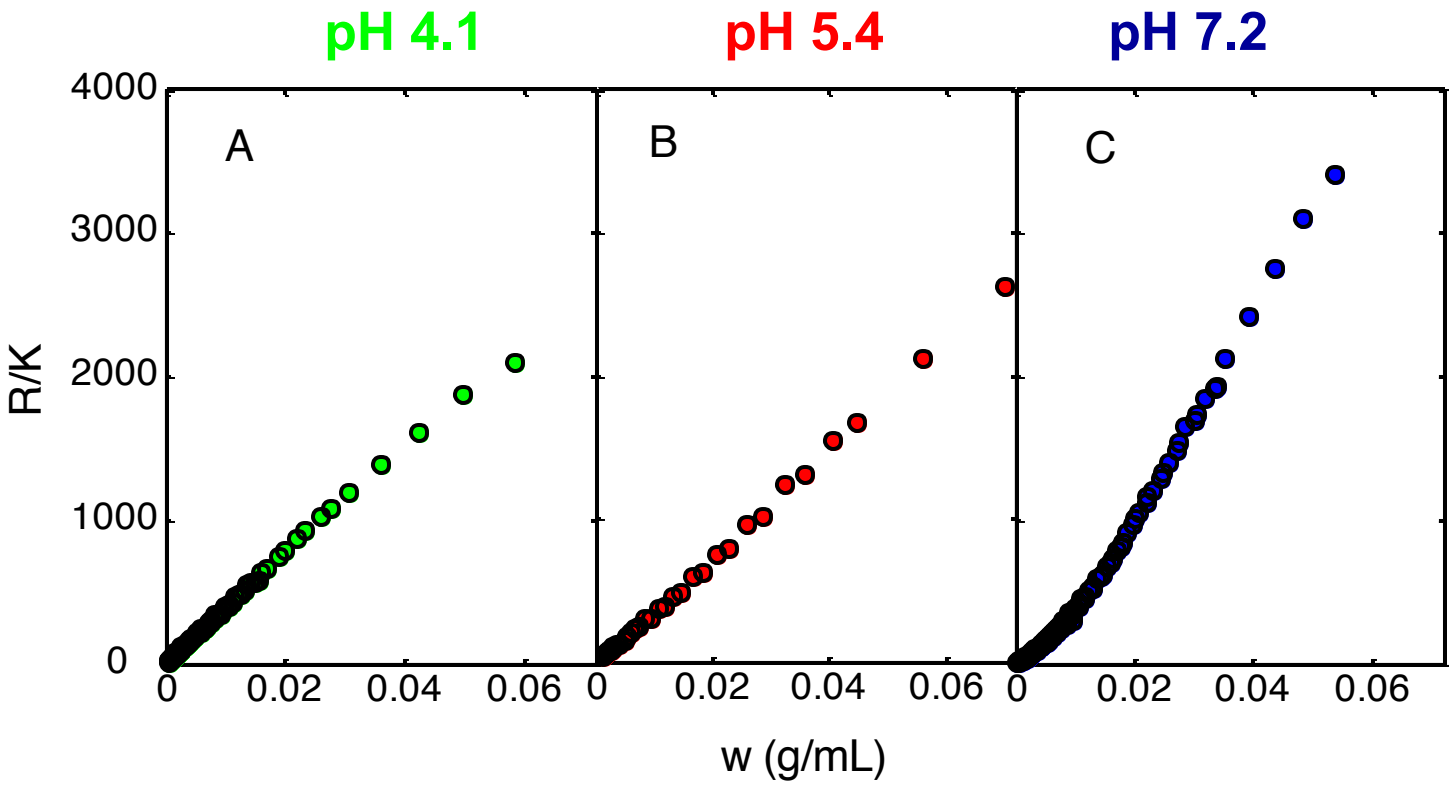
Fernandez & Minton (2010) *J. Phys. Chem. B.* 115:1289-1293

