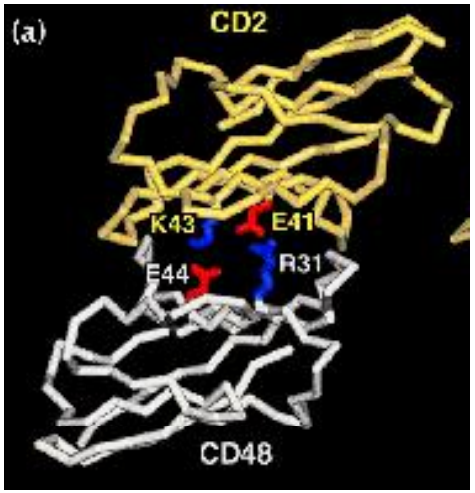
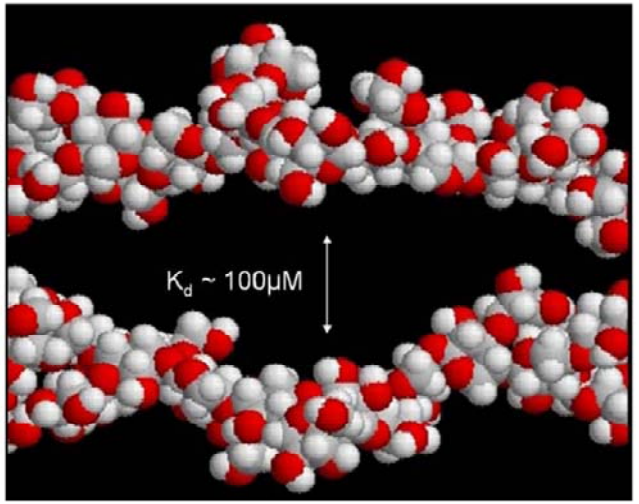
Datum/Zeit	Veranstaltungsort	Thema
Mo, 08.02.2010 10.00-11.30	Hörsaal Institut für Glaschemie Fraunhoferstrasse 6	***Albert Einstein and the Viscosity of Macromolecules***
Mo, 08.02.2010 12.15-13.45	Hörsaal Haus 1,IAAC, August-Bebel-Str. 2	***Light Scattering and SEC-MALLs***
Di, 09.02.2010 12.15-13.45	Institut für Materialwissenschaft und Werkstofftechnologie, HS 124 Löbdergraben 32	***Dynamic Light Scattering***
Mi, 10.02.2010 16.15-17.45	Hörsaal 3 Carl-Zeiss-Str. 3	***Analytical Ultracentrifugation I***
Do, 11.02.2010 14.15-15.45	Döbereiner Hörsaal	***Analytical Ultracentrifugation II: Interactions***

Lecture 5
Analytical Ultracentrifugation II: interactions





Steve Harding

NCMH

The University of Nottingham

Free solution, no immobilisation, columns, membranes etc. needed. Self-association and hetero-interactions, stoichiometry, reversibility and strength (K_{eq}, K_d or ΔG^o)

- *Use of sedimentation equilibrium and sedimentation velocity*
- *Self association or "A-A" interactions, heterologous or "A-B" interactions*
- *How thermodynamic non-ideality problems are now dealt with experimentally & computationally (COVOL)*
- *Polysaccharide interactions, complex formation and mucoadhesion*

Self-association: $A + A \leftrightarrow AA$

Association constant $K_{eq} = [AA]/[A]^2$ - ml/mol, μM^{-1}, M^{-1}

Dissociation constant $K_d = [A]^2/[AA]$ - mol/ml, μM, M

Heterologous association: $A + B \leftrightarrow AB$

Association constant $K_{eq} = [AB]/[A][B]$ - ml/mol, μM^{-1}, M^{-1}

Dissociation constant $K_d = [A][B]/[AB]$ - mol/ml, μM, M

$\Delta G^o = RT\ln K_{eq}$ - J/mol

K_{eq} also given the symbol K_2 or K_a

Interaction strengths commonly represented by K_d values

Strong interactions: $K_d < 1\mu M$

Medium interactions: $1\mu M < K_d < 20\mu M$

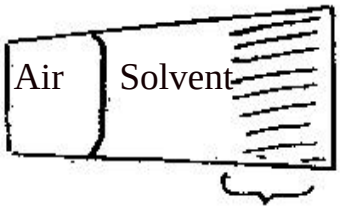
Weak interactions: $K_d > 20\mu M$

Very weak interactions: $K_d > 100\mu M$

NB this applies to reversible interactions

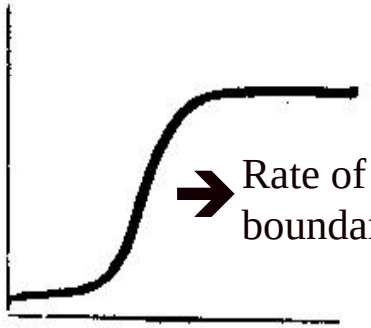
Sedimentation Velocity

Centrifugal force →



Top view, sector of centrifuge cell

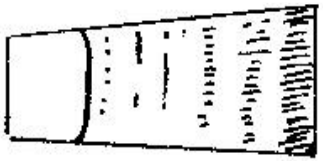
Solution



→ Rate of movement of boundary → sed. coeff

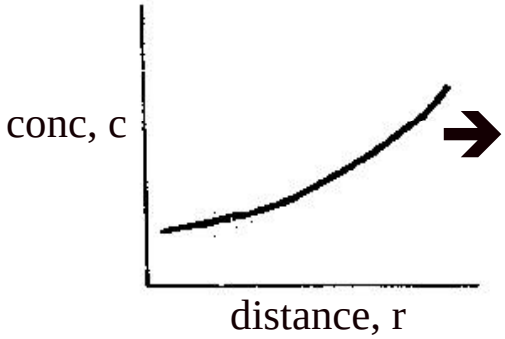
<u>Shape</u>, molecular weight & associated mass-action parameters, K_d etc.

Sedimentation Equilibrium



Centrifugal force →

←Diffusion



→ STEADY STATE PATTERN

Molecular weight & associated mass-action parameters, K_d etc.

Thermodynamic non-ideality

Radial distance

$$c(r) = f(M, B, K, r)$$

(buoyant) molecular weight

Association or dissociation constant(s)

monomer–dimer equilibrium. The apparent weight-average molecular masses, $M_{w,app}$, obtained for interference optics and absorption optics measurements were 21 400 ± 3000 Da and 21 800 ± 4000 Da, respectively.

From these data it is possible to estimate the molar dissociation constant, K_d. For a dimerizing system, correct to first-order in concentration, c (g·mL^{-1}) [58]

$$\frac{1}{M_{w,app}} = \frac{1}{M_1} + 2\left(B_{11} - \frac{K_2}{M_1^2}\right)c \qquad (1)$$

K_2 is the dimerization constant (mL·mol^{-1}), M_1 is the monomer molecular mass and B_{11} is the monomer–monomer second thermodynamic virial coefficient (in mL·mol·g^{-2}). If the system is assumed to be ideal, $B_{11} \approx 0$, then K_2 is simply

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Taking M_1 = 16 626 Da, $M_{w,app}$ = 21 600 ± 3000 Da and c = 60 μM which is equivalent to 1 mg·mol^{-1}, K_2 is calculated to be 1.92 × 10^6 mL·mol^{-1}, and K_d (equivalent to $1/K_2$) is

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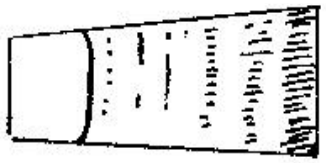
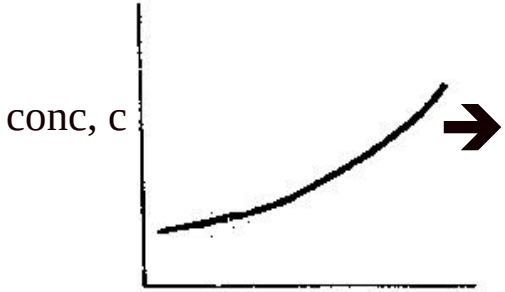


Centrifugal force ➔

➔Diffusion

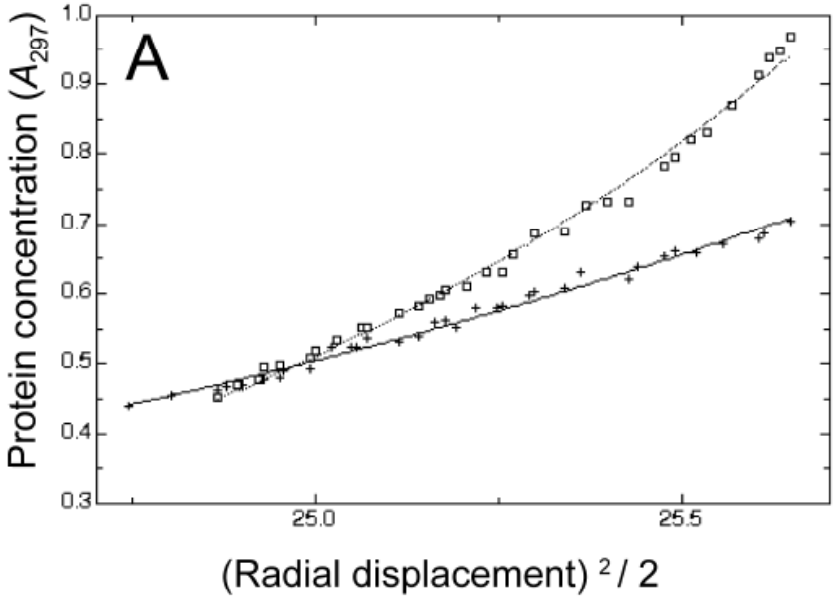


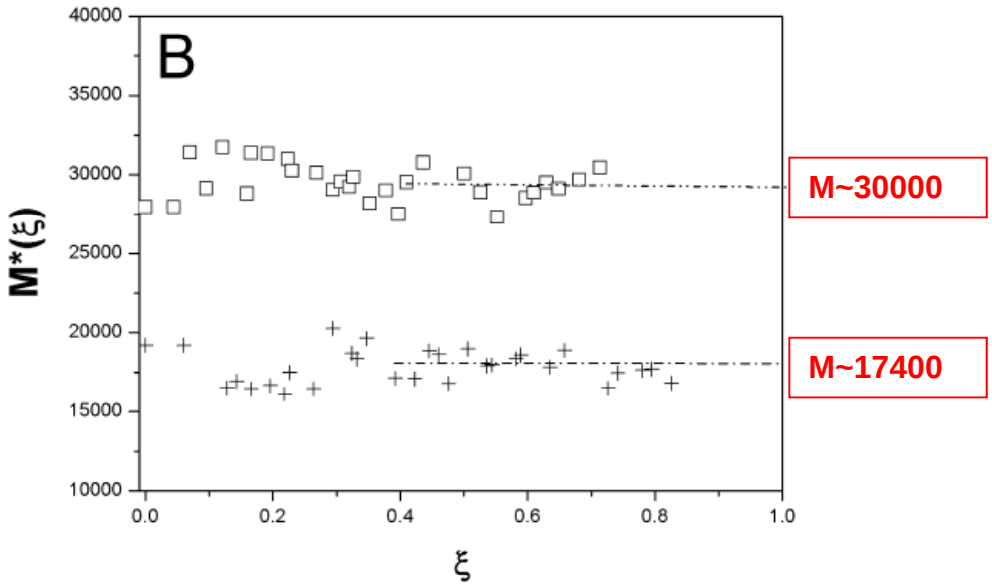
➔ STEADY STATE PATTERN

Molecular weight & associated mass-action parameters, K_d etc.

