



University of HUDDERSFIELD

University of Huddersfield Repository

Harding, S. E., Abdelhameed, A. S. and Morris, Gordon

On the hydrodynamic analysis of conformation in mixed biopolymer systems

Original Citation

Harding, S. E., Abdelhameed, A. S. and Morris, Gordon (2011) On the hydrodynamic analysis of conformation in mixed biopolymer systems. *Polymer International*, 60 (1). pp. 2-8. ISSN 0959-8103

This version is available at <http://eprints.hud.ac.uk/id/eprint/13413/>

The University Repository is a digital collection of the research output of the University, available on Open Access. Copyright and Moral Rights for the items on this site are retained by the individual author and/or other copyright owners. Users may access full items free of charge; copies of full text items generally can be reproduced, displayed or performed and given to third parties in any format or medium for personal research or study, educational or not-for-profit purposes without prior permission or charge, provided:

- The authors, title and full bibliographic details is credited in any copy;
- A hyperlink and/or URL is included for the original metadata page; and
- The content is not changed in any way.

For more information, including our policy and submission procedure, please contact the Repository Team at: E.mailbox@hud.ac.uk.

<http://eprints.hud.ac.uk/>

Mini Review:

On the hydrodynamic analysis of conformation in mixed biopolymer systems

Stephen E. Harding, Ali S. Abdelhameed and Gordon A. Morris

National Centre for Macromolecular Hydrodynamics, University of Nottingham, Sutton Bonington
LE12 5RD, UK

Abstract

A brief review is given of some of the advances in hydrodynamic methodologies for studying the conformation and flexibility of biomacromolecules in mixed systems. We consider first of all evaluation of conformation type and flexibility in polymer systems with a quasi-continuous distribution of molecular weight – using polysaccharides and mucin glycoproteins as our main examples, and then conformation determination in discrete or paucidisperse systems such as aggregated antibody preparations. This paper is based on a presentation given at the European Polymer Congress meeting held in Graz, Austria July 12-17, 2009.

Keywords: Haug triangle; conformation zoning; HYDFIT (combined global analysis); bead modelling

1. Introduction

The last two decades has seen considerable advances in methodology for the analysis of biopolymer systems in an environment where many occur naturally and perform their biological function – a solution environment. These advances include improved ways in which we can ascertain the molecular weight (molar mass) or molecular weight distribution of polymeric systems using size exclusion chromatography coupled to multi angle light scattering [1] and sedimentation equilibrium and sedimentation velocity in the analytical ultracentrifuge [2-4]. There have also been significant advances in the study of macromolecular interactions using hydrodynamic methods again using analytical ultracentrifugation alongside other techniques such as surface plasmon resonance and isothermal calorimetry (see Table 1 for a description of hydrodynamic parameters). The focus of this article is on the ascertainment of conformation in mixed biopolymer systems. We consider first of all evaluation of conformation type and flexibility in polymer systems with a quasi-continuous distribution of molecular weight – using polysaccharides and mucin glycoproteins as our main examples, and then conformation determination in discrete polydisperse systems such as reversibly associating protein systems or aggregated preparations of monoclonal antibody, taking advantage of our ability to resolve useful parameters like the sedimentation coefficient, the intrinsic viscosity and the radius of gyration in such systems.

Table 1 Hydrodynamic parameters and their symbols

<i>Hydrodynamic Parameter</i>	<i>Commonly used Symbol and units</i>
Intrinsic viscosity	$[\eta]$ (ml/g)
Sedimentation coefficient (corrected to standard condition of 20 °C and water)	$s_{20,w}^o$ (S)
Concentration dependence of sedimentation (Gralen parameter)	k_s (ml/g)
Intrinsic sedimentation coefficient	$[s]$ (S)
Radius of gyration (root-mean-square radius)	R_g (cm)
Diffusion coefficient (corrected to standard condition of 20 °C and water)	$D_{20,w}^o$ (cm ² /s)
Molecular weight (molar mass)	M (g/mol)
Weight-average molecular weight (molar mass)	M_w (g/mol)
Flory-Fox constant	Φ (2.86 x 10 ²³ mol ⁻¹)
Avogadro's number	N_A (6.02 x 10 ²³ mol ⁻¹)
Mass per unit length	M_L (g mol ⁻¹ cm ⁻¹)
Persistence length	L_p (cm)
Chain diameter in polysaccharides	d (~8 x 10 ⁻⁸ cm)
Translational frictional ratio	f/f_0
Perrin shape parameter 'frictional ratio due to shape'	P
Time-averaged hydration	δ (g solvent/ g)
Wales – van Holde ratio	R (= $k_s/[\eta]$)
?	Λ
Axial ratio	a/b
Harmonic mean fluorescence anisotropy relaxation time	τ_h
Spherical polar angles	θ and ϕ
Boltzmann constant	k (1.38 x 10 ⁻¹⁶ mol ⁻¹)
Maximum dimension of the particle	D_p

2. Biopolymers with a quasi-continuous distribution of molecular weight

These include for example polysaccharides, glycoconjugates and nucleic acids (which are themselves glycoconjugates). We consider first how hydrodynamic methods can help us assign conformation type and then how they can be used to describe in more detail conformational flexibility.

Conformation type: power law relations.

One of the simplest representations is the Haug triangle [5] where the three extremes of macromolecular conformation – compact sphere, rigid rod and random coil, are placed at the corners of a hypothetical triangle – the conformation of a given macromolecule is then represented by a locus along one of the sides: for example a globular protein would be represented somewhere between the extremes of sphere and rigid rod (Fig. 1), a polysaccharide, DNA or mRNA between the extremes of rod and coil, a denatured protein between the extremes of sphere and coil. To assist with the assignment of conformation type, several power law or “scaling relations” are available for homologous series of polymers (*Table 2*) – these are also popularly called MHKS (Mark Houwink Kuhn Sakurada) relations although this was originally given to denote the viscosity –molecular weight relation (see, e.g. [6]):

$$[\eta] = KM^a; s_{20,w}^o = KM^b; R_g = KM^c; D_{20,w}^o = KM^{b-1} \quad (1)$$

