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Probing hydration and the stability of protein solutions — a colloid science approach

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Abstract

The stability of protein solutions is a topic of much theoretical and practical interest. The theory of Derjaguin, Landau, Verweey and Overbeek (DLVO theory) has long been used by colloid scientists and chemical engineers to define conditions leading to stability of dispersion, but has found very little application by protein chemists. We apply DLVO theory to demonstrate that protein solution stability appears to be closely related to the presence of the hydration layer, rather than to significant inter-molecular net repulsive force. A novel but simple formalism is then developed which indicates, via computation of a newly defined parameter ('site relaxation time'), that estimated 'dwell times' for water molecules on a protein surface are of a magnitude adequate to account for such a role. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Proteins dissolve to form aqueous solutions which have a considerable degree of stability. Indeed, an old but classical distinction has been made in the past between biological macromolecules (especially proteins) which formed the so-called 'lyophilic sols' and other 'colloidal dispersion', for example of silver iodide or dispersed gold, which formed 'lyophobic sols'. The latter were characterised by their limited time stability: on storage, they routinely precipitated. In contrast, protein solutions seemed to have close to unlimited stability in storage, assuming that attack by microorganisms or exposure to 'denaturing' conditions was avoided.

In actual practice, the time stability of protein solutions is far from total. Even under optimal storage conditions, as defined by experience, it is a common observation that gradual aggregation

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often occurs, especially where larger protein assemblies are concerned. This is a topic of considerable importance. As Branch [1] has recently noted 'protein aggregation results in the loss of biological activity and the formation of insoluble deposits represents a serious problem in medicine (e.g. Alzheimers' disease), biotechnology (inclusion body formation) and for the pharmaceutical industry (protein formulation)'. Other areas could easily be added to this list, for example the formation of protein hazes in beverages. Clearly it is unlikely that a single phenomenon is at work here. At the least, the formation of aggregates between partly assembled protein monomers (as in inclusion bodies) is likely to proceed through mechanisms different from those which, for example, limit the shelf life of an acellular, protein vaccine preparation. It is the latter mechanism, involving usually irreversible changes leading to aggregation and possible precipitation, which are the main object of our own interest, and which, from the standpoint of protein chemistry, we are seeking to elucidate.

We must first enquire as to why so many proteins, whose primary structure contains so very many hydrophobic motifs, are highly soluble in aqueous solution? The familiar textbook answer is the 'oil-drop' model, in which hydrophobic groups are buried within the interior, whilst the charged, hydrophilic side-chains are mostly exposed on the surface. The assumption — mostly unstated — is that this must lead to charge repulsion between molecules, thus stabilising the solution (dispersed) state.

Even at the most naïve level, this theory is questionable. The net surface charge on the surface of a typical globular protein is not particularly large (see later), and compares poorly with that found in most hydrophobic sols [2,3]. Yet it is the latter which lack stability. At a more sophisticated level, the concept of net repulsion is inconsistent with the long established view, buttressed by extensive experimental evidence, that even in concentrated solutions protein molecules mostly behave as hard spheres (see a recent review by Minton [4]).

Are we able to define, in an at least semi-quantitative manner, the factors which govern protein solubility and solution stability? Colloid scientists have for decades used the theory of Derjaguin, Landau, Verweey and Overbeek [5–7] to define the stability of dispersions. In this 'DLVO' theory, the net balance of attractive and repulsive potential energies at any point in the separation between simple model particles (or surfaces) is computed, based upon knowledge of parameters including the particle surface potential, the dielectric constant and ionic strength of the medium, and the effective 'Lifhsitz–Hamaker constant' of the particle in that medium. The Lifshitz–Hamaker constant defines the strength of the van der Waals forces, and hence of the particle attraction. DLVO theory is described at length in texts and monographs on colloid science [6,7]. The precise degree of quantitative validity of DLVO theory has long been a matter for some dispute [8,9], to an extent because — at least in its basic form — the theory considers important parameters such as surface potential and dielectric constant to be single-valued and independent of particle separation. Nonetheless, for particle separation distances which are not too small, where the precise location of charges becomes important, DLVO theory offers a simple and reasonably precise approach to the understanding of particle–particle interactions, and their effect on dispersion stability.

The theory has, however, found only limited application, predominantly by chemical engineers, in the understanding of the properties of protein solutions. For example, in recent years Kuehner et al. [10] and Coen et al. [11] have applied it to solutions of protein in strong salt solutions, Bowen and Williams to the estimation of osmotic pressure of electrostatically stabilised dispersions and in generating a model for ultrafiltration of proteins [12,13], Bowen et al. to the prediction of equilibrium constants for ionic exchange of protein [14], MolinaBolivar et al. to the aggregation of protein-coated particles [15] and Müller et al. from the standpoint of structural biology, have described the interaction between purple membrane and a probe tip [16] under a range of ionic conditions, as part of a study involving atomic force microscopy. We have sought to re-visit the use of DLVO theory as applied to particle–par-

ticle interaction in simple globular protein solutions under 'physiological' conditions of ionic strength and pH. We find that it gives a simple yet robust account of some of the factors which determine solution stability, and that it can identify the likely function of the surface 'hydration layer' which is a property largely unique to proteins. The precise meaning of a 'hydration layer' as an entity which contributes to the hydrodynamic particle has, however, traditionally been far from clear. In an attempt to give a more precise meaning to the concept we develop a new formalism, predictive of the conditions under which water molecules can be considered as contributing to such a layer.