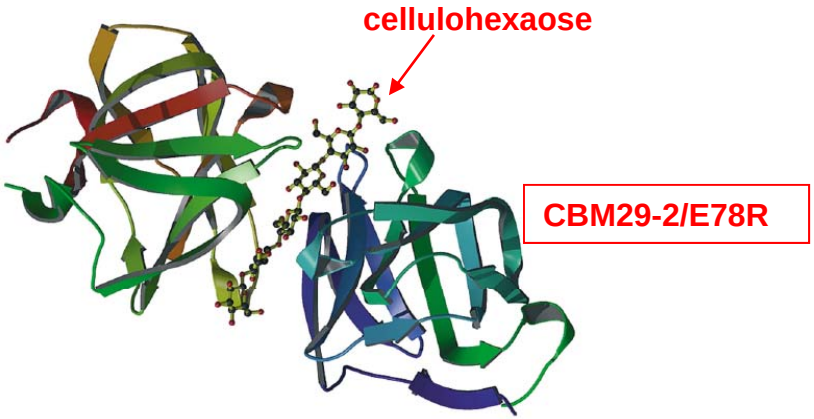
Carbohydrate induced dimerisation of a protein
M ~ 17.4kDa, K_d ~ (120)μM

MSTAR analysis





$$M^*(r) = j(r) / \left\{ kJ(a)(r^2 - a^2) + 2k \int_a^r r \cdot j(r)\,\mathrm{d}r \right\}$$



doi:10.1016/j.jmb.2003.12.081 *J. Mol. Biol.* (2004) **337**, 417–426

JMB

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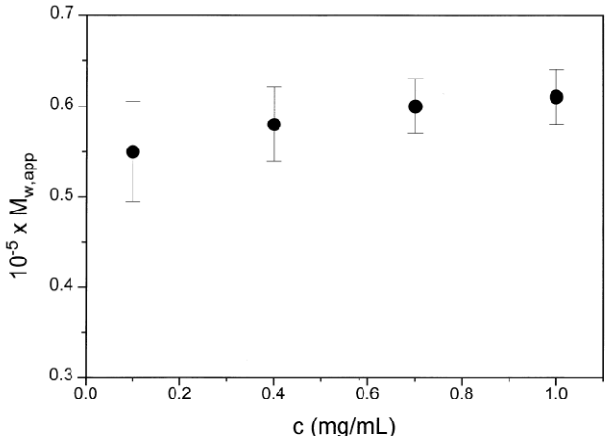
Ligand-mediated Dimerization of a Carbohydrate-binding Module Reveals a Novel Mechanism for Protein–Carbohydrate Recognition

James Flint[1], Didier Nurizzo[2], Stephen E. Harding[3], Emma Longman[3] Gideon J. Davies[2], Harry J. Gilbert[1] and David N. Bolam[1*]

Sedimentation equilibrium - Electron Transfer Flavoprotein-heterodimer, M ~ 63kDa, K_d ~ (1.5±0.1)μM

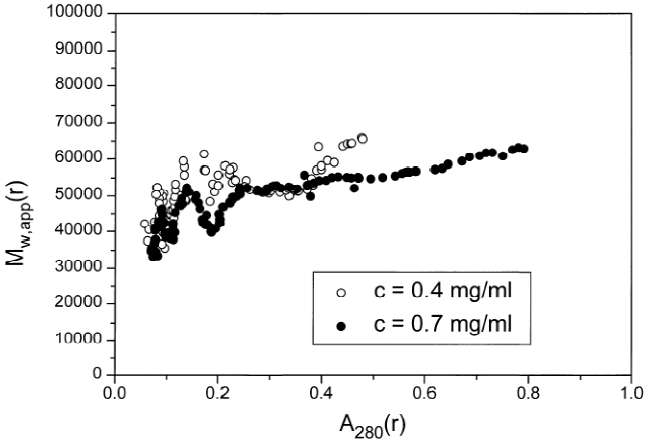


Helmut Cölfen · Stephen E. Harding
Emma K. Wilson · Nigel S. Scrutton
Donald J. Winzor

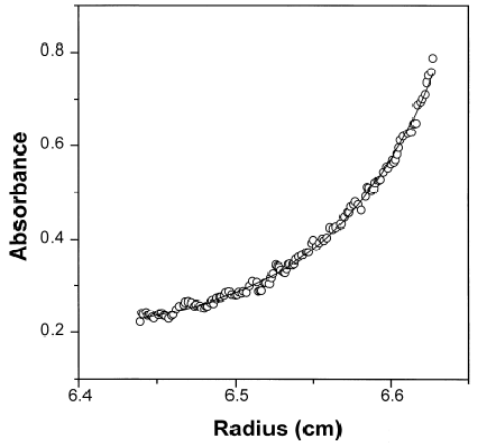
European Biophys. J. (1997)

Low temperature solution behaviour of *Methylophilus methylotrophus* electron transferring flavoprotein: a study by analytical ultracentrifugation

1. $M_{w,app}$ analysis vs loading conc - stoichiometry.



2.$M_{w,app}$ (r) analysis - reversibility



3. Fitting the concentration distribution – K_d

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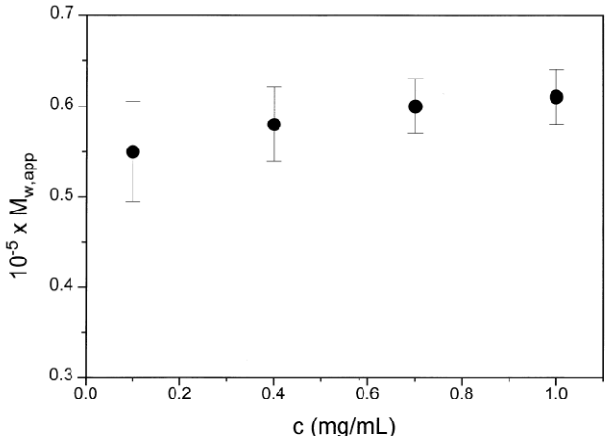

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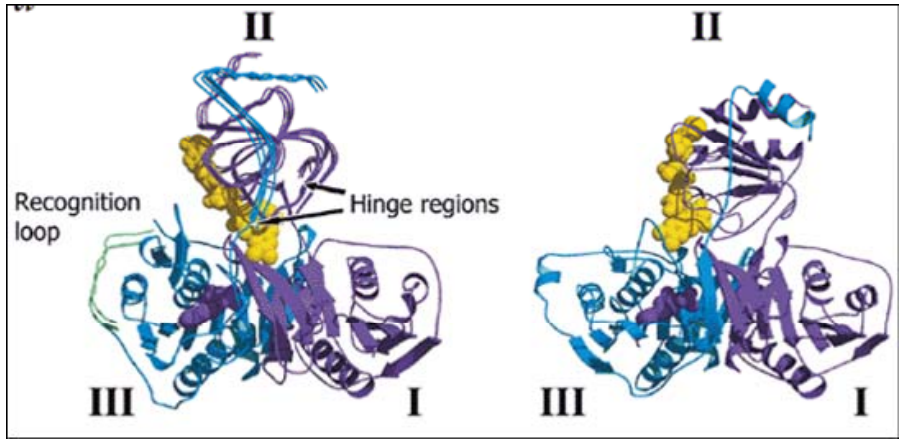
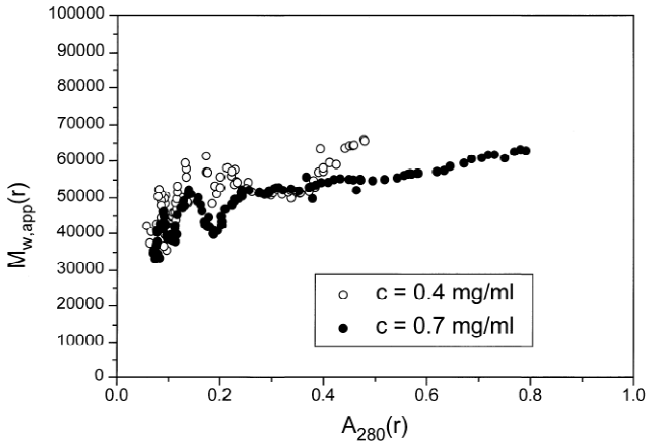


Blue chain: 29kDa; Purple chain: 34kDa



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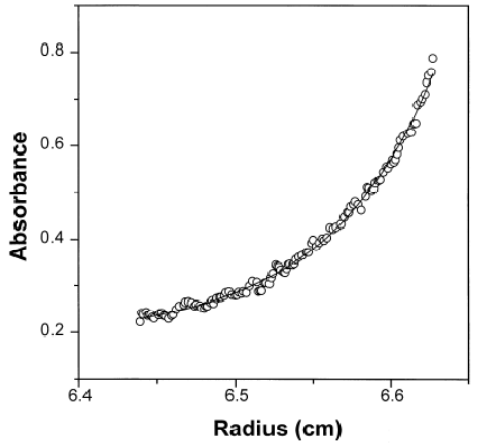

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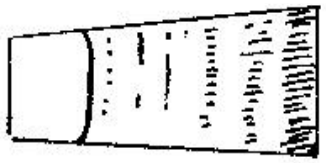
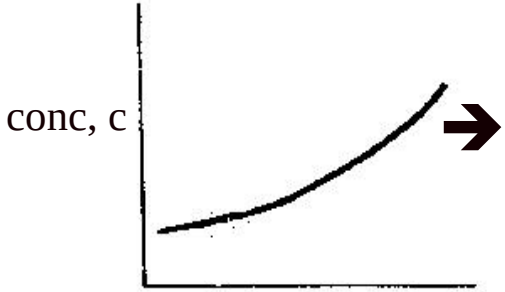


Centrifugal force ➔

➔Diffusion



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Molecular weight & associated mass-action parameters, K_d etc.

B = f(excluded volume, charge)

Ignoring B leads to incorrect K_d

Choice:

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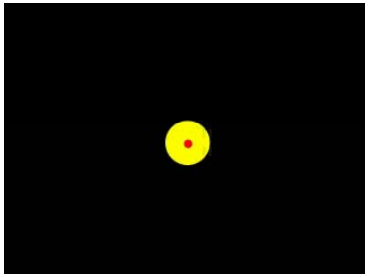

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Excluded Volume for Pairs of Triaxial Ellipsoids at Dominant Brownian Motion

J. M. RALLISON* AND S. E. HARDING†

*University of Cambridge, Department of Applied Mathematics and Theoretical Physics, Silver Street, Cambridge CB3 9EW, and †University of Cambridge, Department of Biochemistry, Tennis Court Road, Cambridge CB2 1QW, England

Received May 4, 1984; accepted July 19, 1984

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$$S = \frac{8}{3}\int_0^{\pi/2}\int_0^{\pi/2} \cos u\,du\,dv \left\{\left(\frac{bc}{a}+\frac{ca}{b}+\frac{ab}{c}\right)\Delta - \sin^2 v\cos^2 v\cos^2 u\Delta^3 c\left(\frac{b}{a}-\frac{a}{b}\right)\left(\frac{1}{a^2}-\frac{1}{b^2}\right)\right.$$
$$\left. - \sin^2 u\cos^2 u\Delta^3\left(\frac{\cos^2 v}{a^2}+\frac{\sin^2 v}{b^2}-\frac{1}{c^2}\right)\left[c\left(\frac{b\cos^2 v}{a}+\frac{a\sin^2 v}{b}\right)-\frac{ab}{c}\right]\right\} \quad [3]$$

and

$$R = \frac{2}{3\pi}\int_0^{\pi/2}\int_0^{\pi/2} \cos u\,du\,dv \left\{\left(\frac{a}{bc}+\frac{b}{ac}+\frac{c}{ab}\right)\Delta^2\right.$$
$$- \sin^2 v\cos^2 v\cos^2 u\,\Delta^4\left(\frac{1}{a^2}-\frac{1}{b^2}\right)\frac{1}{c}\left(\frac{b}{a}-\frac{a}{b}\right)$$
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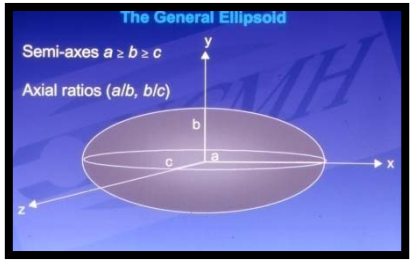

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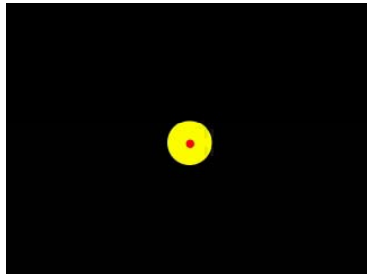

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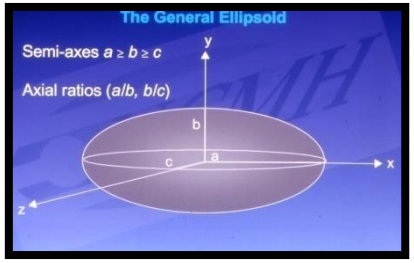

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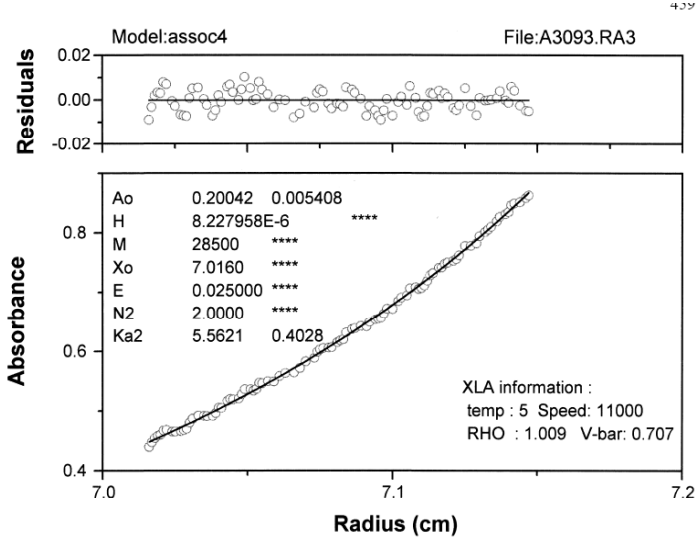
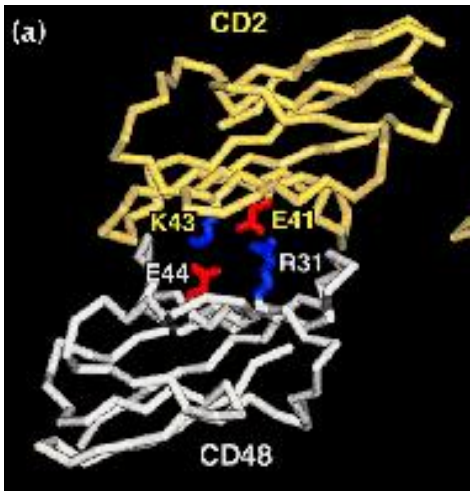
COVOL – calculates B from size, shape and charge