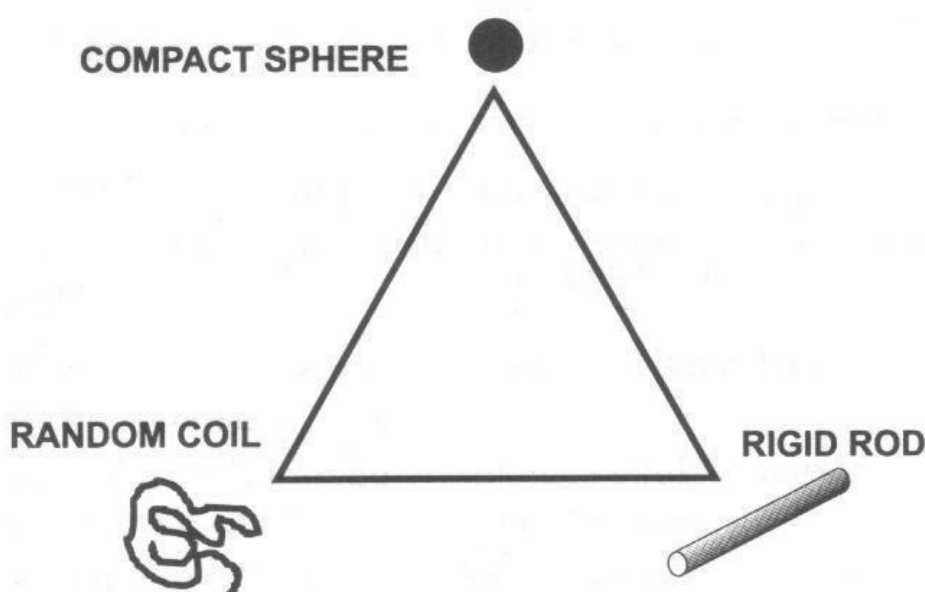


Fig. 1. Haug triangle representation of the extremes of conformation [5]

Table 2 Power law coefficients (see .g. ref 6 and references cited therein)

<i>Sphere</i>	<i>Rod</i>	<i>Coil</i>
$[\eta] \sim M^0$	$[\eta] \sim M^{1.8}$	$[\eta] \sim M^{0.5-0.8}$
$s_{20,w}^o \sim M^{0.67}$	$s_{20,w}^o \sim M^{0.15}$	$s_{20,w}^o \sim M^{0.4-0.5}$
$R_g \sim M^{0.33}$	$R_g \sim M^{1.0}$	$R_g \sim M^{0.5-0.6}$
$D_{20,w}^o \sim M^{-0.33}$	$D_{20,w}^o \sim M^{-0.85}$	$D_{20,w}^o \sim M^{-(0.5-0.6)}$

Perturbations can result from intra-molecular chain co-exclusion effects. Draining effects have also been considered but these are usually small compared with the strong hydrodynamic interactions between segments of a macromolecule [7]. The power law relations are particularly useful for assessing conformation change in macromolecular systems as shown for example for amylose by Rollings [8].

Conformation zoning

In an extension of this concept, Pavlov and coworkers [9] developed an extended conformation zoning protocol to cover 5 conformation types: extra-rigid rod – a rod with no flexibility (zone A), rigid-rod (with a very limited amount of flexibility – zone B), a semi-flexible coil (zone C), random coil (zone D) and finally a heavily branched or globular structure (zone E). A semi-empirical method was developed involving the concentration dependence of the sedimentation coefficient k_s , the intrinsic sedimentation coefficient, $[s]$ and mass per unit length, M_L (measurable by for example by electron microscopy or from knowledge of the composition of the polymer). Fig. 2 shows such a zoning plot illustrated with three examples, glycogen (zone E), pullulans (zone D) and citrus pectins (zone C), all consistent with expectation.