2. Theory and results

2.1. The net repulsive potential energies between model spherical protein particles

Proteins dissolve to form aqueous solutions which have a considerable degree of time stability, indeed they possess a very extensive time stability as compared to dispersion of classical 'lyophobic' sols. To what extent is it possible to account for this in terms of the balance of attractive and repulsive potential energies between the individual protein particles? DLVO theory — which is extensively considered elsewhere [6,7] and so needs only be briefly summarised — sees the stability of a dispersion of particles as being determined by the simple algebraic sum of the potential energies leading to repulsion (V_R), which are electrostatic in nature and associated with the diffuse double layer which surrounds charged particles in ionic solutions, and the potential energies leading to attraction (V_A) which arise from 'van der Waals forces', a name given to the sum of permanent and transient dipole interactions. In the latter, the transient (London dispersion) forces predominate. Put at its simplest, dispersion stability requires that the condition $V_R \gg V_A$ be met at all plausible interaction distances. Dispersions of inorganic sols in water do indeed show net repulsive potential energies of the order of 50–100 kT [6,7]. This is routinely associated with the pres-

ence of a significant surface charge, normally associated with the presence of a surface layer of adsorbed ions. This latter has been described as the 'Stern layer' [6,7], and the effective surface charge, as measured by the so-called ζ-potential, considered to lie just slightly (a fraction of an ionic radius) outside of the mid-plane of these adsorbed ions. Such dispersions are normally destabilised by the addition of neutral salt at the level found in biological systems (approx. 100 mM), and double layer calculations confirm that net repulsion is very much reduced under such conditions [6,7]. Whilst (as noted in Section 1) the detailed quantitative application of DLVO theory has been criticised, on grounds which are likely to be sound, there is no doubt that at a descriptive and at least semi-quantitative level this theory has found enormous application over several decades.

The model described above suggests problems in its application to proteins. These have a generally modest level of surface charge, bind only a very limited number of ions from the solvent, and yet are stable under conditions (100 mM salt, minimal surface charge) where DLVO theory, applied naively, would seem to imply otherwise. To seek to shed light on this apparent anomaly, we compute the repulsive and attractive potential energies for a simple model system, namely a spherical protein of diameter 3 nm with a surface charge of 30 mV, a value typical for a globular protein in 100 mM salt at neutral pH. The value assumed for the effective Lifshitz−Hamaker constant is critical to the calculation: we use a recent value for protein in aqueous solution of 0.754×10^{-20} [16]. Other conditions used are as follows.

2.1.1. Repulsive potential energies

Repulsive potential energies have been computed using the approximation of Reerink and Overbeek [6,7], in which the Debye−Hückel approximation is not made, but it is assumed that the total potential at any point is equal to the algebraic sum of all contributing potentials. A particular problem with proteins concerns the radial location of the surface charge and of the 'Outer Helmholtz Plane' (OHP) where the diffuse double layer can be considered to commence. We have begun with the simplest possible assump-

tions. The surface charge, which arises predominantly from intrinsic charge (amino acid side chains) is located co-incident with the spherical surface. This is probably no worse an assumption than the basic assumption of sphericity. We have also assumed the counter-ions to be point charges, making the OHP also co-incident with the spherical surface. A value for the dielectric constant equal to that of bulk water is assumed to apply even at small separations. We later relax these assumptions for both the location of the OHP and the simple distance-invariant dielectric constant, to explore their significance. The repulsive ('Born') element of the Lennard−Jones forces have not been incorporated in our treatment, as they are operative only at very short separation values.

2.1.2. Attractive potential energies

Attractive potential energies have been computed following the original treatment of Hamaker [6,7] for spherical particles and the value for the Lifshitz−Hamaker constant noted above. A consistent assumption has been made that particle separations are small, additionally enabling retardation effects to be neglected.

Fig. 1 shows plots of the values computed for V_R and V_A and for $(V_R − V_A)$ as a function of H (separation distance of the surfaces of the spheres). It is immediately clear that even at its maximal value (at approx. $H = 0.75−0.8$ nm) the net repulsive potential energy is very small indeed, being of the order of < 1 kT. This value is negligibly small in comparison to those which are considered to stabilise dispersion of inorganic sols.

Could this be a problem associated with the simplifying assumptions made in our treatment? To test this we have re-computed the net repulsive potential energy on two alternative further assumptions: (1) that the OHP is located at a radial distance of approximately half of an ionic radius beyond the surface; and (2) that the there is a distance dependent dielectric operational, such that this parameter decays exponentially from the value operative for protein ($= 80$) to that for water ($= 4$) with a decay constant of 0.2 nm.