CD2-CD48 cell recognition heterodimer: a weak interaction





2BM (from COVOL programme) = 10.4 ml/g

K_d = 100±30 μM

K_d(from SPR) = 70-90 μM (Anton van der Merwe)

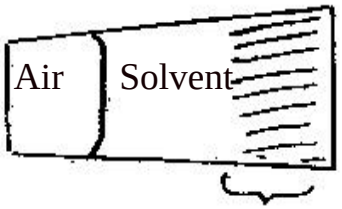
Helena Silkowski · Simon J. Davis · A. Neil Barclay
Arthur J. Rowe · Stephen E. Harding · Olwyn Byron

European Biophys. J. (1997)

Characterisation of the low affinity interaction between rat cell adhesion molecules CD2 and CD48 by analytical ultracentrifugation

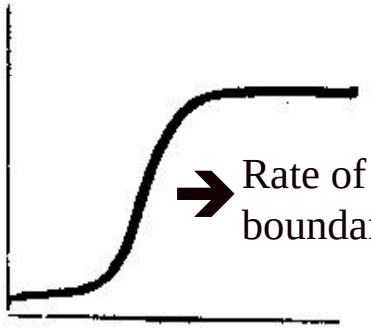
Sedimentation Velocity

Centrifugal force →



Top view, sector of centrifuge cell

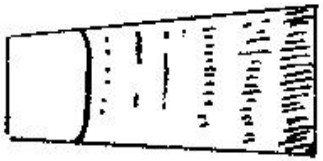
Solution



→ Rate of movement of boundary → sed. coeff

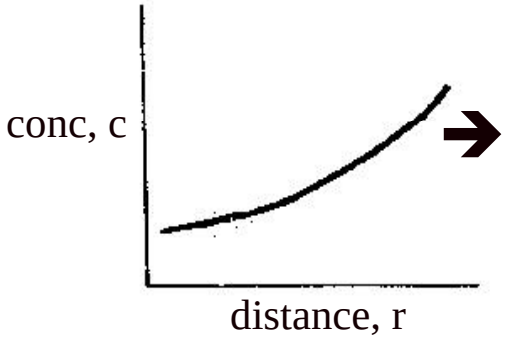
<u>Shape</u>, molecular weight & associated mass-action parameters, K_d etc.

Sedimentation Equilibrium



Centrifugal force →

←Diffusion



→ STEADY STATE PATTERN

Molecular weight & associated mass-action parameters, K_d etc.

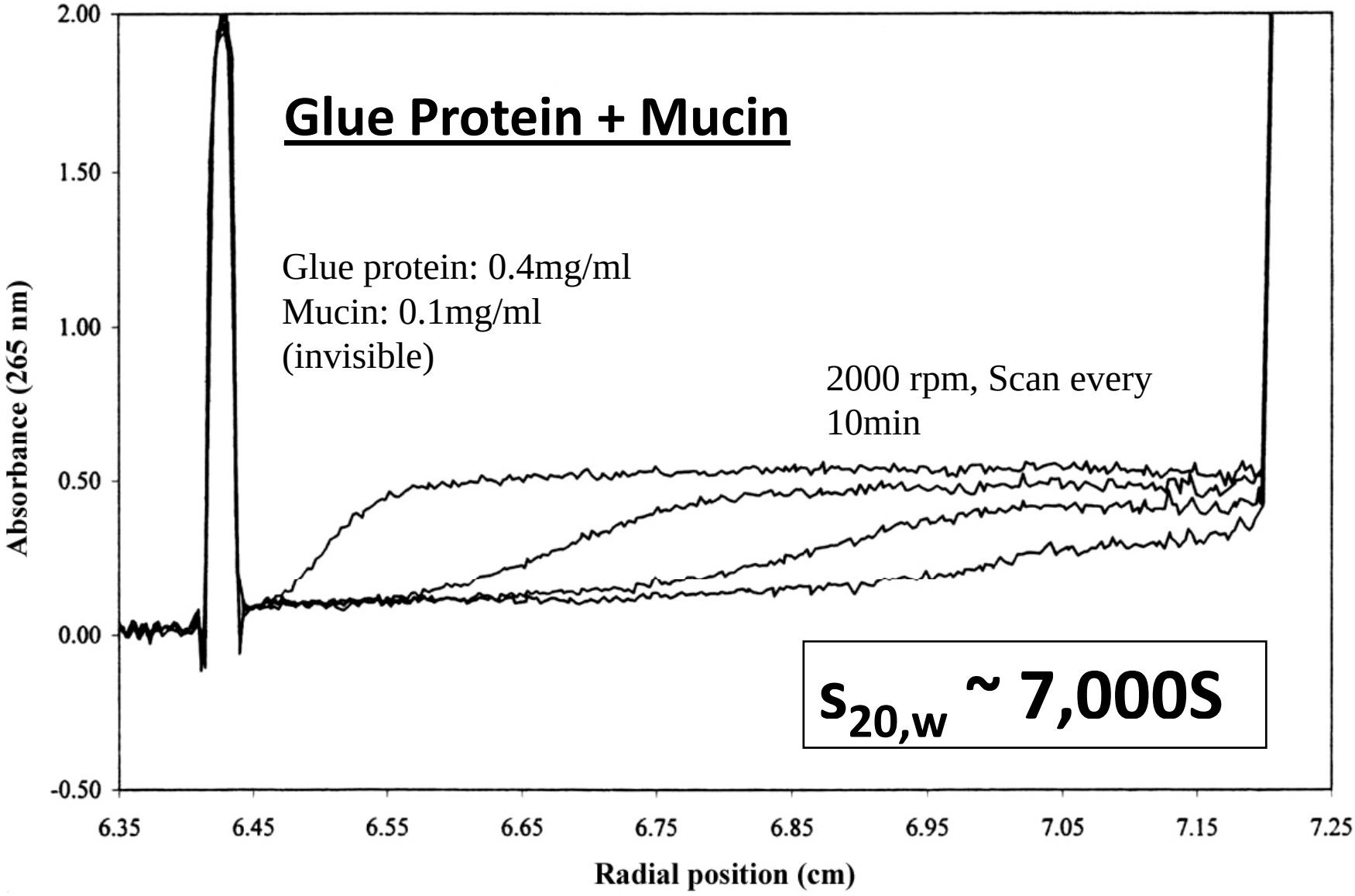
Structure and Mucoadhesion of Mussel Glue Protein in Dilute Solution†

Matt P. Deacon,‡ Stanley S. Davis,§ J. Herbert Waite,|| and Stephen E. Harding*,‡

NCMH Unit, School of Biological Sciences, University of Nottingham, Sutton Bonington, LE12 5RD, U.K., School of Pharmacy, University of Nottingham, Nottingham, NG7 2RD, U.K., and Department of Chemistry & Biochemistry, University of Delaware, Newark, Delaware 19716

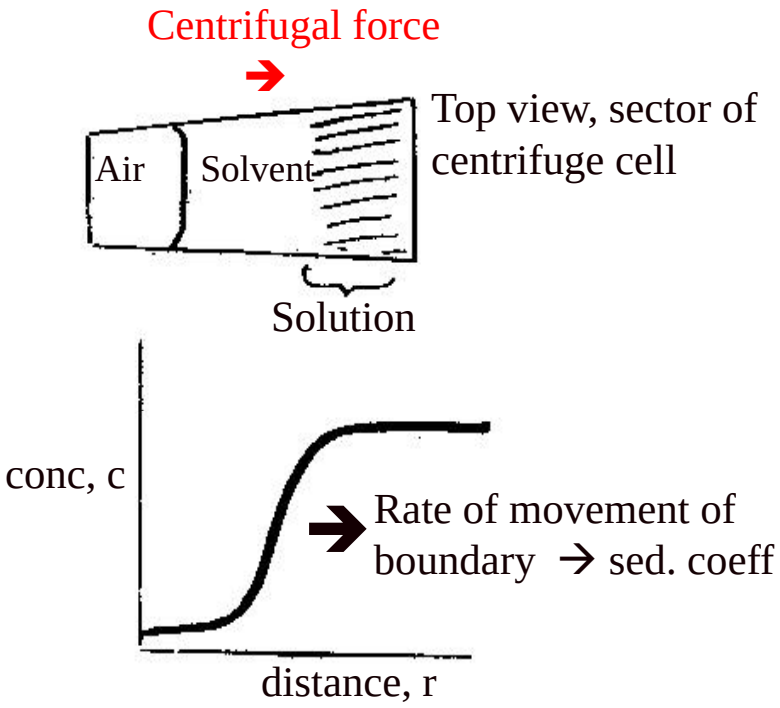
Glue Protein

Biochemistry (1998)



Biochemistry (1998)

Sedimentation Velocity



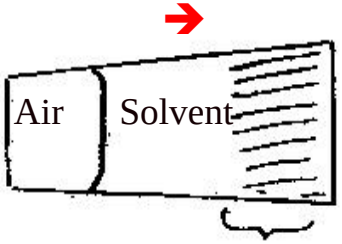
***Shape*, molecular weight & associated mass-action parameters, K_d etc.**

$$c(r,t) = f(f/f_o\ M,\ k_s,\ K,\ r,\ t)$$

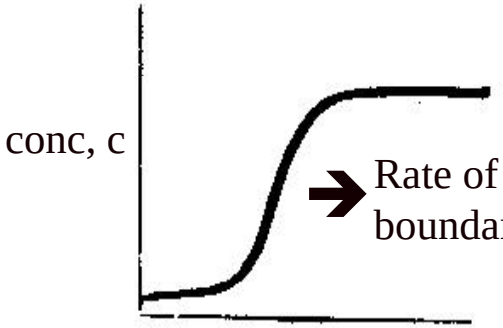
- frictional ratio
- Hydrodynamic non-ideality
- molecular weight
- Association or dissociation constant(s)

Sedimentation Velocity

Centrifugal force



Top view, sector of centrifuge cell



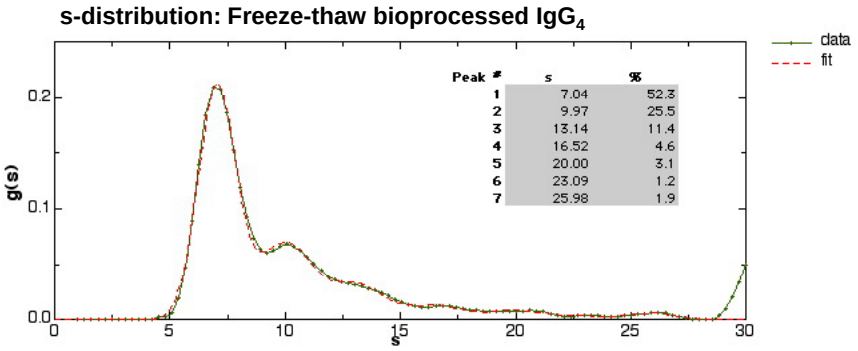
Rate of movement of boundary → sed. coeff

***Shape*, molecular weight & associated mass-action parameters, K_d etc.**

$$c(r,t) = f(f/f_o\ M,\ k_s,\ K,\ r,\ t)$$

- frictional ratio
- Hydrodynamic non-ideality
- molecular weight
- Association or dissociation constant(s)

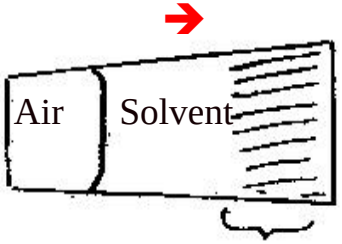
- numerical solutions to Lamm equation (1923)



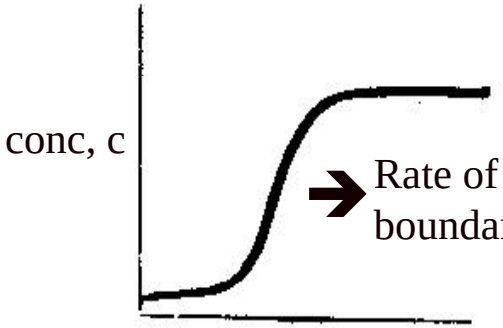
- nb single peak does not necessarily mean single solute! (Gilbert & Jenkins 1958)

Sedimentation Velocity

Centrifugal force



Top view, sector of centrifuge cell



Rate of movement of boundary → sed. coeff

<u>Shape</u>, molecular weight & associated mass-action parameters, K_d etc.