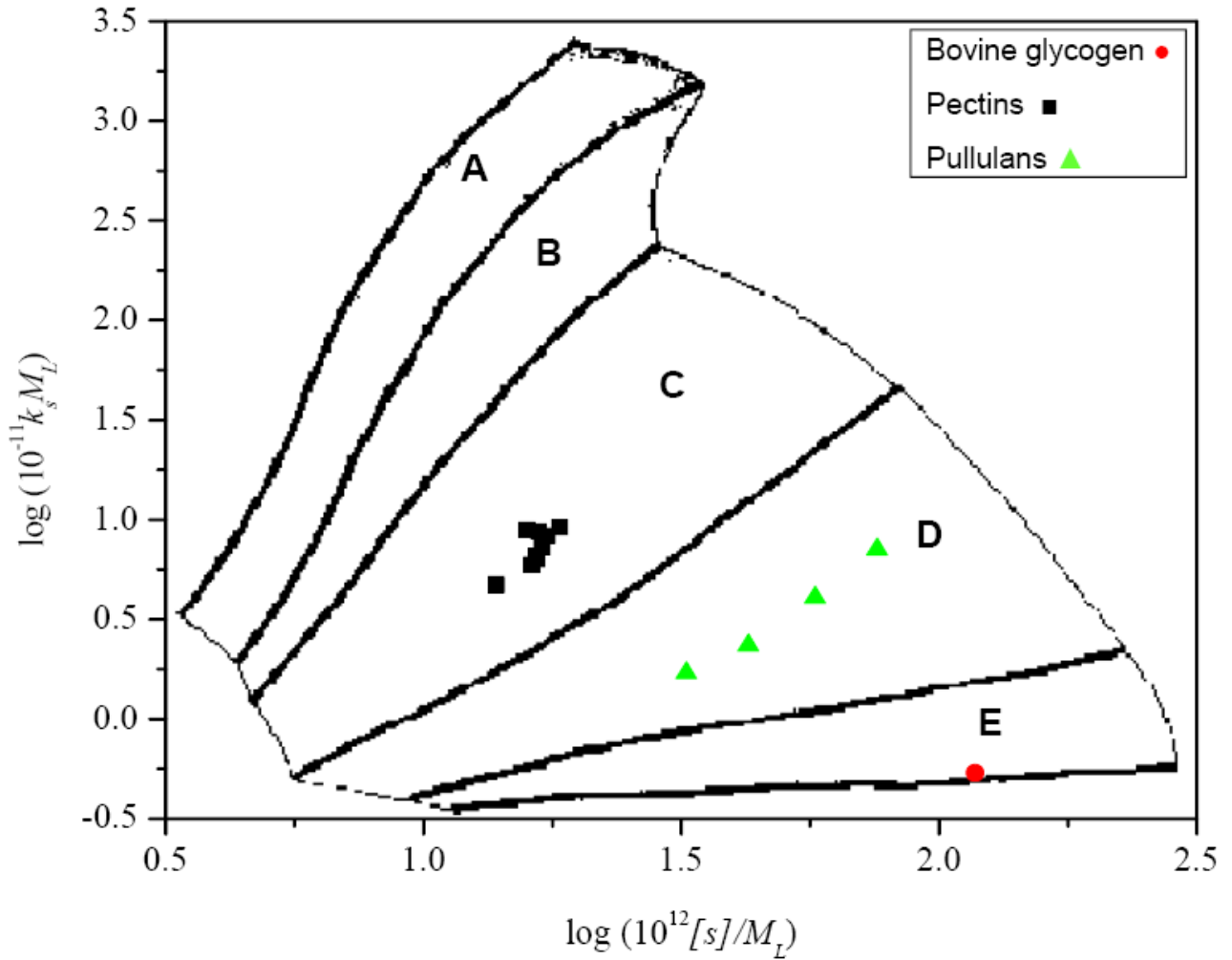


Fig. 2 Conformation zoning plot. Zone A: extra rigid rod, Zone B: rigid rod, Zone C: semi-flexible coil, Zone D: random coil, Zone E: globular/branched. $[s] = \frac{s_{20,w}^0 \eta_{20,w}}{(1 - \bar{v} \rho_{20,w})}$ where $s_{20,w}^0$ is the sedimentation coefficient normalized to standard conditions (the viscosity and density of water at 20°C, $\eta_{20,w}$ and $\rho_{20,w}$ respectively) and extrapolated to zero concentration. Red circle - glycogen [10], green triangles – pullulans [adapted from ref. 11], black squares – citrus pectins [12].

Flexibility: Global or “HYDFIT” estimations of the persistence length L_p .

More complex forms of the relations between hydrodynamic parameters and molecular weight have been given in terms of the persistence length L_p , which is now a popularly used measure of chain flexibility. For example Bushin [13] and independently Bohdanecky [14] have given this expression for the intrinsic viscosity – molecular weight relationship:

$$\left(\frac{M_w^2}{[\eta]}\right)^{1/3} = A_0 M_L \Phi^{-1/3} + B_0 \Phi^{-1/3} \left(\frac{2L_p}{M_L}\right)^{-1/2} M_w^{1/2} \quad (2)$$

where M_L is the mass per unit length, N_A is Avogadro's number, Φ the Flory-Fox constant ($2.86 \times 10^{23} \text{ mol}^{-1}$) and A_0 and B_0 are tabulated coefficients [14], and Yamakawa and Fujii have [15] have given the corresponding relation for the sedimentation coefficient:

$$s^0 = \frac{M_L(1-\bar{\nu}\rho_0)}{3\pi\eta_0 N_A} \times \left[1.843 \left(\frac{M_w}{2M_L L_p}\right)^{1/2} + A_2 + A_3 \left(\frac{M_w}{2M_L L_p}\right)^{-1/2} + \dots \right] \quad (3)$$

where $A_2 \sim -\ln(d/2L_p)$ and $A_3 \sim 0.1382$ provided that L_p is much higher than the chain diameter d . An analogous expression to (2) and (3) exists for the radius of gyration-molecular weight relation. Ortega and Garcia de la Torre [16] have introduced a global procedure (HYDFIT) for estimating L_p and M_L by combining these relations together with their corresponding sets of data and performing a global weighted minimization of a target function. The target function is calculated using equivalent radii, where the equivalent radius (a_x) is defined as the radius of an equivalent sphere having the same value as the determined property. In this paper, we are mainly interested in the equivalent radii resulting from the sedimentation coefficient *i.e.* translational frictional coefficient (a_T) and from the intrinsic viscosity (a_I). Therefore this target function is a dimensionless estimate of the agreement between the theoretical calculated values for the sedimentation coefficient and intrinsic viscosity for a particular molar mass, persistence length and mass per unit length and the experimentally measured parameters [16], An estimate for the chain diameter d is also required although the minimization procedure is not generally sensitive to the value chosen. Fig. 4 gives an example evaluation [17]. In cases where M_L is known then the minimization procedure can yield a better defined value for L_p . Table 3 gives a comparison of values obtained with the global method. Practically the limits are ~ 1 -2 nm for a randomly coiled polysaccharide like pullulan, and ~ 200 nm for a rod-like triple helical polysaccharide like scleroglucan or xanthan.