- Non self-associating proteins
- Self-associating proteins

α-chymotrypsin

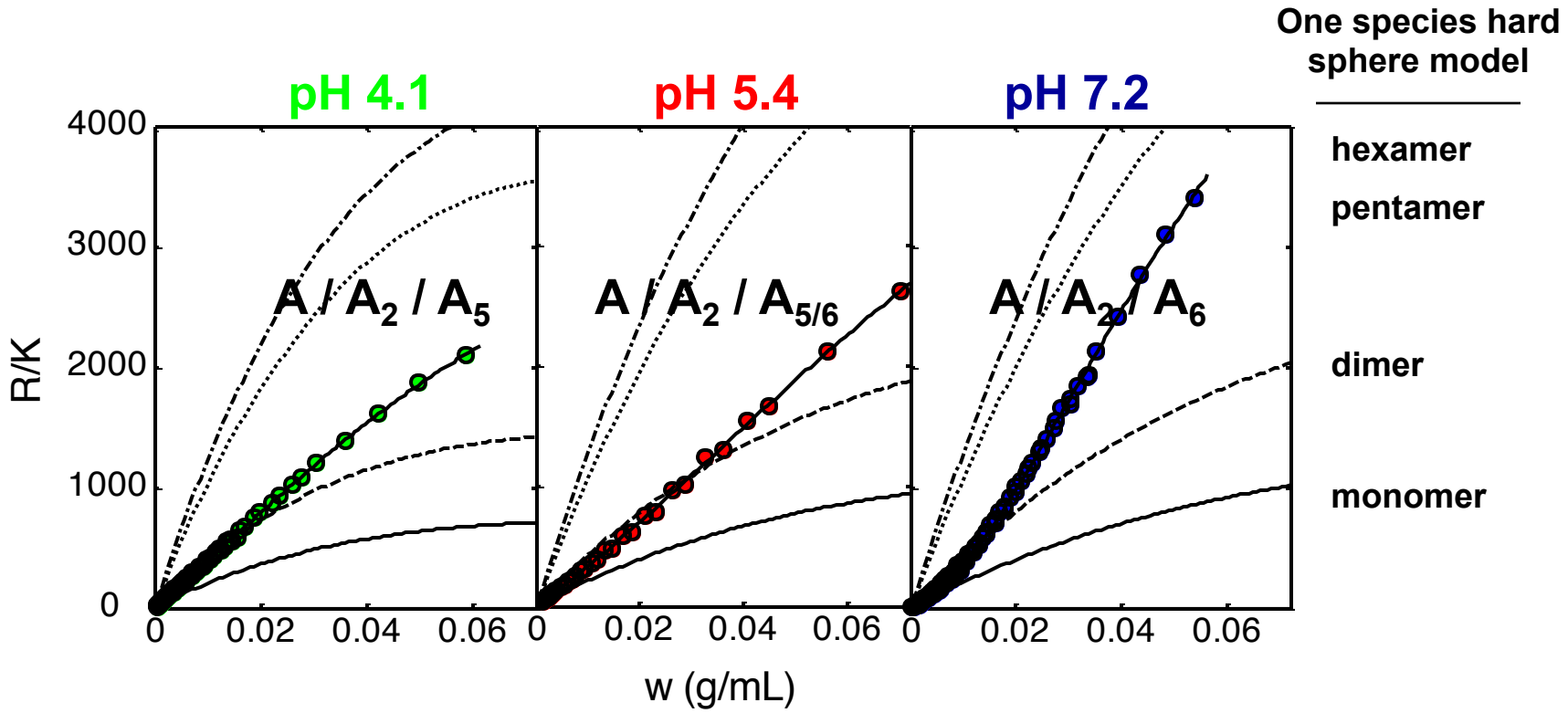


$$M + M \xleftrightarrow{K} D$$



Chymotrypsin self-associates with an affinity that changes with pH

Detection and characterization of low affinity self-association at high concentration



Repulsive solute-solute interaction

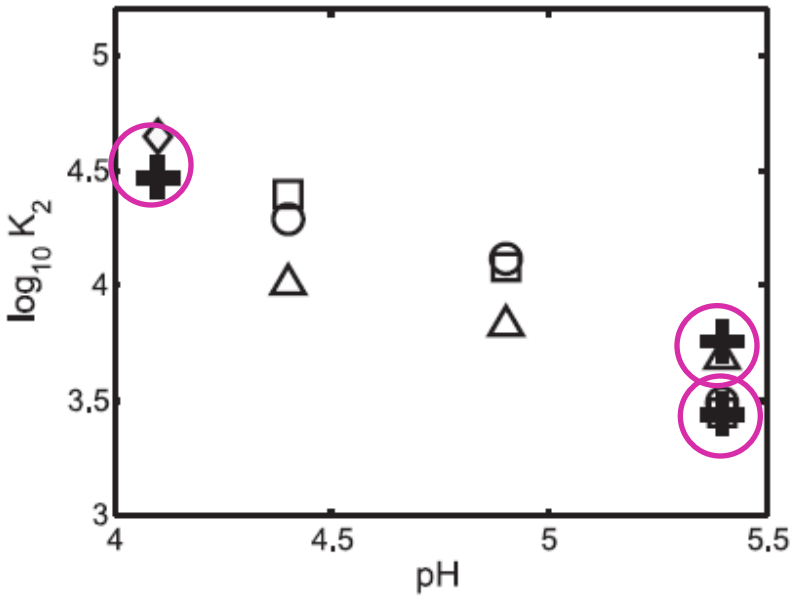
Attractive interactions are treated as association equilibrium:

$$iA \xleftrightarrow{K} A_i \qquad K = \frac{\gamma_i c_i}{\gamma_1^i c_1^i}$$

TABLE 2 Best-fit values of model parameters assuming various association schemes

pH	Self-association scheme	log K_2 (M^{-1})	log K_n (M^{-n+1})	v_{eff} (cm^3/g)
7.2	Monomer-dimer- hexamer ($n = 6$)	3.45 (−0.15, +0.15)	16.67 (−0.29, +0.33)	0.86 (−0.10, +0.10)
5.4	Monomer-dimer- hexamer ($n = 6$)	3.76 (−0.17, +0.18)	16.49 (−0.37, +0.44)	1.58 (−0.20, +0.18)
	or	3.44 (−0.15, +0.14)	12.36 (−0.36, +0.24)	1.00 (−0.36, +0.20)
	Monomer-dimer-pentamer ($n = 5$)			
4.1	Monomer-dimer- pentamer ($n = 5$)	4.47 (−0.15, +0.17)	14.94 (−0.31, +0.34)	1.50 (−0.18, +0.14)

M_1 was fixed at a value of 23000 on the basis of results obtained from experiments carried out at low total concentration. Indicated uncertainties correspond to 1 standard error of estimate.

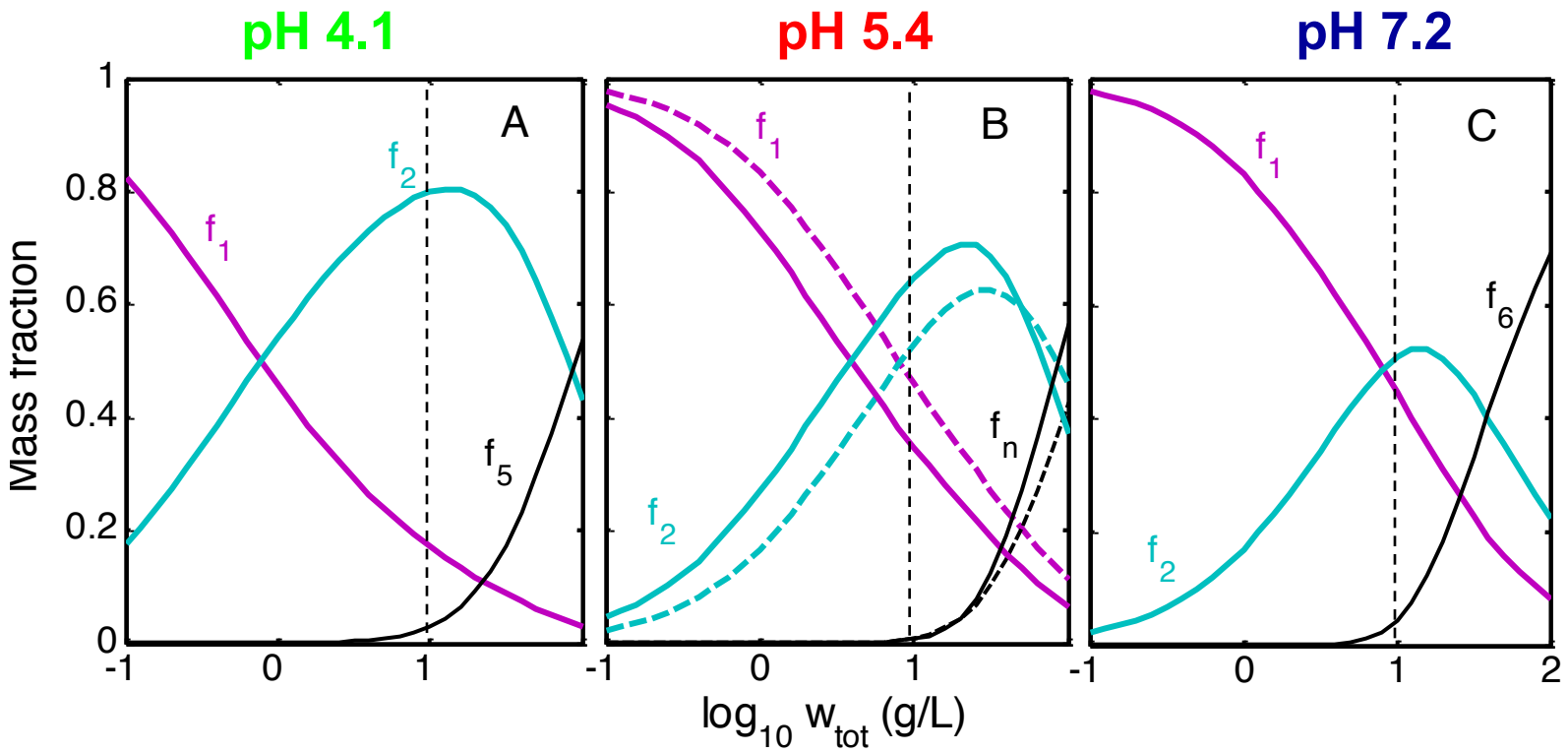


Comparison of values of the pH-dependent monomer-dimer equilibrium constant

✚ **Values presented in Table 2**

Fernandez & Minton (2009) *Biophys. J.* 96:1992-98

α-chymotrypsin



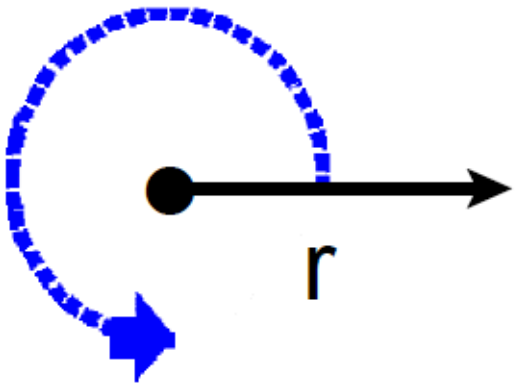
f_1 → monomer
f_2 → dimer
f_5 → pentamer
f_6 → hexamer

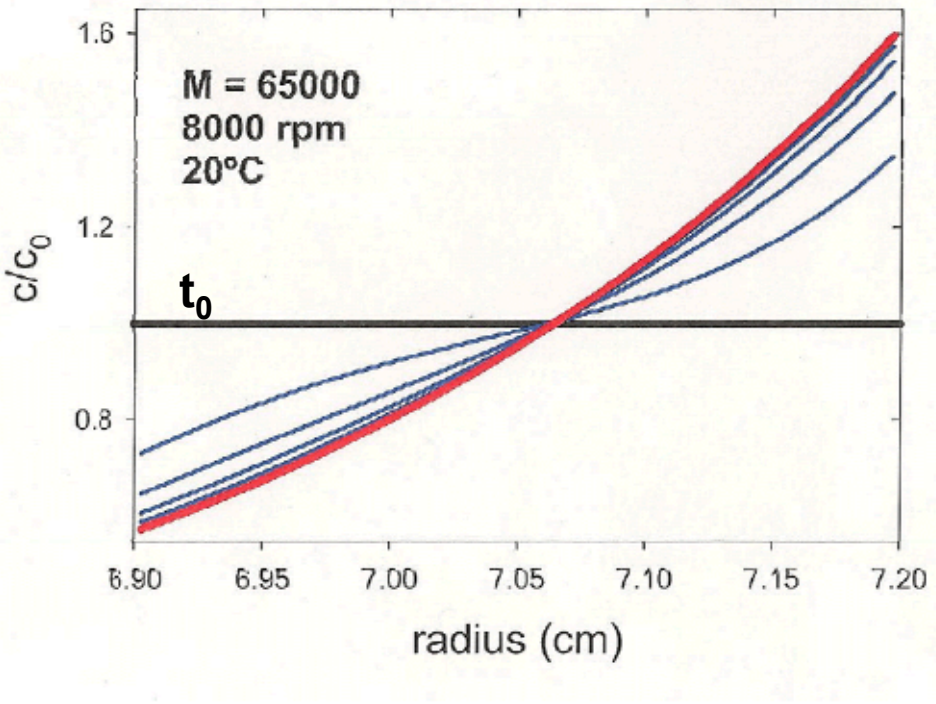
Experimental techniques especially suited for characterization of macromolecular interactions in concentrated solution