frictional ratio

Hydrodynamic non-ideality

$$c(r,t) = f(f/f_o\ M,\ k_s,\ K,\ r,\ t)$$

molecular weight

Association or dissociation constant(s)

c(s) method of P. Schuck attempts to correct for diffusion broadening

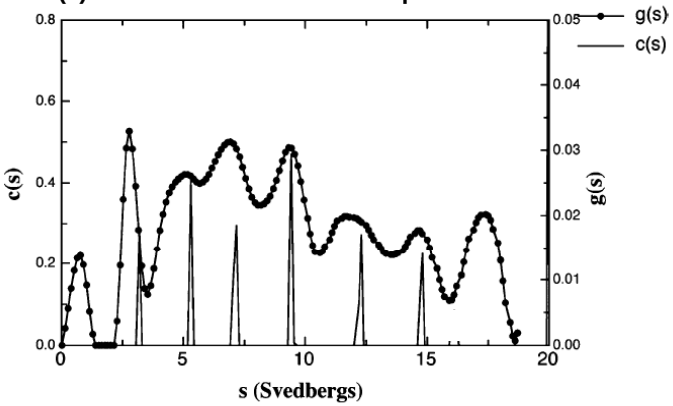
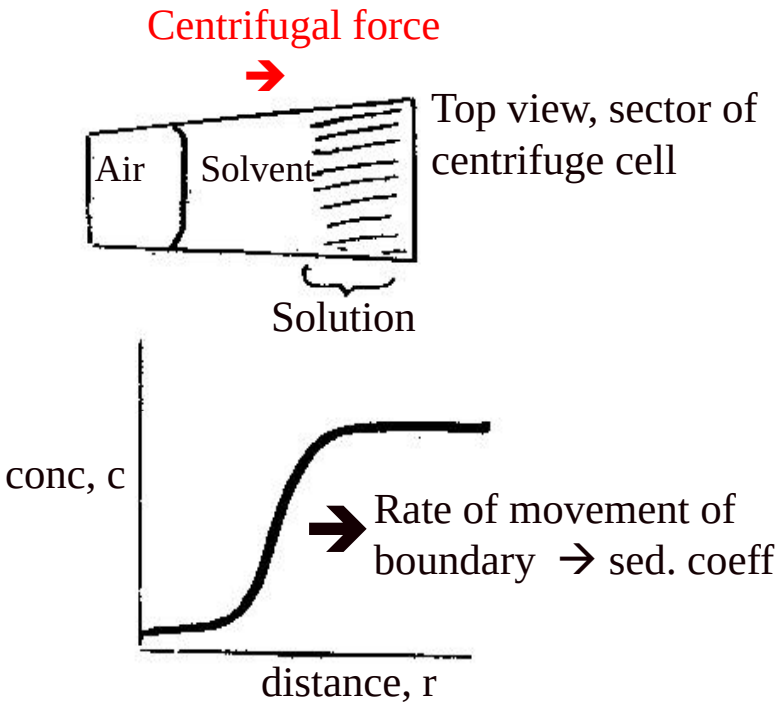


Fig. 3 Overlay of the $c(s)$ and $g(s)$ distribution data for sedimentation velocity analysis of HPL-F at a loading concentration of 1 mg/ml. The spikes represent $c(s)$ peaks used to identify the s values of each species present

Sedimentation Velocity



<u>Shape</u>, molecular weight & associated mass-action parameters, K_d etc.

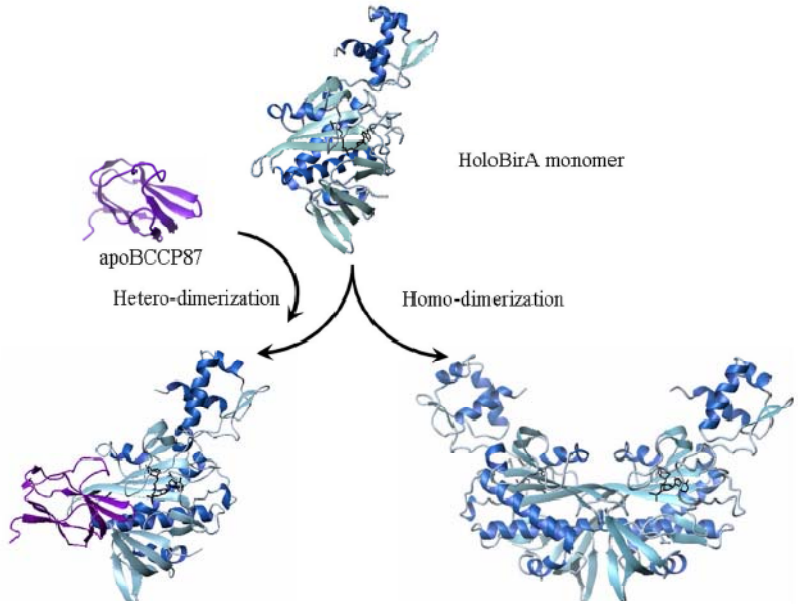
<u>Choice of software</u>:

1.SEDFIT (P. Schuck): analysis of c(r,t) in terms of stoichiometry, reversibility

2.SC-ISOTHERM (A. Rowe et al) analysis of weight average sed.coeff. vs conc. in terms of stoichiometry & K_d

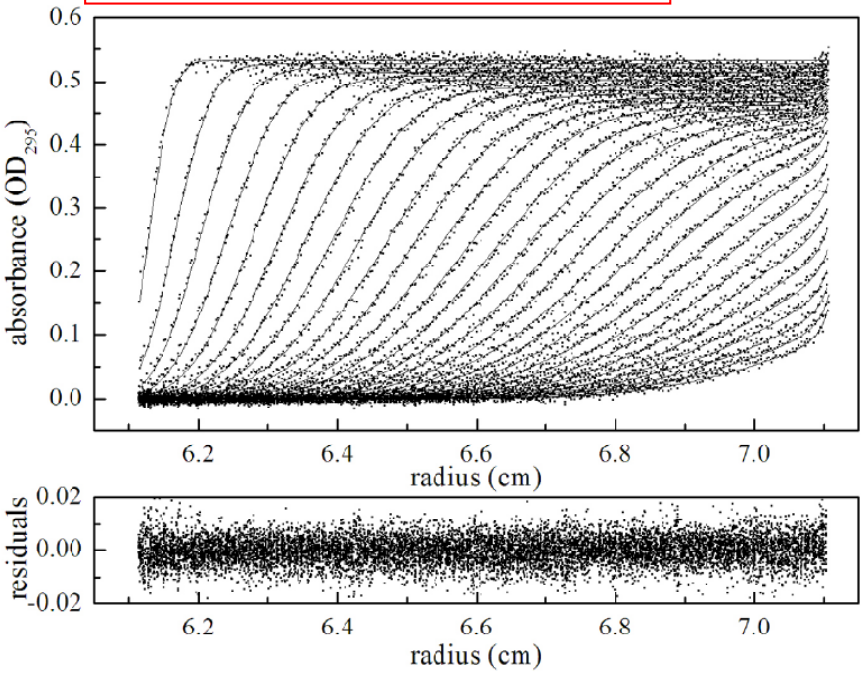
3.SEDANAL (W. Stafford): global analysis of several c(r,t)'s in terms of K_d, k_{off} (& k_{on})

Demonstration of a protein switch: homodimerisation to heterodimerisation:



metabolism **transcription repression**

Absence of apoBCCP87: homo-dimerisation



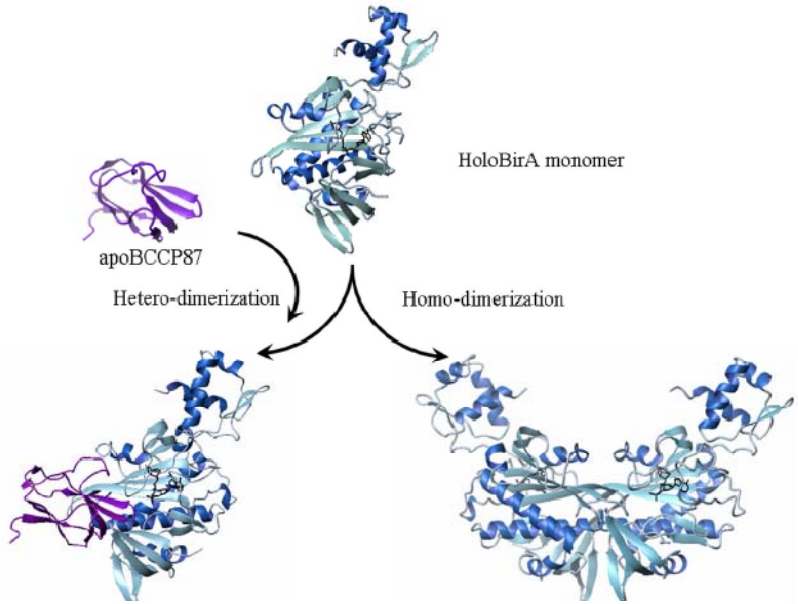
Top: fit by SEDFIT analysis
Bottom: residuals

J Mol Biol. 2008 June 27; 380(1): 223–236. doi:10.1016/j.jmb.2008.04.068.

Kinetic Partitioning Between Alternative Protein:Protein Interactions Controls a Transcriptional Switch

Huaying Zhao and **Dorothy Beckett***
Department of Chemistry & Biochemistry, Center for Biological Structure & Organization, University of Maryland, College Park, MD 20742

Demonstration of a protein switch: homodimerisation to heterodimerisation:



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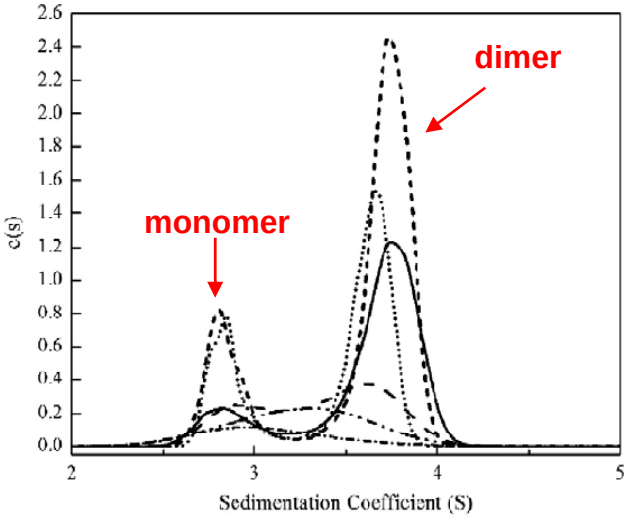

…as concentration increases, proportion of dimers increases

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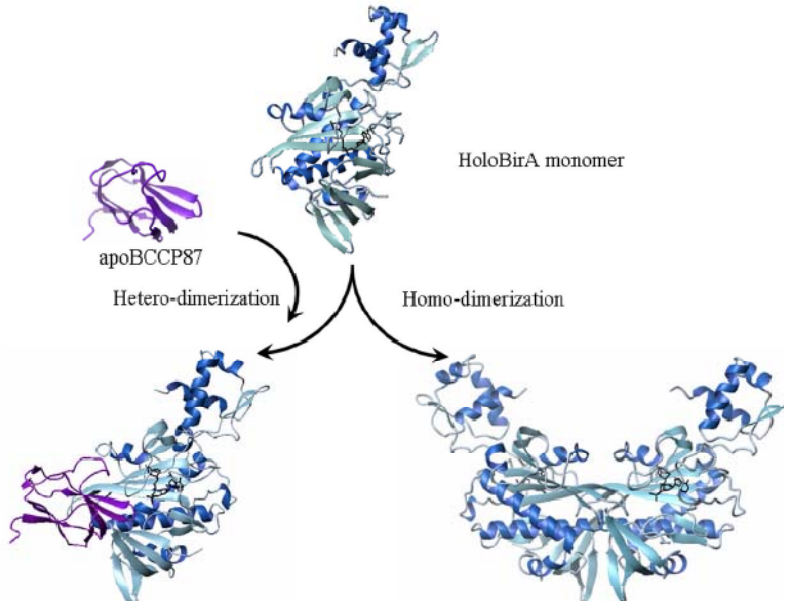

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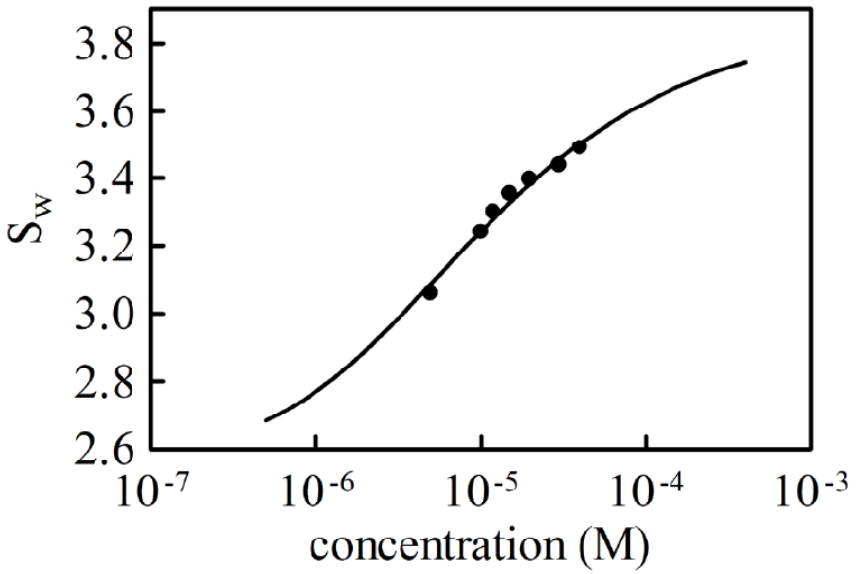