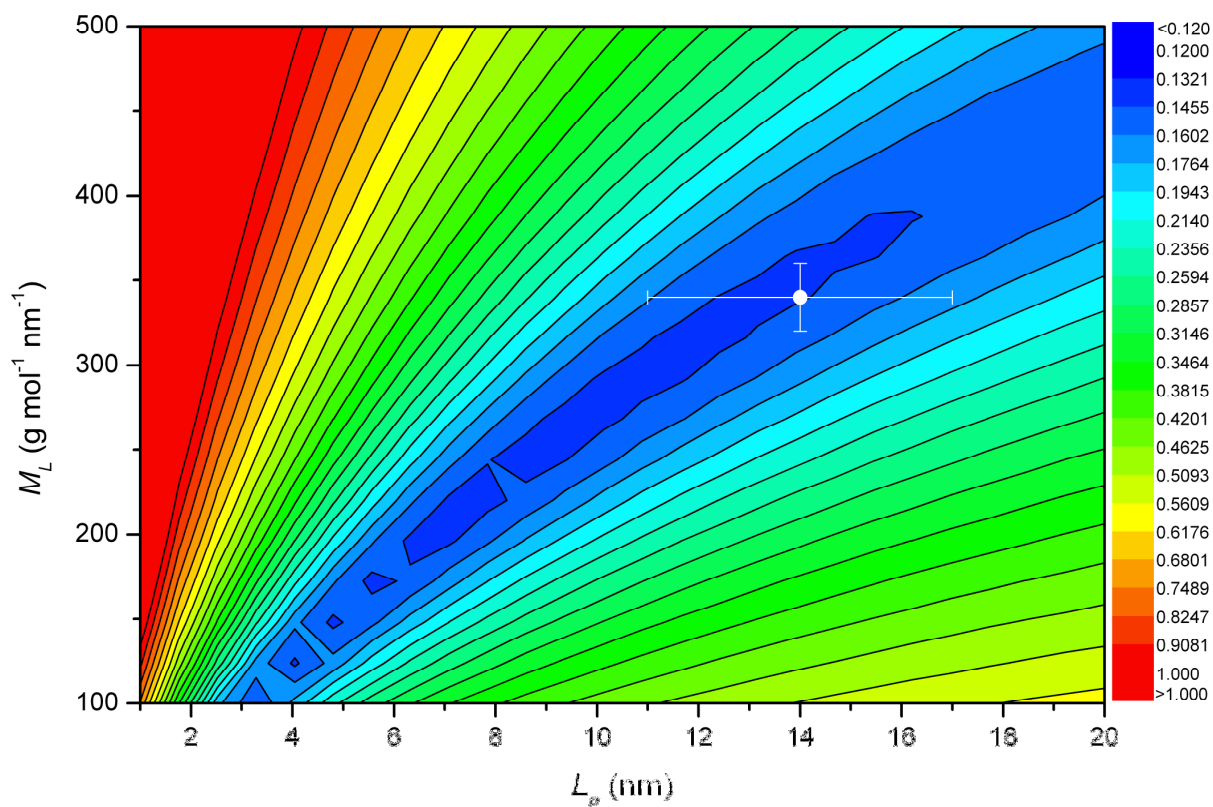


Fig 3. Global hydrodynamic analysis of methyl cellulose [adapted from 17]. The minimum value of a target function (colour bar calibration on the right) is shown by the white cross of values. L_p is $\sim (14 \pm 3)$ nm corresponding to a semi-flexible molecule.

Table 3 Comparative estimates for the persistence length for different polysaccharides obtained via the global HYDFIT procedure

Carbohydrate Polymer	Persistence length L_p (nm)	Ref
Pullulan	1 - 2	from 11
Deoxy-azido cellulose	2 - 4	18
Xyloglucan	7 ± 1	19
Guar	8 ± 1	20
Locust bean gum	9 ± 1	20
Pectins	12 ± 1	12
Konjac Glucomannan	13 ± 1	21
Methyl cellulose	14 ± 3	17
Chitosan	16 ± 2	22

3. Conformation determination in paucidisperse protein systems

The resolving power of modern hydrodynamic methods such as sedimentation velocity in the analytical ultracentrifuge can be used to good effect to estimate conformation in a mixed non-interacting system of proteins [23]. In the case of some non-interacting systems it is possible to fractionate or partially fractionate components preparatively – a recent example has been for wheat protein gliadins. After fractionation the principal fractions, namely α , γ , ω_{slow} , ω_{fast} were studied using sedimentation velocity in the analytical ultracentrifuge and their (weight average) molecular weights M_w defined by sedimentation equilibrium. The translational frictional ratio (ratio of the frictional coefficient of a molecule to the frictional coefficient of a spherical particle of the same anhydrous mass) can be obtained from M_w and $s_{20,w}^0$ via:

$$\frac{f}{f_0} = \frac{M_w (1 - \bar{v} \rho_{20,w})}{(N_A 6\pi\eta_{20,w} s_{20,w}^0)} \left(\frac{4\pi N_A}{3 \bar{v} M_w} \right)^{1/3} \quad (4)$$

This depends on shape and (time averaged) molecular hydration. The Perrin shape parameter, P (or 'frictional ratio due to shape' [26]), can then be calculated from f/f_0 by assigning a hydration value, δ , using the expression

$$P = \left(\frac{f}{f_0} \right) \left[1 + \left(\frac{\delta}{v \rho_{20,w}} \right) \right]^{-1/3}$$

(5)

Since values for δ were not known a range of plausible values (from 0.35 to 1.0) (27, 28) were chosen to specify a range of P values for each and corresponding (prolate) ellipsoidal axial ratios a/b were calculated using the routine ELLIPS1 [25]: all the proteins are extended molecules with axial ratios ranging from ~ 10 -30 with α appearing the most extended and γ the least (Fig. 4).