1. Concentration gradient – static light scattering

2. Non-ideal tracer sedimentation equilibrium

Sedimentation equilibrium





Sedimentation
⇄
Diffusion

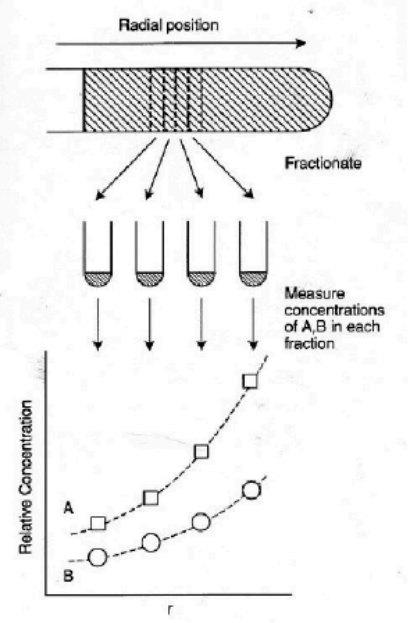
Tracer sedimentation equilibrium

- A tracer is defined as a solute component present at low concentration relative to other solute components, the equilibrium concentration gradient of which may be reliably quantified independent of the gradients of other solute components.

- A particular component can qualify as a tracer if it has a uniquely detectable signal, or if it can provided with a unique signal by means of labeling.

Analitical ultracentrifuge

Preparative ultracentrifuge + microfractionation



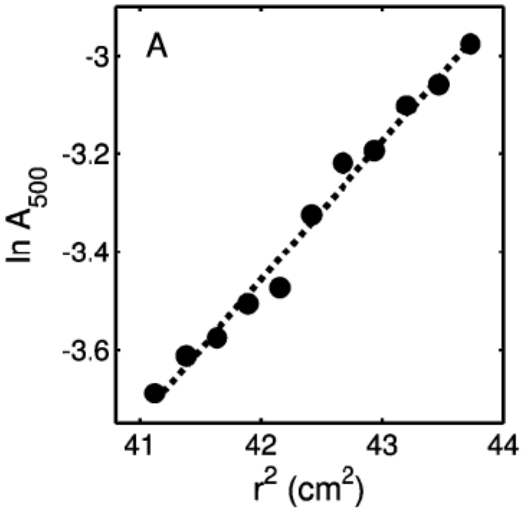
Depending upon the type of tracer, the experiment can be carried out using an analytical or a preparative ultracentrifuge.

Attri & Minton (1986)
Darawshe, *et al*. (1993)

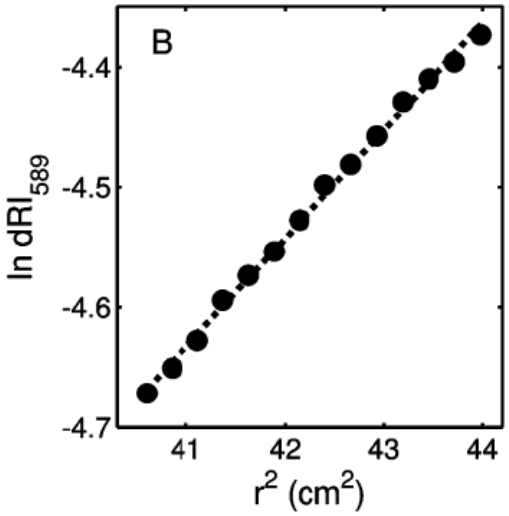
Sample gradients measured at sedimentation equilibrium

Fodeke & Minton (2010)

Absorbance gradient of FITC-BSA (2 g/l) in Ficoll 70 (80 g/l)



Refractive index gradient of Ficoll 70 (80 g/l)



$$M^*_{i,app} \equiv \frac{2RT}{\omega^2}\frac{d\ln S_i}{dr^2}$$

Apparent buoyant molar mass

$$M^*_{BSA,app} = 13250$$

$$M^*_{Fic,app} = 3800$$

Nonideal sedimentation equilibrium (Rivas et al, 1999)