SC-Isotherm analysis:
K_d ~ (10±1) μM

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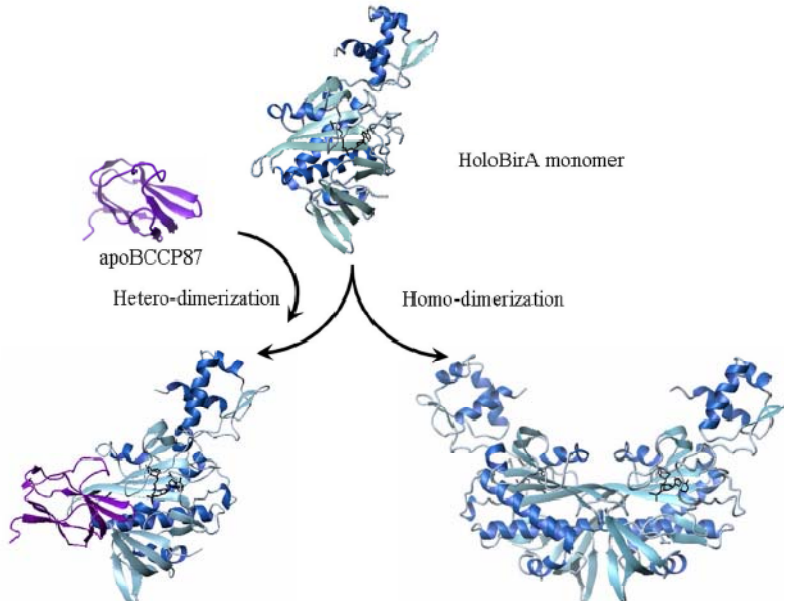

metabolism **transcription repression**

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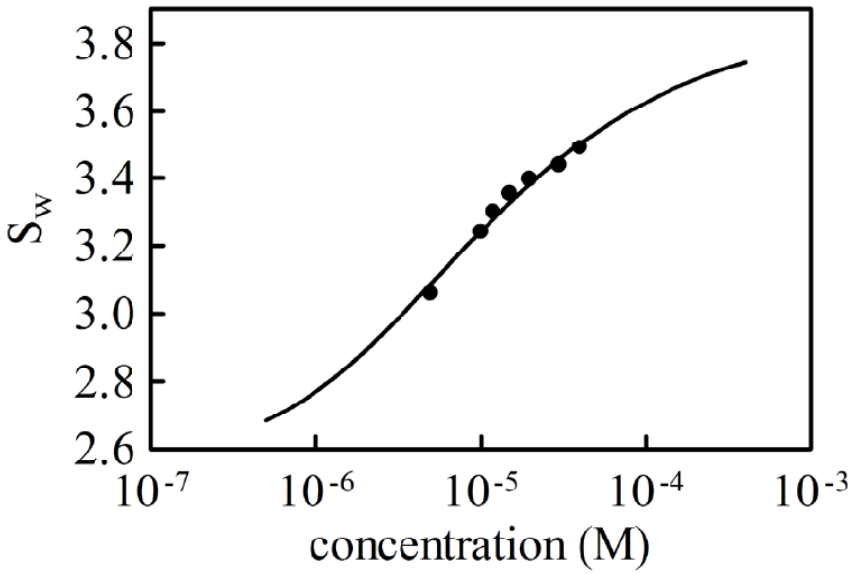

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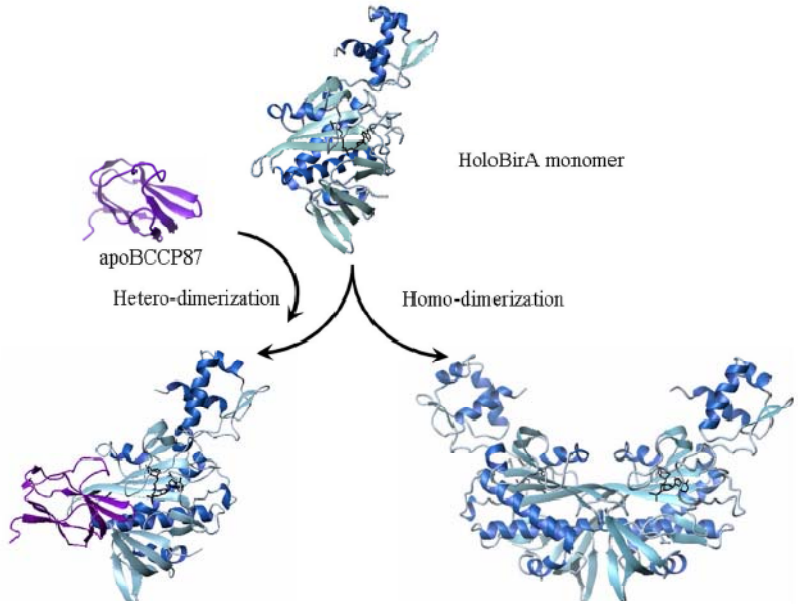
SC-Isotherm analysis:
K_d ~ (10±1) μM

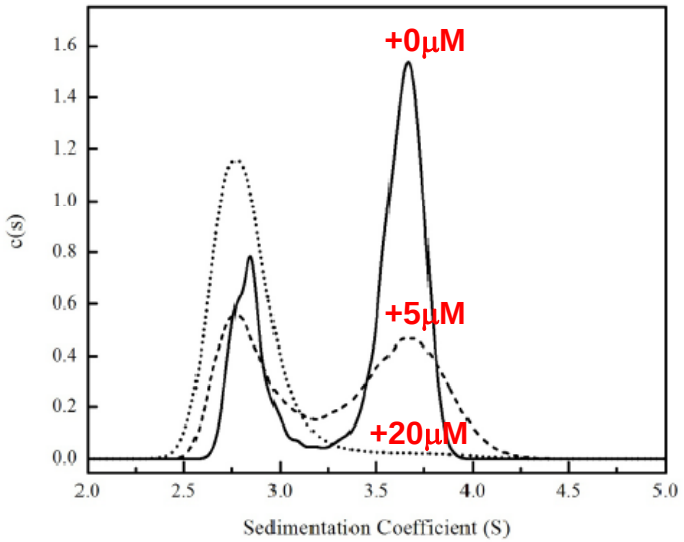
SEDANAL analysis:
***k_{off}* = (2.7±0.5)x$10^{-4}s^{-1}$**

anything faster than 0.01 per second is considered as instantaneous. Too slow – anything slower than 0.0001 per second won't distort the boundary enough

Demonstration of a protein switch: homodimerisation to heterodimerisation:



Addition of apoBCCP87: switch from homo to hetero-dimerisation



J Mol Biol. 2008 June 27; 380(1): 223–236. doi:10.1016/j.jmb.2008.04.068.

Kinetic Partitioning Between Alternative Protein:Protein Interactions Controls a Transcriptional Switch

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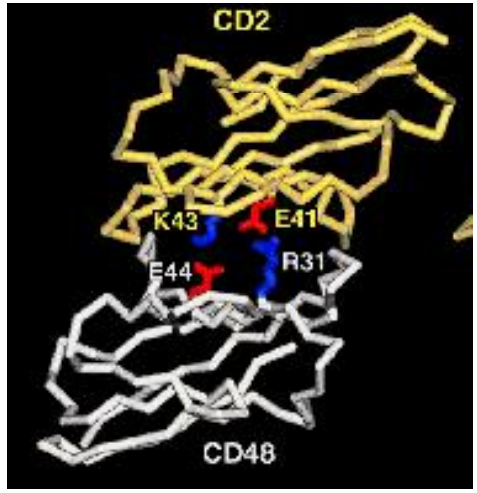
K_d (hetero-dimerisation) ~(2.4±0.4) μM

SC-ISOTHERM analysis CD2:CD48



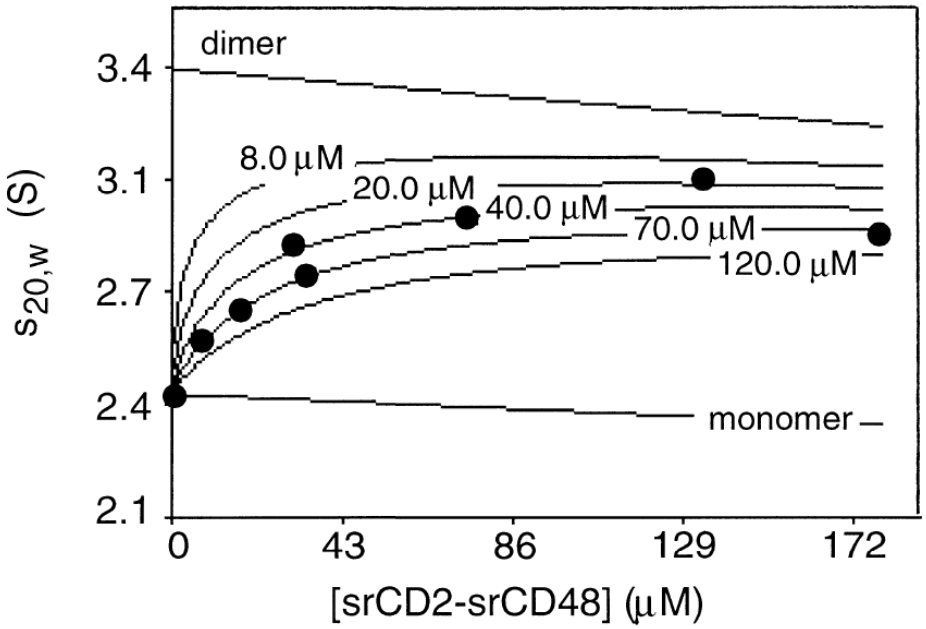


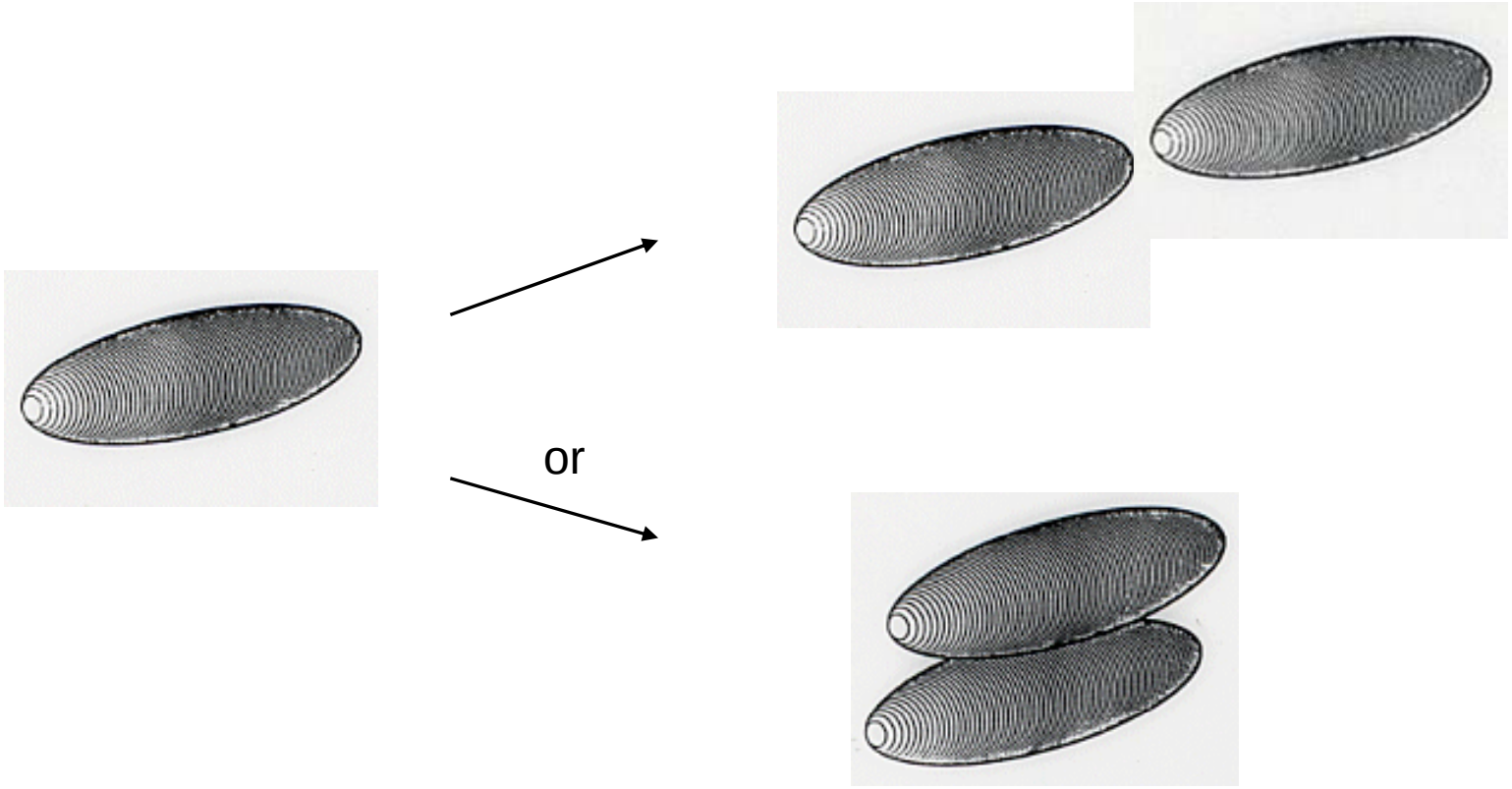
Fig. 6 Weight-average sedimentation coefficients (●) modelled iteratively to Eqs. (7)–(11) [with k_s (monomer) set as 5 ml/g; k_s (dimer as 8.5 ml/g)], for values of the dissociation constant K_d in the range 8–120 µM using the software *SA-Plot*