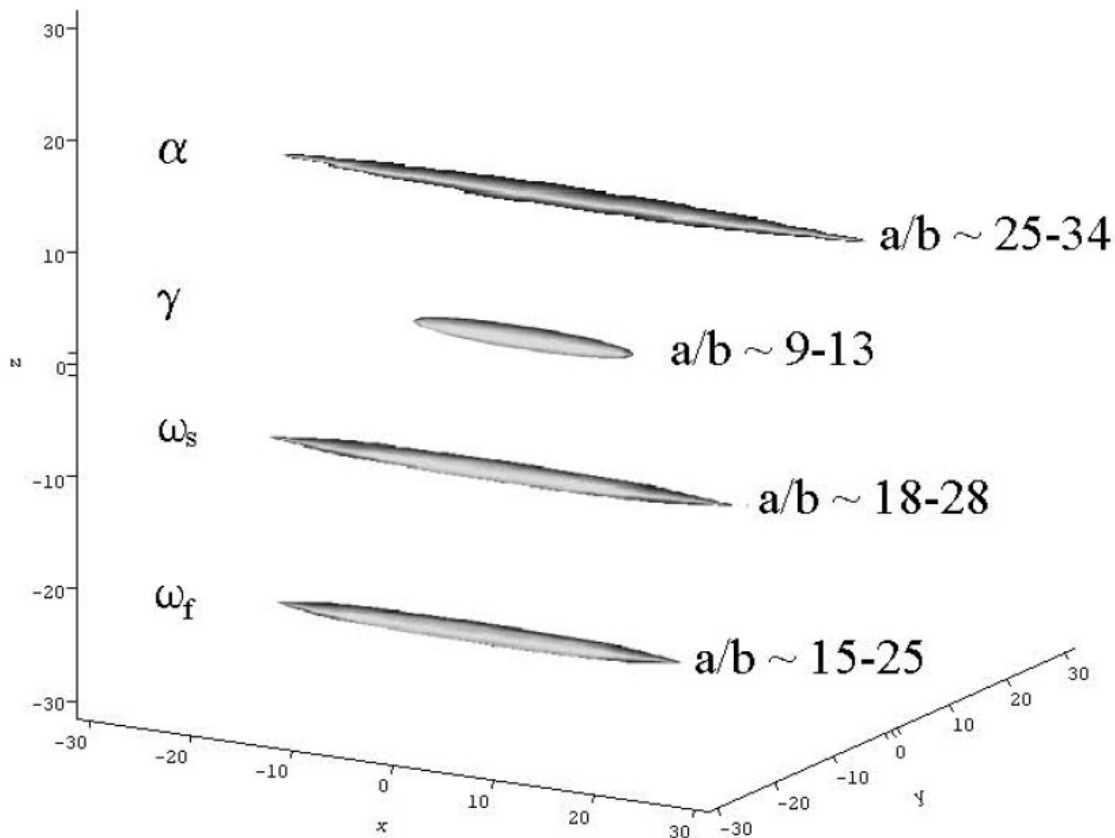


Fig 4. Axial ratios of wheat protein gliadins estimated from sedimentation velocity in the analytical ultracentrifuge and analysed using ELLIPS1 (adapted from [23])

4. Conformation determination in a dimerising protein system

A quite different case to gliadin is the neural protein neurophysin – this is a reversible monomer-dimer system although it was possible to define conditions so that the intrinsic viscosity $[\eta]$, the concentration dependence sedimentation coefficient k_s and the harmonic mean fluorescence anisotropy relaxation time τ_h for both the monomer and dimeric state could be defined [26, 27]. This allowed the evaluation of 2 hydration-independent combined shape functions, R [28] and Λ [29]:

$$R = k_s/[\eta] \quad (6)$$

$$\Lambda \equiv \frac{3\eta_0[\eta]M}{N_A k T \tau_h} \quad (7)$$

Where η_0 is the viscosity of the solvent at temperature, T (K) and k is the Boltzmann constant. The relationship of these with the *two* axial ratios (a/b , b/c) defining a general ellipsoid are known – each defines a line solution of possible values of (a/b , b/c) but when combined together graphically (allowing for experimental error) can define the conformation using a routine ELLIPS3 [25]. Using this procedure the monomer species appears as a prolate ellipsoid of (a/b , b/c) \sim (4.0, 1.0). Had the dimerisation process been end to end one would expect this to increase to (a/b , b/c) \sim (8.0, 1.0), instead a value of \sim (2.7, 2.0) was obtained, indicating that the association was side-to-side (Fig. 5)

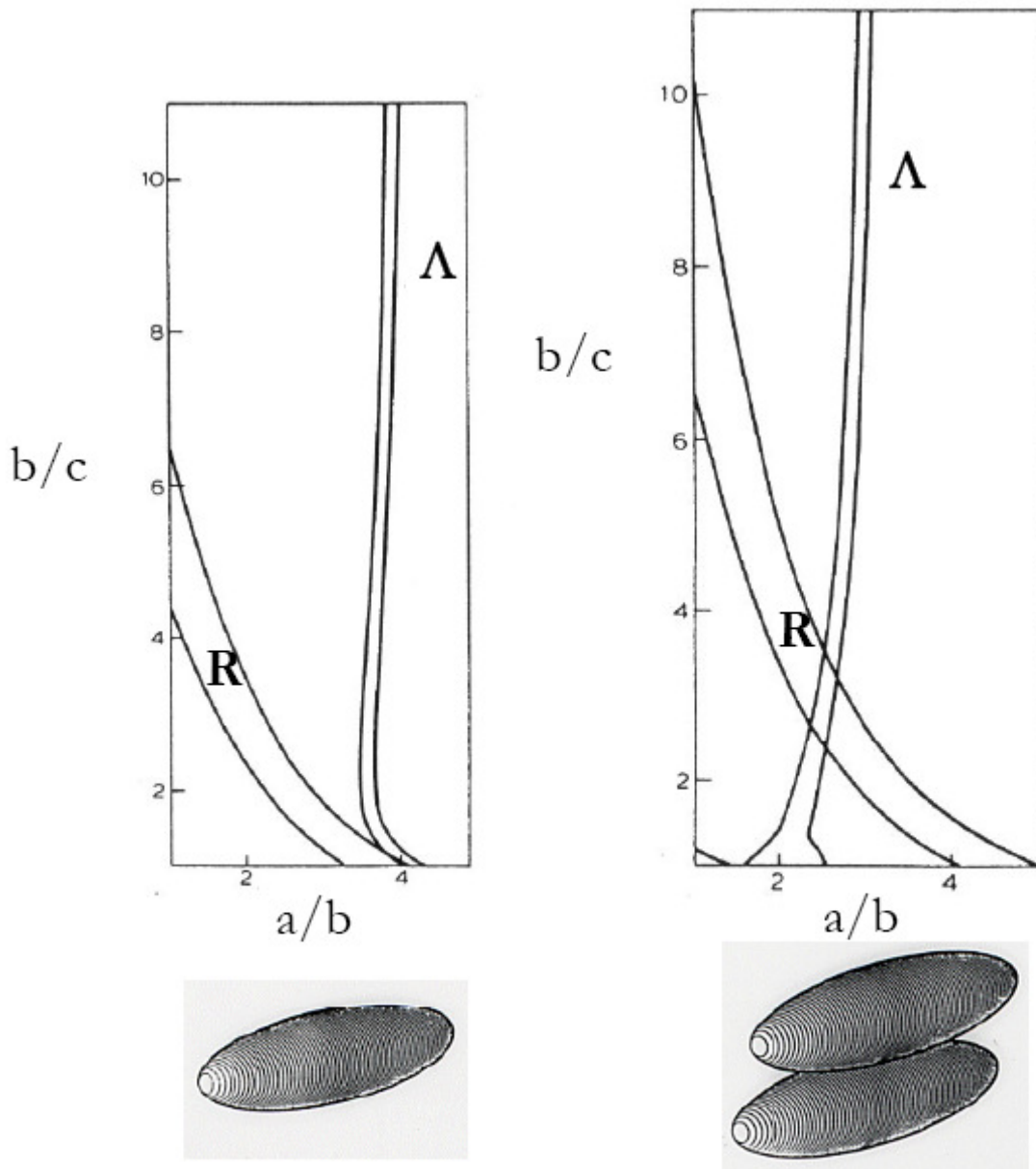


Fig. 5. Conformation assignment of a dimerising protein system: neurophysin. Left: monomers, $(a/b, b/c) \sim (4.0, 1.0)$; dimers $(a/b, b/c) \sim (2.7, 2.0)$, based on the intersection of the hydration independent R function (from sedimentation and viscosity measurements) and Λ (from harmonic mean rotational relaxation time and viscosity measurements). Adapted from ref [28].