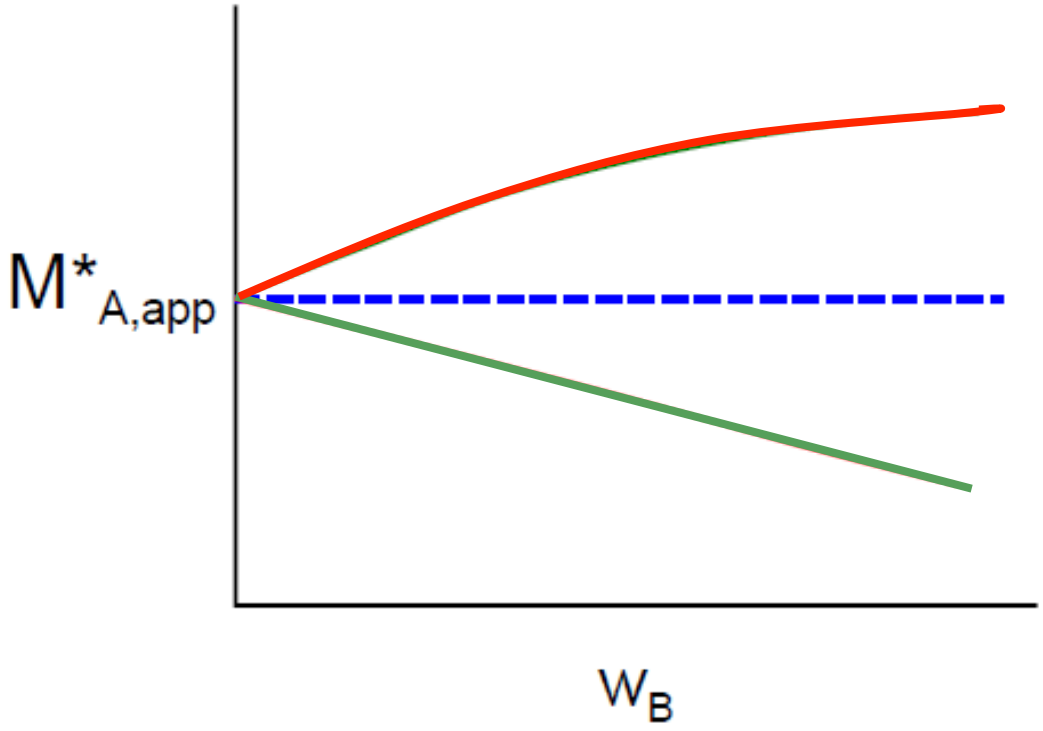
$$M^*_{i,app} \equiv \frac{2RT}{\omega^2}\frac{d\ln S_i}{dr^2}$$

$$M^*_{i,app} = M^*_i - \sum_j w_j \left(\frac{d\ln\gamma_i}{dw_j}\right) M^*_{j,app}$$