Helena Silkowski · Simon J. Davis · A. Neil Barclay
Arthur J. Rowe · Stephen E. Harding · Olwyn Byron

Characterisation of the low affinity interaction between rat cell adhesion molecules CD2 and CD48 by analytical ultracentrifugation

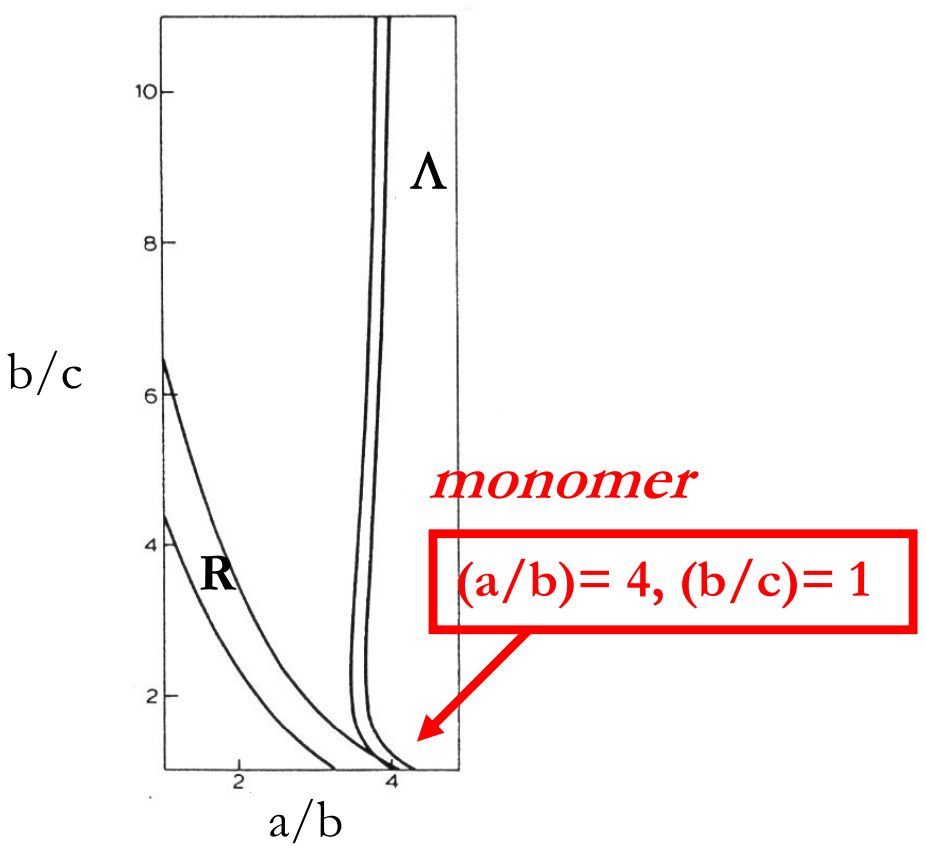
Conformation analysis in associating systems

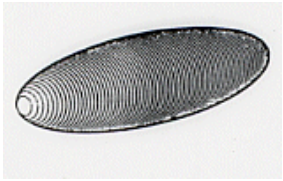
e.g. take the case of a dimerising globular protein system

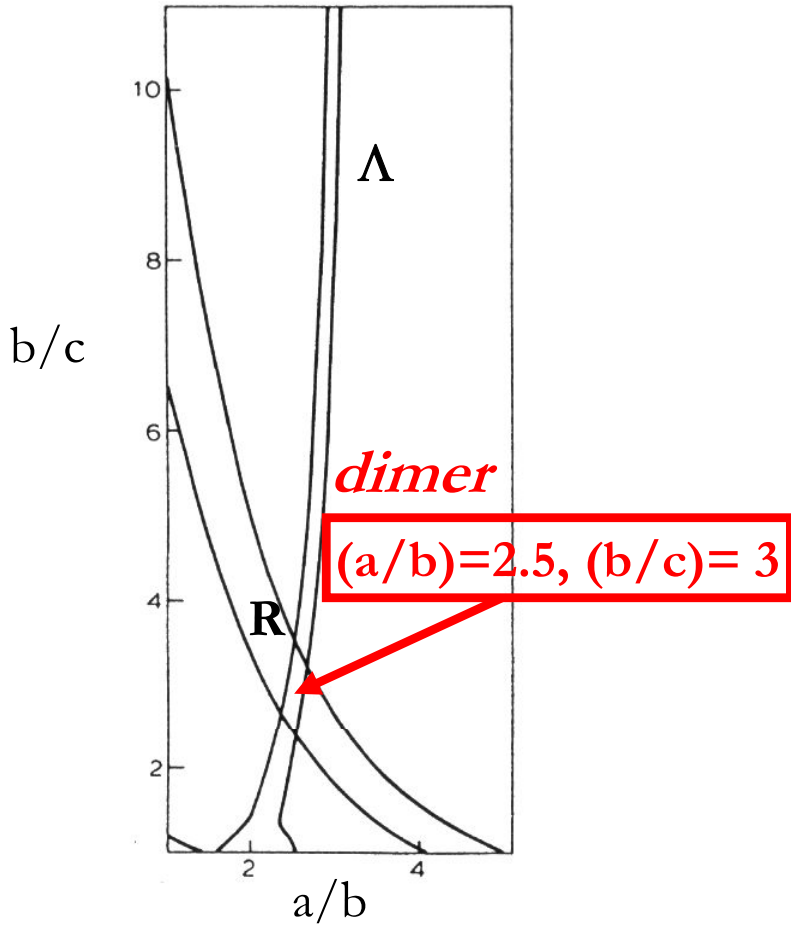


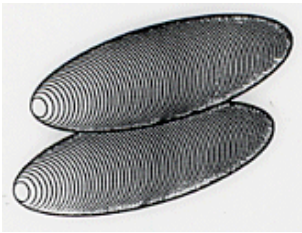
?

Neurophysin dimerises side by side - triaxial contour mapping (ELLIPS3)



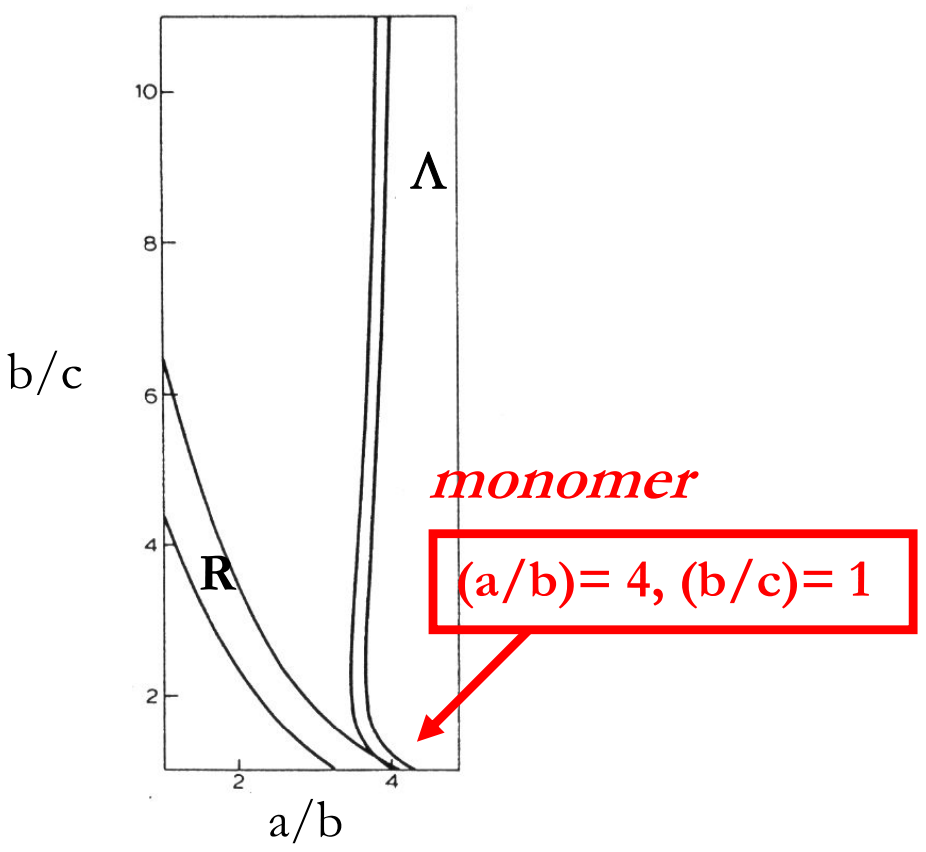


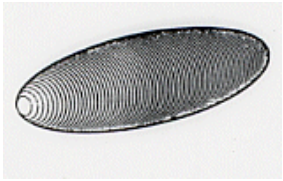


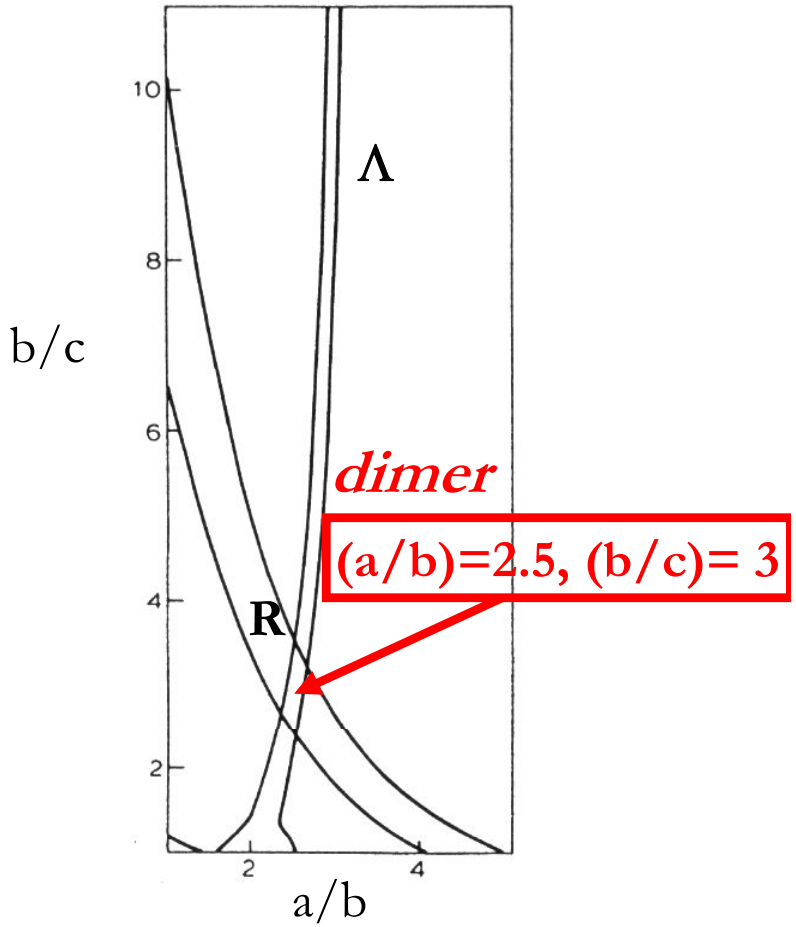


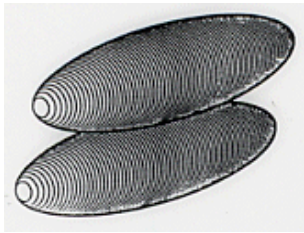
a>b>c: triaxial semi-axes (sphere a=b=c)
R: hydration independent function from viscosity & sedimentation
Λ: " " " " viscosity & fluorescence depolarisation

Neurophysin dimerises side by side - triaxial contour mapping (ELLIPS3)