5. Conformation determination in an aggregated antibody system

Ellipsoids – either simple ellipsoids of revolution defined by a single axial ratio a/b , or more general triaxial ellipsoids defined by two – are not sufficient to define the conformation of irregularly shaped molecules that are neither axially or centrally symmetric. An example of this conformation type are immunoglobulins or antibodies which (apart from the class IgM) are defined by 2 Fab

regions (which themselves can be approximated as prolate ellipsoids), and Fc region (~oblate ellipsoid) and depending on the antibody class/subclass, a flexible hinge region. Because of their flexibility on a small number with relatively short hinge regions have been crystallized and had their structures determined. In the case of the others it is possible to estimate conformation in terms of the orientation of the domains – which dictate the hydrodynamic properties of these substances [31-33]. To do this requires knowledge of the shape of the Fab and Fc domains (from x-ray crystallography), knowledge of the hydrodynamic properties of the domains and the hydrodynamic properties of the intact antibody. Hydrodynamic bead modelling – can be used to represent the orientation of the domains relative to each other in terms of the spherical polar angles θ and ϕ (Fig. 6a). Combination of 2 or more hydrodynamic measurements together, along with knowledge of the domain shape can then help define the angles & allowing for complications like hydration.

Besides the Perrin function P (eq. 5) from the sedimentation coefficient (or translational diffusion coefficient), others include the viscosity increment ν .

$$\nu = \frac{[\eta]}{\left(\frac{\delta}{\rho_0} + \nu \right)}$$

(8)

and x-ray scattering functions such as the radius of gyration R_g and the maximum dimension of the particle D_p . The method has been successfully applied to near monodisperse preparations of IgG3 and hinge mutants (Fig 6b,c). Current interest is in trying to define the conformation of the monomer species in the presence of aggregates – this is highly relevant for the case for monoclonal antibodies because in their production for therapies storage, freeze thaw processes etc. can lead to aggregate formation. The distribution of sedimentation coefficient from sedimentation velocity experiments analysed using the SEDFIT procedure [34] helps ascertain the extent of aggregation [35] (Fig 7a). This procedure involves analysis of the evolution of the whole concentration, c , versus sedimentation distance, r , and versus time, t , distribution, $c(r,t)$, in the ultracentrifuge cell, via numerical solution of the differential equation describing sedimentation and diffusive transport known as the Lamm equation. Measurement of the effect of change in concentration on the extent of aggregation also helps us also to assess the reversibility of the process (increase in concentration should increase the relative proportion of aggregates – this is usually not the case). More

appropriately it is possible to assign a value for the sedimentation coefficient of the monomer species in the presence of the aggregates as Fig 7a illustrates quite well.

It is also possible to measure the intrinsic viscosity in the presence of aggregates (Fig 7b) using viscometers based on a differential pressure principle and coupled on-line to size exclusion columns [36]. Although research is currently in progress, this combination provides the possibility of not only assessing the effect of bioprocessing on the conformational orientation of the monomer but also to assess if there is a link between conformational change and the state of aggregation.