Thermodynamic interaction between species *i* and *j*

- : **Repulsive interaction**

+ : **Attractive interaction**



$\partial \ln \gamma_A / \partial w_B < 0$

attractive interaction between A and B

$\partial \ln \gamma_A / \partial w_B = 0$

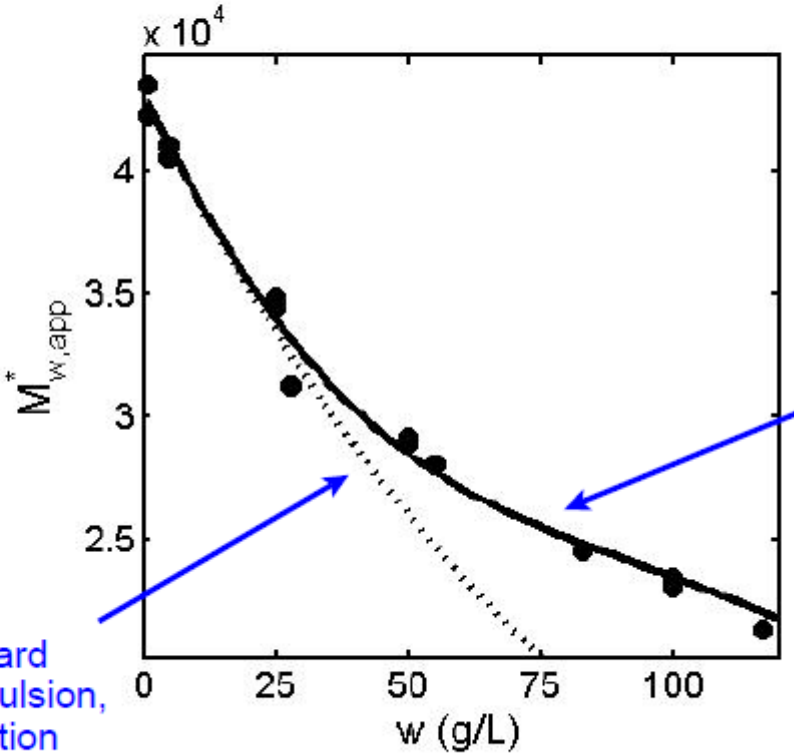
no interaction between A and B

$\partial \ln \gamma_A / \partial w_B > 0$

repulsive interaction between A and B

Human polyclonal IgG in PBS (pH 7.4, 150 mM NaCl)

Jiménez et al (2007)

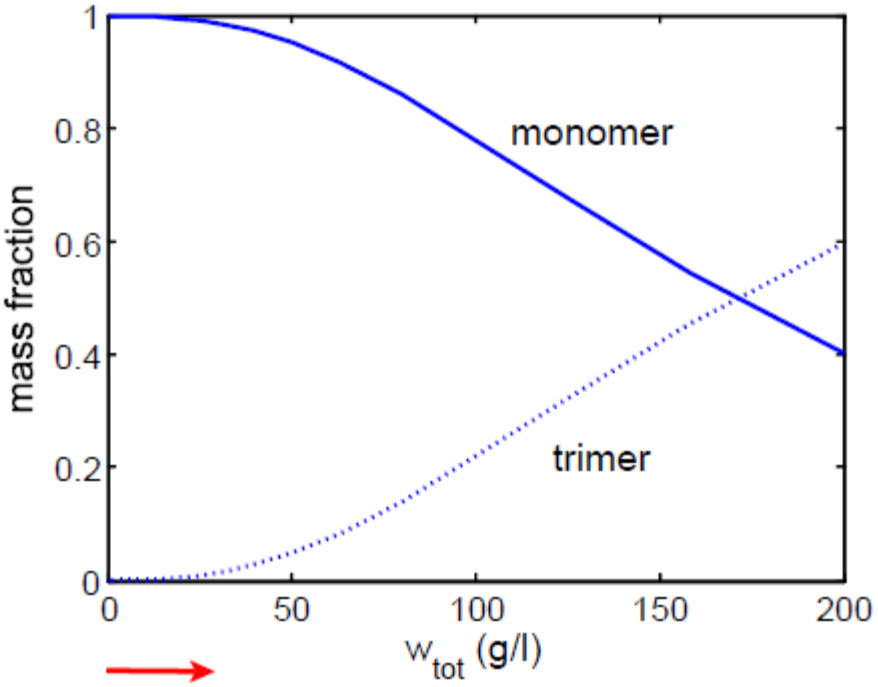


$$M^*_{i,app} = M^*_i - \sum_j w_j \left(\frac{d \ln \gamma_i}{dw_j} \right) M^*_{j,app}$$

$\frac{d \ln \gamma_i}{dw_j}$ **The values are calculate using relationships specified by the effective hard particle model, with each species represented by an effective sphere.**

Human polyclonal IgG in PBS (pH 7.4, 150 mM NaCl)

Jiménez et al (2007)