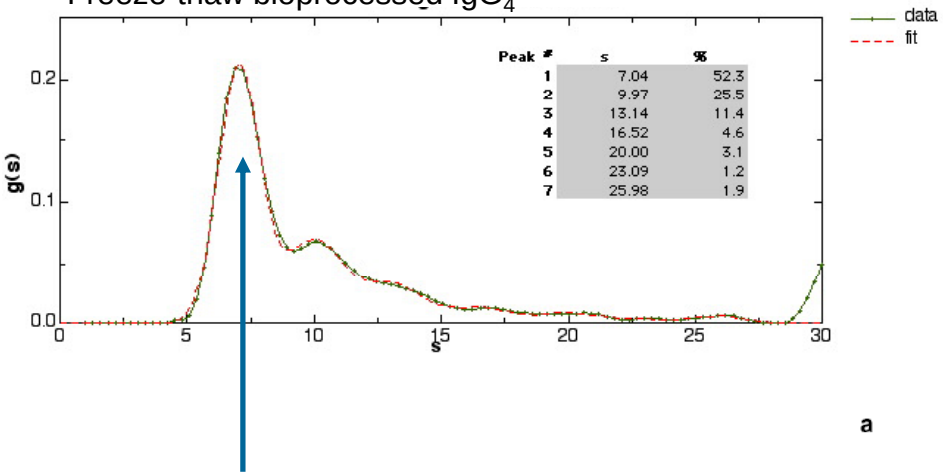




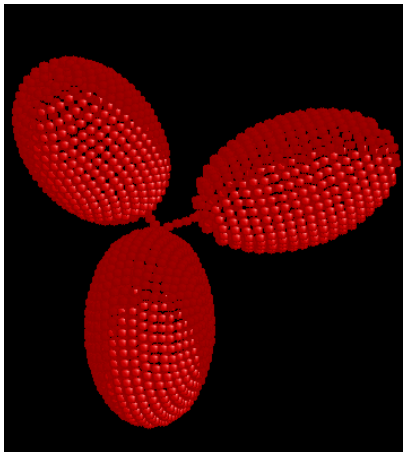
a>b>c: triaxial semi-axes (sphere a=b=c)
R: hydration independent function from viscosity & sedimentation
Λ: " " " " viscosity & fluorescence depolaristion & viscosity

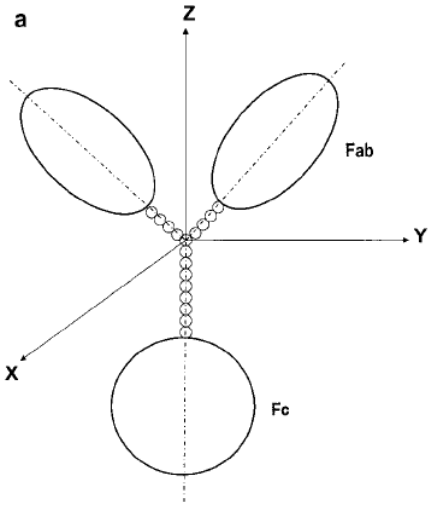
and is bioprocessing-induced aggregation in antibodies linked to a conformation change in the monomer?…

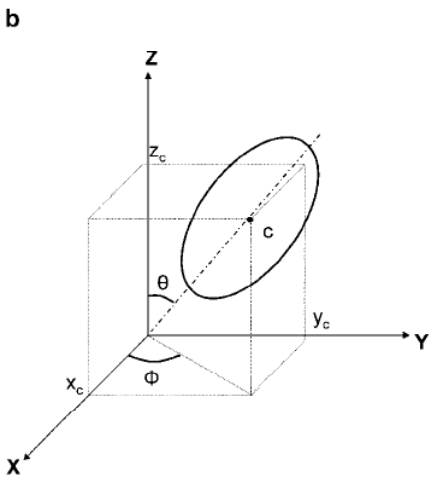
Freeze-thaw bioprocessed IgG_4



Monomer, s=7.0S







Requires measurement of properties of the monomer <u>in the presence of aggregates:</u>

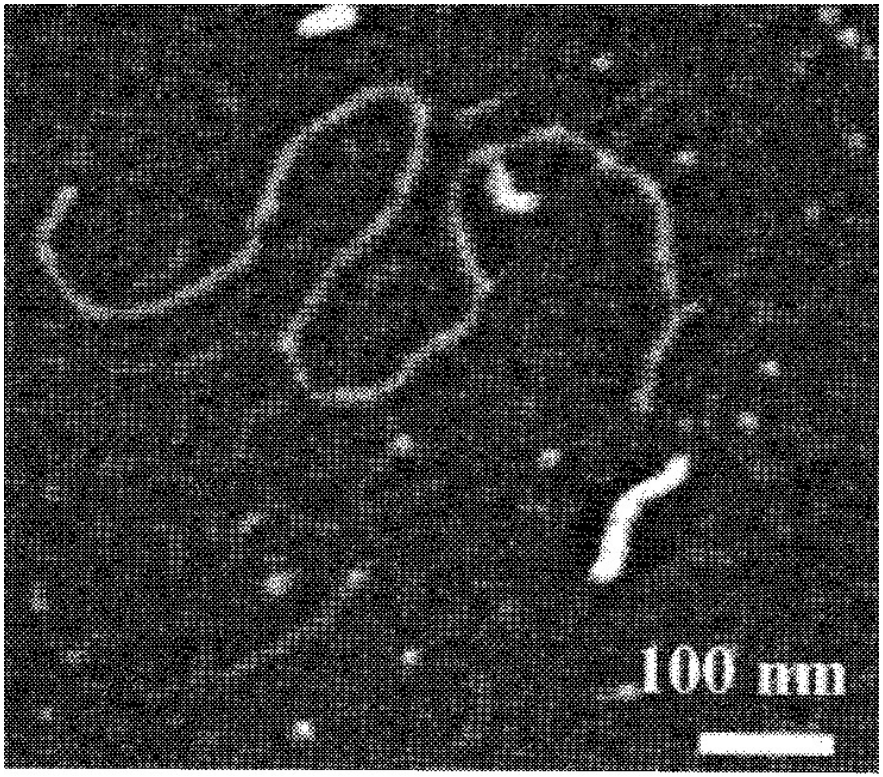
s, [η] (see Lecture 1) and other properties, modelled using SOLPRO (see Lecture 4) – an active area for current research!

Polysaccharide interactions

- *Mucoadhesive interactions - chitosan*
- *Weak dimerisation – arabinoxylan*
- *Self association of amino-celluloses*