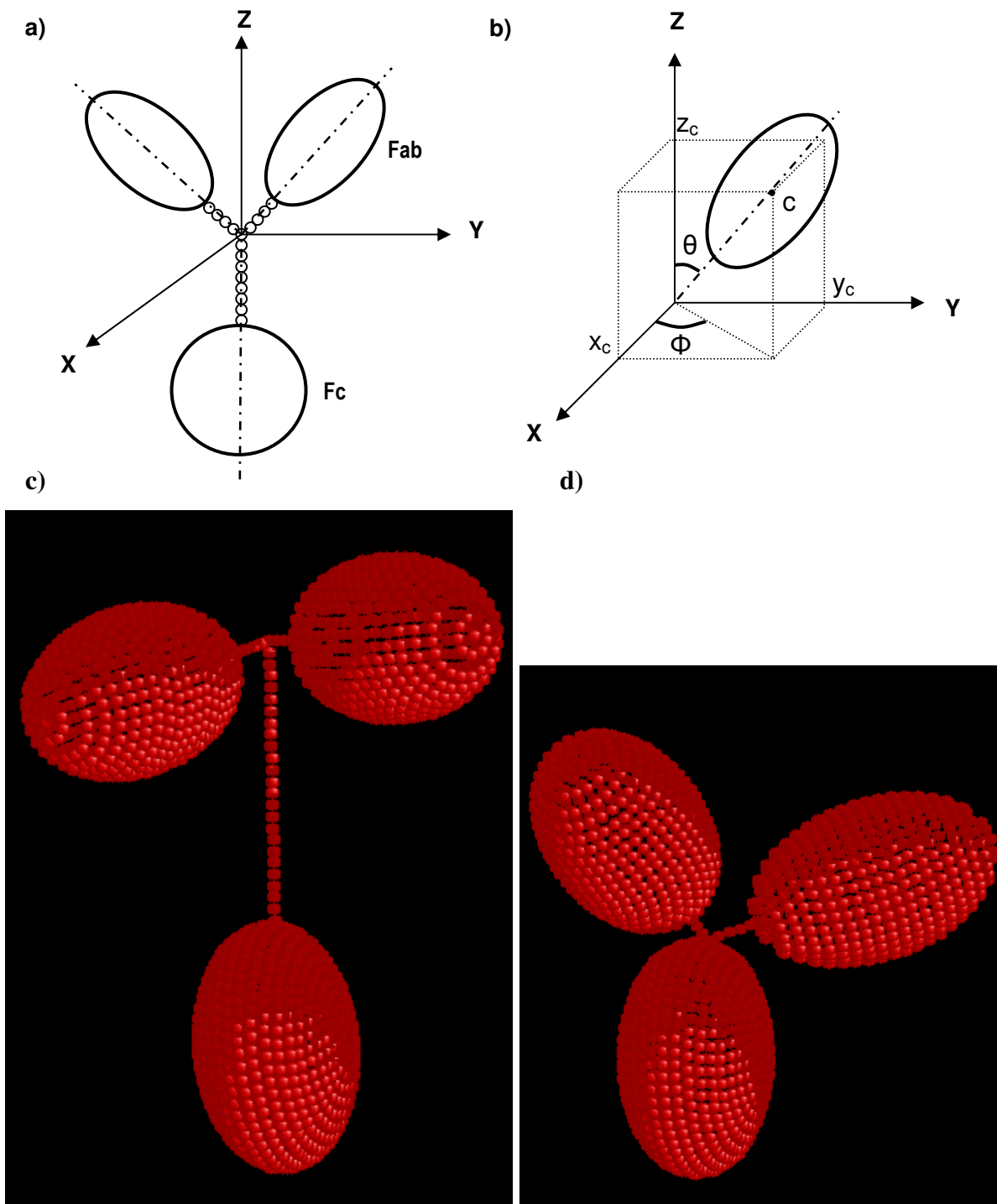
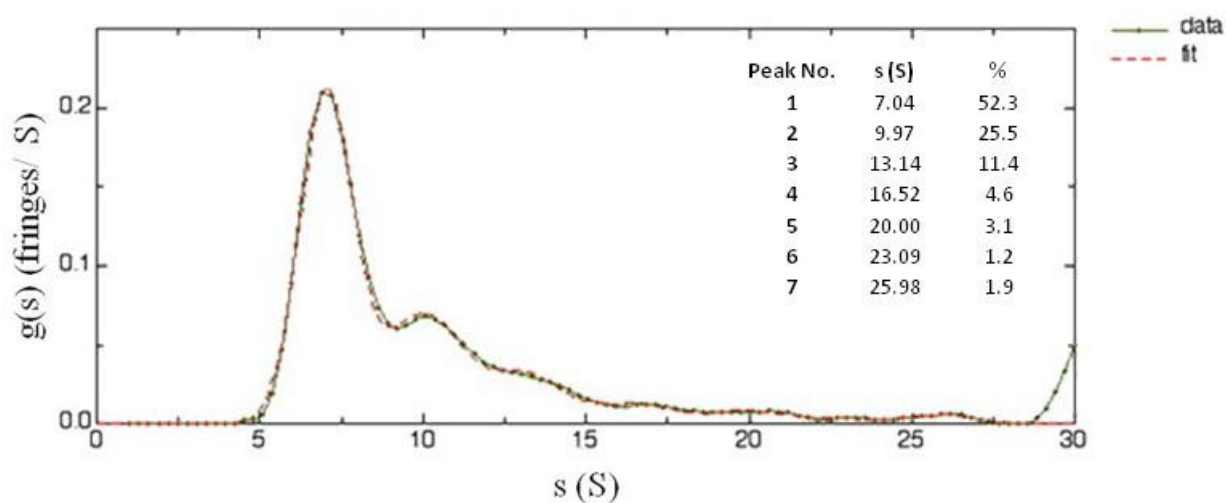


Fig. 6. Bead-shell models for antibodies a) antibody is modeled in terms of symmetrical prolate ellipsoid domains (Fab regions) and oblate ellipsoid (Fc region) linked by a hinge region. b) arrangement of the spherical polar angles θ and ϕ . c) model for wild-type IgG3 and d) a mutant IgG3 with a shorter hinge region (right).

a)



b)

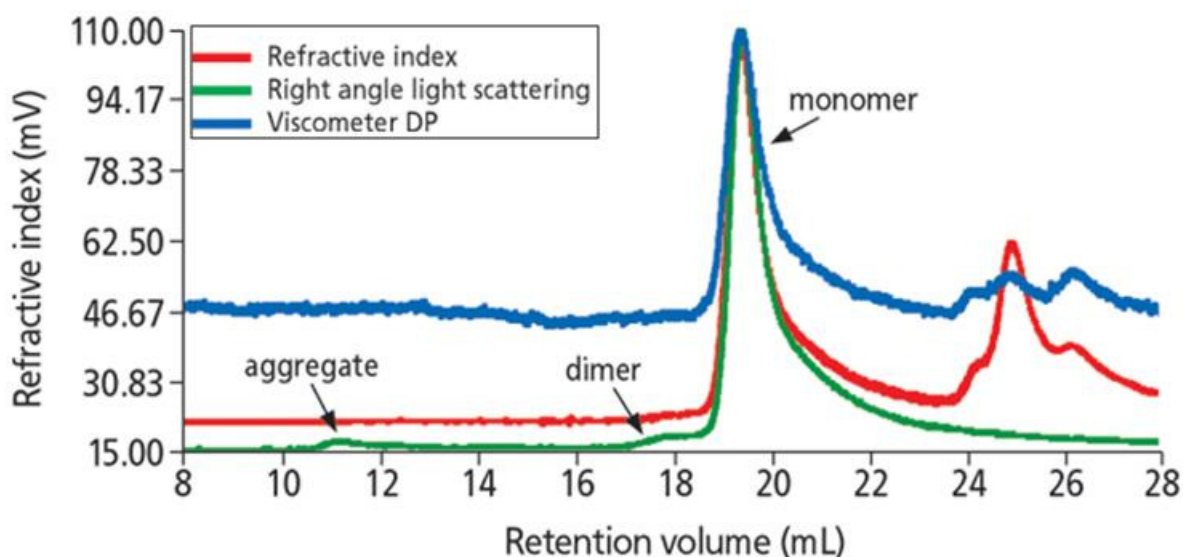


Fig. 7 (a) Sedimentation coefficient distribution of an aggregated preparation of IgG4, s (monomer) = 7.04 S; (b) Relative viscosity distribution of IgG3 wild type antibody in the presence of dimers and aggregates measured using a “pressure imbalance” or differential pressure viscometer: $[\eta]$ (monomer) = 9.9 ml/g [36].