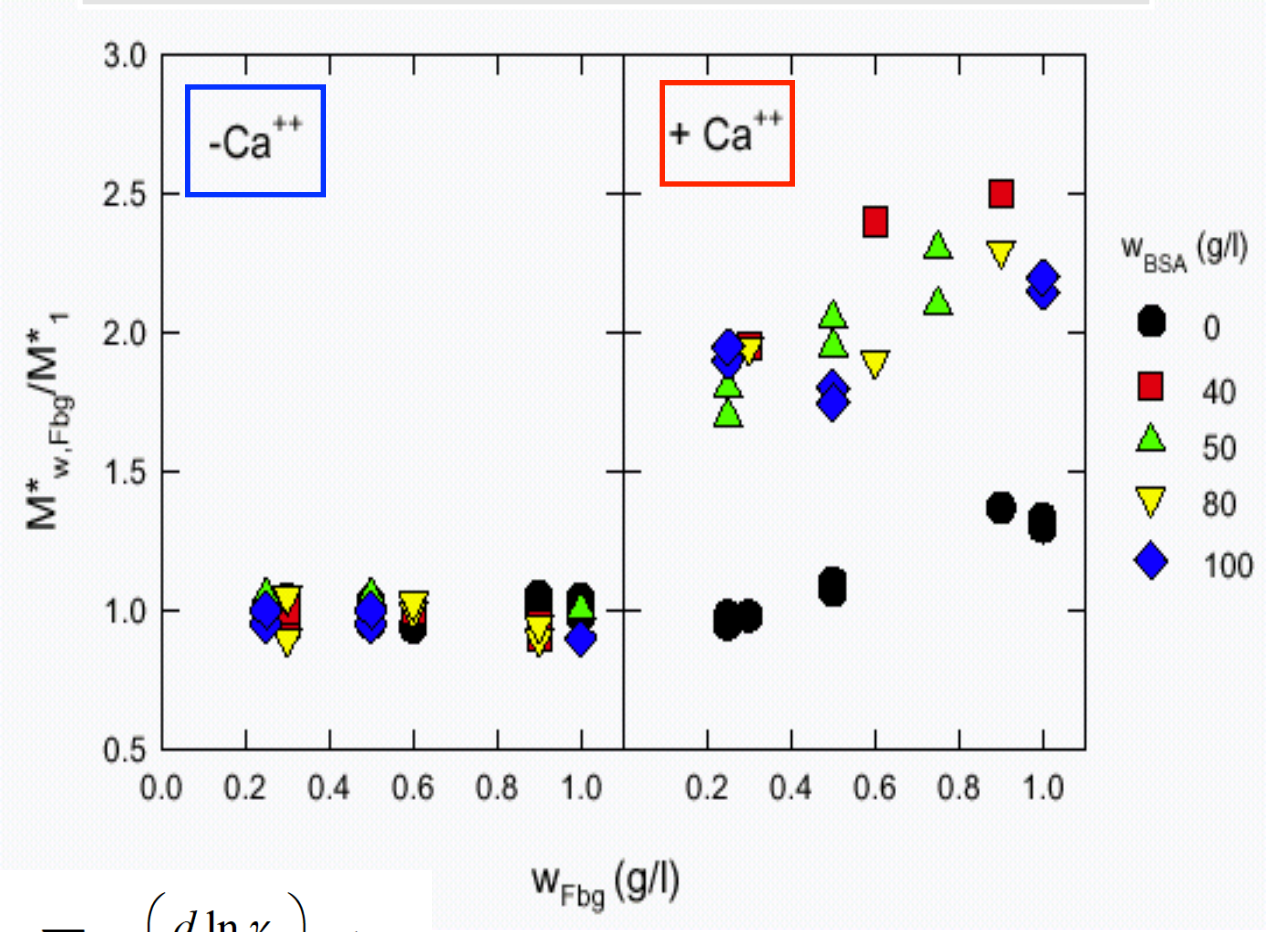


Below ~30 g/l, significant amounts of reversibly formed oligomer are not evident - must go to substantially higher concentrations to detect and characterize it.

Non-Ideal Tracer Sedimentation Equilibrium

Self-association of dilute proteins in the presence of inert macromolecules at high concentration via NITSE

Crowding-induced Fibrinogen oligomerization



$$M^*_{i,app} = M^*_i - \sum_j w_j \left(\frac{d \ln \gamma_i}{dw_j} \right) M^*_{j,app}$$

Rivas et al. (1999) *Biochemistry* 38:9379

SUMMARY

1. Approximate theories have been developed for interpretation of the sedimentation equilibrium and static light scattering of solution mixtures of interacting macromolecules at arbitrarily high concentration.

2. Instrumental methods have been developed for quantitative measurement of sedimentation equilibrium and static light scattering of arbitrarily concentrated solutions of macromolecules.

3. Experimental measurements of the sedimentation equilibrium and static light scattering have yielded self-consistent quantitative information about macromolecular self-and heteroassociation and repulsive interactions between macromolecular solutes in concentrated or crowded solutions with a total macromolecular concentration exceeding 100 g/l.

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Nonideal tracer sedimentation equilibrium

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