Mucoadhesive interactions - chitosan

1. Image by atomic force microscopy



2. Sedimentation velocity result

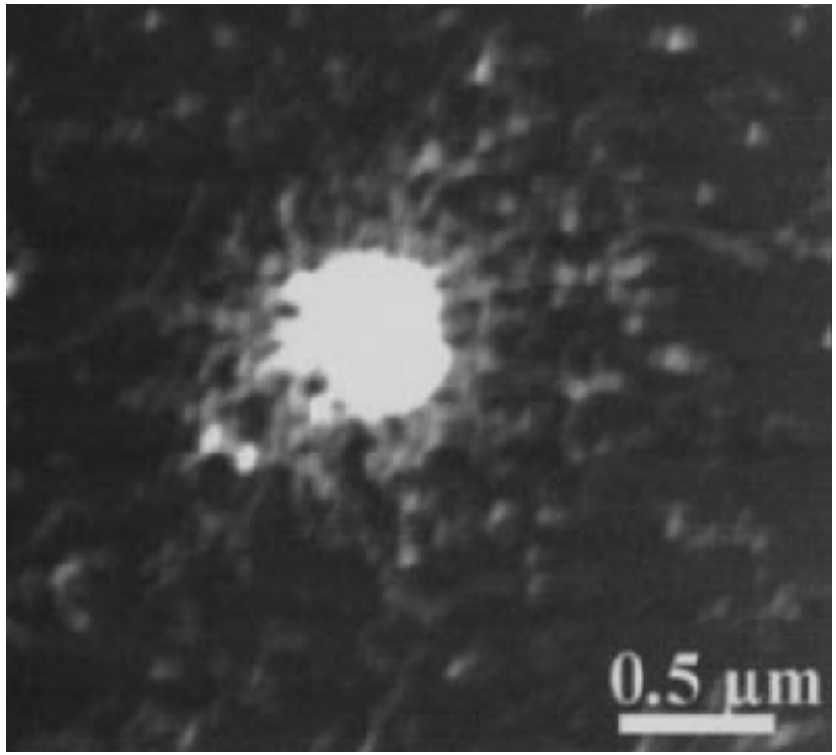
Sedimentation coefficient

$s^{o}_{20,w} \sim 1S$

Deacon et al, *Biochem. J*. 2000

Chitosan – mucin complex

1. Image by atomic force microscopy



2. Sedimentation velocity result

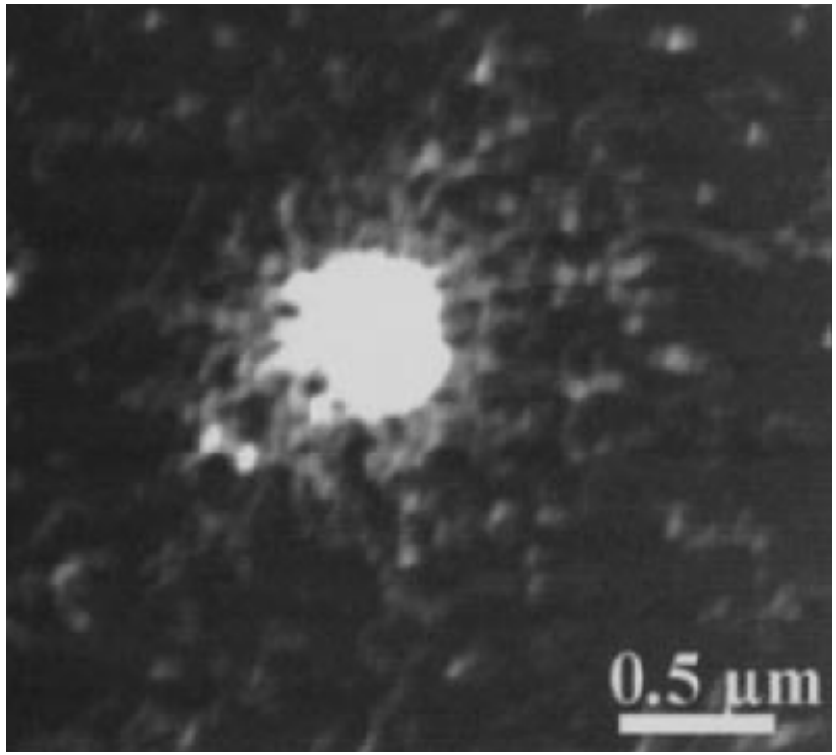
Sedimentation coefficient

$s^{o}_{20,w}$ ~ 2000S

Deacon et al, *Biochem. J*. 2000

Chitosan – mucin complex

1. Image by atomic force microscopy



2. Sedimentation velocity result

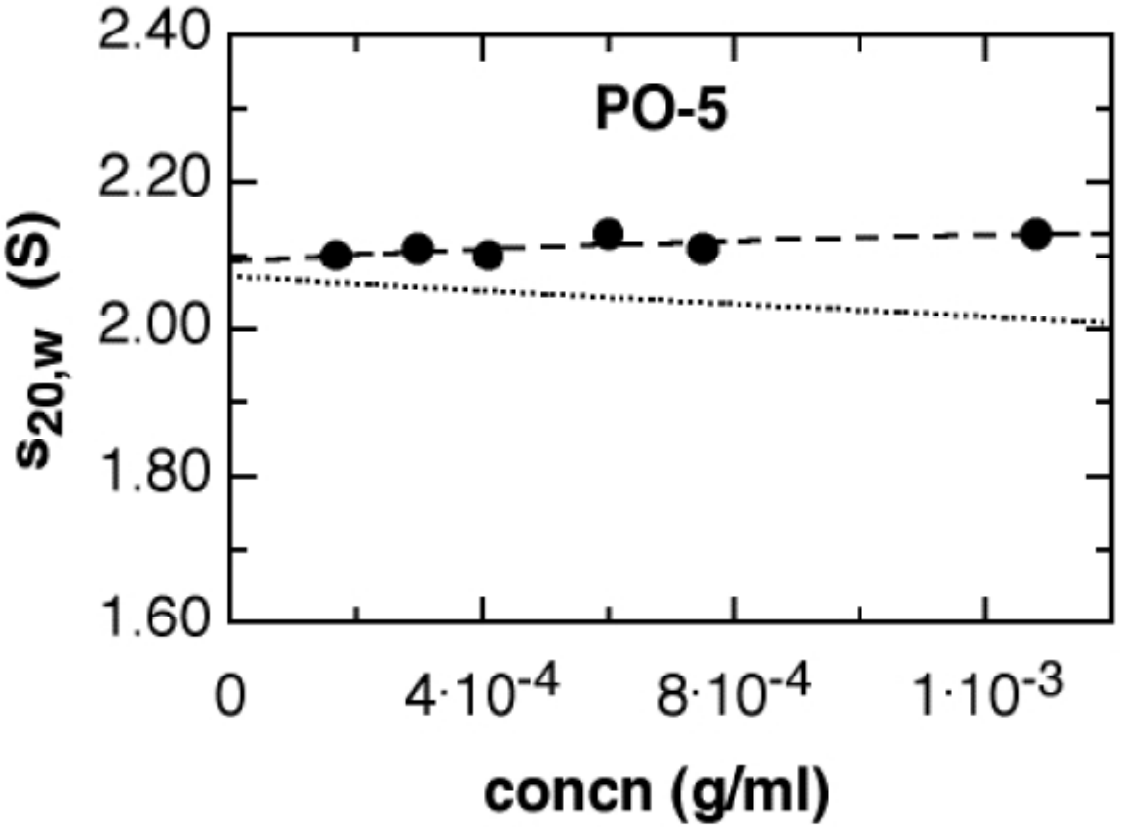
Sedimentation coefficient

$s^{o}_{20,w}$ ~ 2000S

very strong, irreversible interaction

Deacon et al, *Biochem. J*. 2000

A very weak carbohydrate interaction: arabinoxylan dimerisation

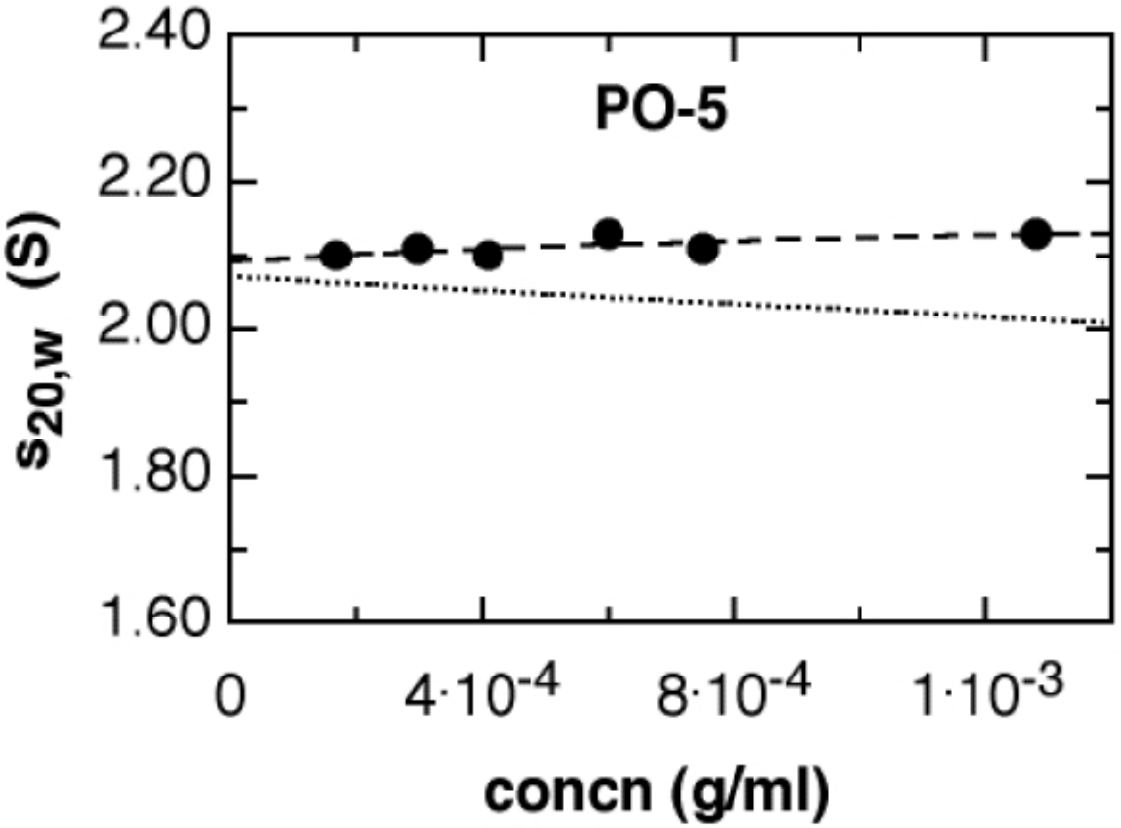


⟸ Expected behaviour for a non-interacting system

very weak, reversible interaction

Patel et al, *Biophys. J.* 2007

A very weak carbohydrate interaction: arabinoxylan dimerisation



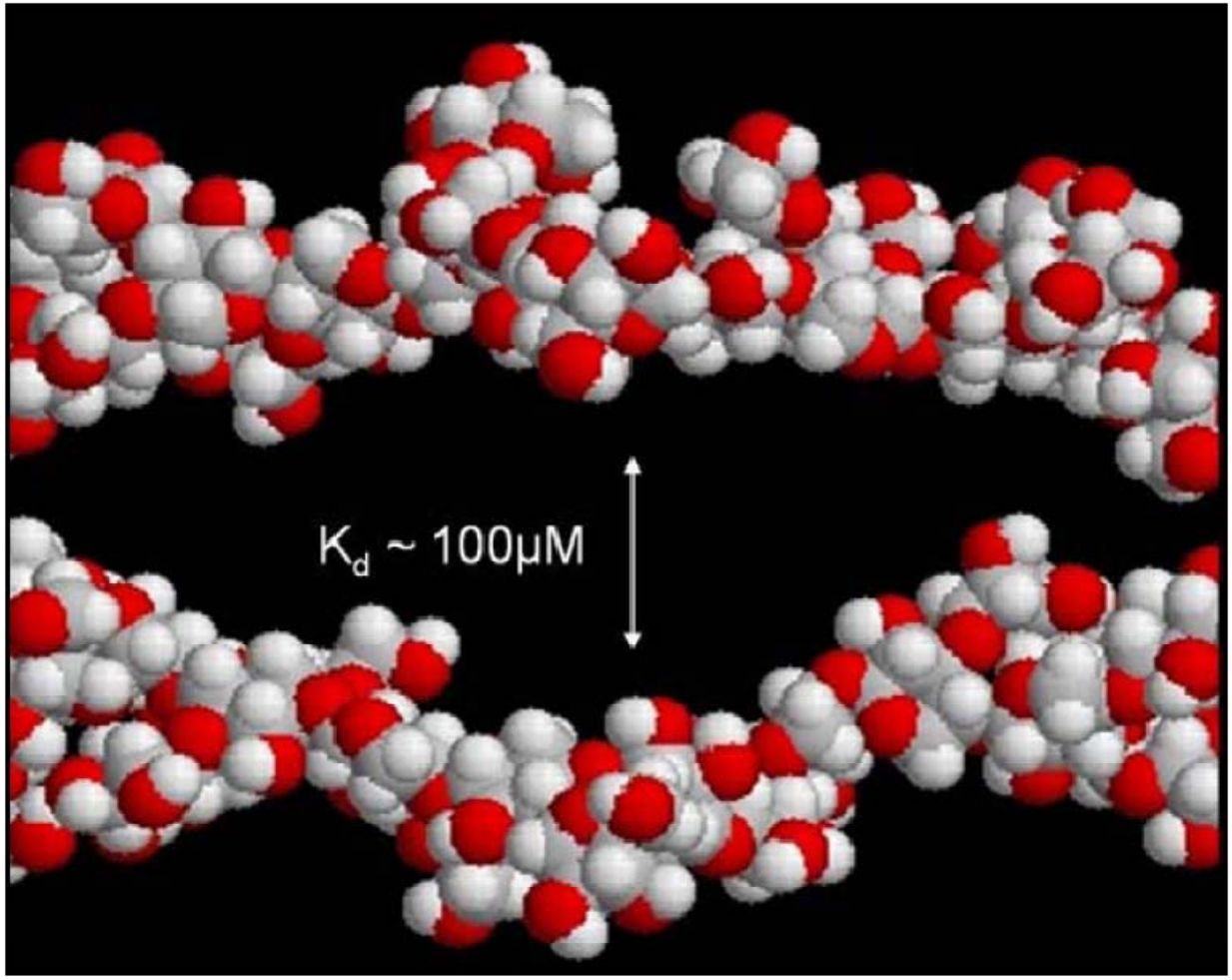
⟸ But it gives a +ve slope

⟸ Expected behaviour for a non-interacting system

very weak, reversible interaction

Patel et al, *Biophys. J.* 2007

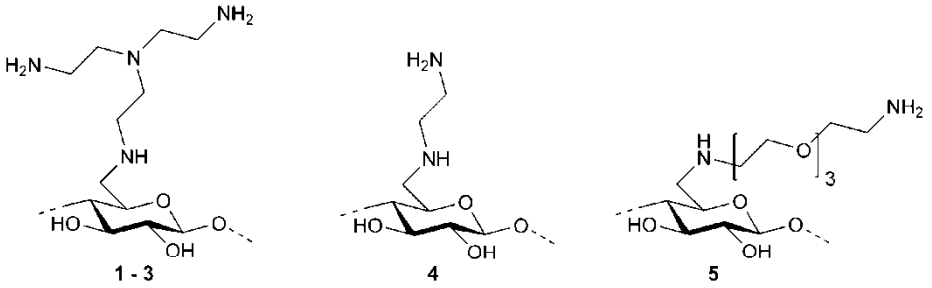
A very weak carbohydrate interaction: arabinoxylan dimerisation



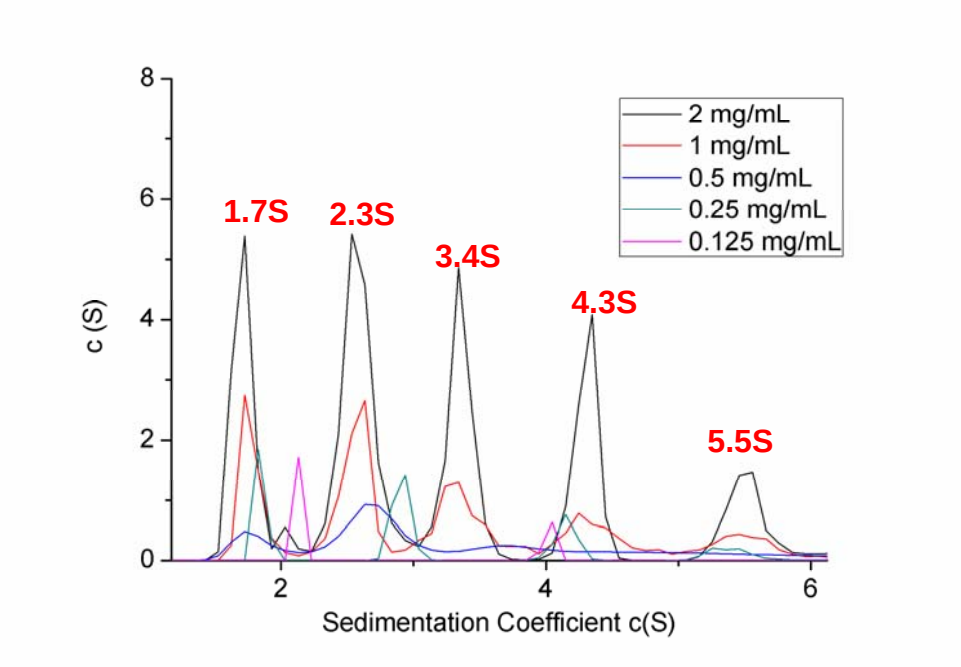
very weak, reversible interaction

Patel et al, *Biophys. J.* 2007

Protein-like self-association in amino-celluloses!



all M_1 ~ 20kDa



Seems to associate into dimers, trimers, tetramers and pentamers, and with b ~0.7, more like a protein than a polysaccharide (0.2-0.5)

$s \sim M^b$

Daus et al, *(mss in preparation)*

Summary

- **free solution technique *for K_d's from <1μM to >100μM, and irreversible aggregation (e.g. antibodies) and complex formation phenomena (e.g. mucoadhesive complexes)***
- **major advances in both sedimentation velocity and equilibrium for stoichiometry, reversibility and strengths, self and hetero-associations and conformation analysis**
- **for reversible associations, complications through non-ideality, often need to be considered – COVOL, for prediction of B.**

Follow up bibliography:

1. Serydyuk, I.N., Zaccai, N.R. and Zaccai, J. (2006) *Methods in Molecular Biophysics*, Cambridge, Chapter D4
2. Harding, S.E., Rowe, A.J. and Horton, J.C., Eds (1992) *Analytical Ultracentrifugation in Biochemistry and Polymer Science,* Royal Soc. Chem. Cambridge
3. Scott, D.J. et al (2005) *Analytical Ultracentrifugation. Techniques and Methods*, Royal Soc. Chem. Cambridge
4. Brown, P.H., Balbo A. and Schuck, P. (2008) Characterizing protein-protein interactions by sedimentation velocity analytical ultracentrifugation. *Current Protocols in Immunology*, 18, 18-15
5. Harding, S.E., Horton, J.C. and Winzor, D.J. (1998) COVOL: an answer to your thermodynamic non-ideality problems?" *Biochem*. *Soc. Trans*. 26 737-741