6. Summary and Perspective

The many significant advances in solution conformation and flexibility analysis of linear polymers now allow quite detailed information to be provided – conformation type or zone and conformational flexibility in terms of reliable estimates of the persistence length using combined approaches. The same is true for discrete or paucidisperse protein systems such as wheat gliadins, dimerising protein systems and aggregated systems of antibody. With regard the latter it is crucial

to have measurements on a number of different parameters where sedimentation coefficients and intrinsic viscosities for monomer species can now be resolved. These, combined with other resolvable hydrodynamic data – such as fluorescence anisotropy relaxation times (a measure of rotational diffusion behaviour) [37] offer further strong possibilities which are now being explored.

These advances now offer the real potential of linking conformation – and conformation change with fundamental functional properties of macromolecules, including the possible link between conformation change and aggregation processes following for example the stresses caused by bioprocessing of materials in the Biopharmaceutical and Food Industries.

7. References

1. Wyatt PJ, in *Laser Light Scattering in Biochemistry* (Harding SE, Sattelle DB and Bloomfield VA eds), Royal Society of Chemistry, Cambridge, pp35-58 (1992),
2. Harding SE, Abdelhameed AS and Morris GA, *Macromol Biosci* (2010, in press),
3. Harding SE, Rowe AJ and Horton JC, *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, Royal Society of Chemistry, Chemistry (1992),
4. Scott DJ, Harding SE and Rowe, AJ, *Analytical Ultracentrifugation. Techniques and Methods*. Royal Society of Chemistry (2005)
5. Smidsrød O and Andresen IL, *Biopolymerkjemi*, Tapir Press, Trondheim (1979?).
6. Harding SE, Vårum KM, Stokke BT, Smidsrød O, *Advances in Carbohydrate Analysis* (C.A. White ed.) **1**:63 (1991).
7. Tanford CA, *Physical Chemistry of Macromolecules* (Wiley & Sons, New York), p? (1961)
8. Rollings JE, in *Laser Light Scattering in Biochemistry* (Harding SE, Sattelle DB and Bloomfield VA eds), Royal Society of Chemistry, Cambridge, pp275-293 (1992)
9. Pavlov GM, Rowe AJ, Harding SE, *Trends Analyt Chem* **16**: 401 (1997)
10. Morris GA, Ang S, Hill SE, Lewis S, Schäfer B, Nobbmann U and Harding SE, *Carbohydr Polym* **71**: 101 (2008)
11. Kawahara K, Ohta K, Myamoto H and Nakamura S, *Carbohydr Polym* **4**:335 (1984)
12. Morris GA, García de la Torre J, Ortega A, Castile J, Smith A and Harding SE, *Food Hydrocoll* **22**:1435 (2008)
13. Bushin SV, Tsvetkov VN, Lysenko JB, Emel'yanov VN, *Vysokomol Soedin* **A23**:2494 (1981)
14. Bohdanecky M, *Macromolecules* **16**:1483 (1980).
15. Yamakawa H and Fujii M, *Macromolecules* **6**:405 (1973).
16. Ortega A and Garcia de la Torre J *Biomacromolecules* **8**:2464 (2007).
17. Patel TR, Morris GA, Garcia de la Torre J, Ortega A, Mischnick P and Harding SE, *Macromol. Biosci* **8**:1108 (2008)
18. Pohl, M, Morris GA, Harding, SE, Heinze T, *Eur Polym J*, **45**:1098 (2009).
19. Patel TR, Morris GA, Ebringerová A, Vodenicarová M, Velebný V, Ortega A, Garcia de la Torre JG, Harding SE, *Carbohydr Polym* **74**:845 (2008)
20. Morris GA, Patel TR, Picout SR, Ross-Murphy SB, Ortega A, Garcia de la Torre J and Harding SE, *Carbohydr Polym* **72**:356 (2008).
21. Kök MS, Abdelhameed AS, Ang S, Morris GA and Harding SE, *Food Hydrocoll* **23**:1910 (2009)

22. Morris GA, Castile J, Smith A, Adams GG and Harding SE, *Carbohydr Polym* **76**: 616 (2009)
23. Ang S, Kogulanathan J, Morris GA, Kök MS, Shewry PR, Tatham AS, Adams GG, Rowe AJ and Harding SE, *Eur Biophys J* **39**:255 (2010).
24. Harding SE *Carbohydr Res* **340**:811 (2005)
25. Harding SE, in Scott DJ, Harding SE and Rowe, AJ, *Analytical Ultracentrifugation. Techniques and Methods*. Royal Society of Chemistry, Cambridge pp468-483 (2005).
26. Harding SE, Horton JC and Colfen H, *Eur Biophys J* **25**:347 (1997)
27. Rholam M and Nicolas P *Biochemistry* **20**:5837 (1981)
28. Harding SE and Rowe AJ, *Int J Biol Macromol*, **4**:357 (1982)
29. Rowe AJ *Biopolymers* **16**:2595 (1977)
30. Harding SE *Biochem J* **189**:359 & corrigenda to vol 189 (1980)
31. Carrasco B, Garcia de la Torre J, Davis KG, Jones S, Athwal D, Walters C, Burton DR and Harding SE, *Biophys Chem* **93**:181 (2001)
32. Lu Y, Longman E, Davis K, Ortega A, Grossmann JG, Michaelsen TE, García de la Torre J and Harding SE *Biophys J* **91**, 1688 (2006)
33. Lu Y, Harding SE, Michaelsen TE, Longman E, Davis KG, Ortega A, Grossmann JG, Sandlie I and Garcia de la Torre J, *Biophys J* **93**: 3733 (2007).
34. Schuck P, in Scott DJ, Harding SE and Rowe AJ (Eds.). *Analytical Ultracentrifugation Techniques and Methods*, Royal Society of Chemistry, Cambridge, pp26-50 (2005)
35. Lu Y, Harding SE, Rowe AJ, Davis KG, Fish B, Varley P, Gee C and Mulot S, *J Pharmaceutic Sci* **97**:948 (2008)
36. Longman E, Harding SE and Marheineke N (2006) *LCGC North America* **24**: 64
37. Serydyuk, I.N., Zaccai, N.R. and Zaccai, J. (2006) *Methods in Molecular Biophysics*, Cambridge University Press.