Fig. 2 illustrates the effects of these further assumptions. Clearly there is a discernible effect in either case, but in no way to a level which would invalidate our basic conclusion: which is that, expressed in terms of kT, the net repulsive
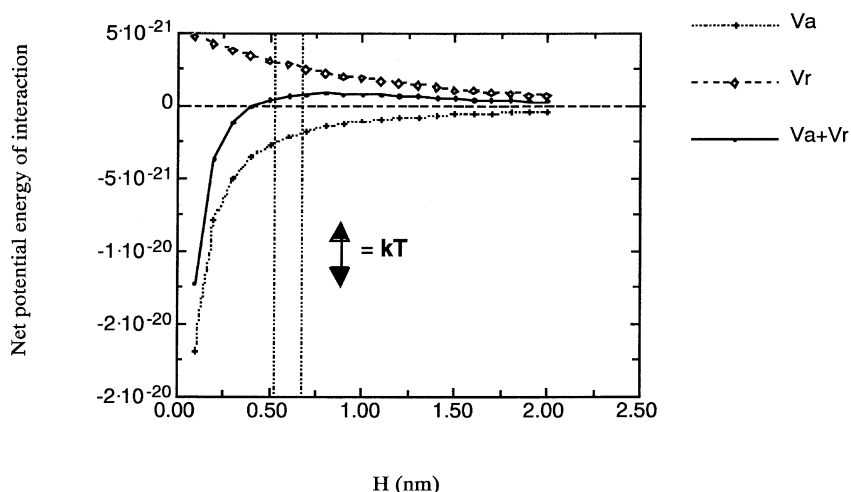

Fig. 1. The net potential energy as a function of inter-particle separation distance (H) for a dispersion of spherical particles of diameter 3 nm and surface charge −30 mV (see text for other conditions and assumptions). The vertical lines indicate the approximate range of location of the (double) hydration layers, considering only a monolayer to be present on each surface. The vertical double arrow represent the range of potential energy = 1 kT.
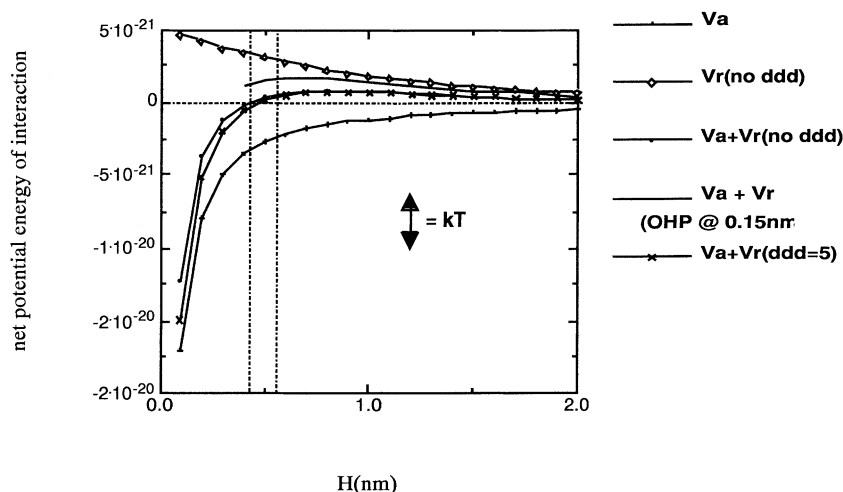
Fig. 2. The net potential energy as a function of inter-particle separation distance (H) for a dispersion of spherical particles (see Fig. 1) for which alternative calculations have been performed, assuming either (a) an outer Helmholtz plane located at approximately half an ionic radius beyond the surface (a constant value for the dielectric constant of the medium has been used in computation of V_R); or (b) a distance-dependent dielectric(for details see text). The vertical lines indicate the approximate range of location of the (double) hydration layers, considering a monolayer to be present on each surface. The vertical double arrow represent the range of potential energy = 1 kT.

potential energy between our model spherical protein particles is negligible in terms of providing an explanation for the stability of protein solutions. This conclusion we have found is also robust in relation to moderate variations in particle diameter or surface potential. As is described below (Section 3), the only simple explanation is that in the context of a close to even balance of attractive and repulsive potential energy at separations which are dictated by the presence of hydration layers on the particles, it is precisely the presence of that hydration layer which is critical for the maintenance of stability of the solution state.

2.2. A novel formalism for the description of the hydration layer of protein

Given that the presence of a hydration layer is critical for the maintenance of stability of the solution state, we are forced to enquire as to precisely what is meant by water molecules — which are clearly not covalently bound to protein — being located in such a layer. There is long-established evidence from hydrodynamics which indicates that the plane of shear extends appreciably beyond the protein surface (see a summary of a wide range of evidence by Squire and Himmel [17]). Various other approaches, especially using NMR, have indicated that the 'dwell time' of water molecules close to the protein surface is extended as compared to the average dwell time at any given locus in free solution. Depending upon the precise form of NMR employed, average values in the range of 0.1 ns (100 ps) upwards have been reported [18,19], and strong indications are currently being given as to the nature of the chemical groups to which the water is transiently attached [20]. These NMR estimates are consistent with estimates computed using molecular dynamics (MD) algorithms, which range from approximately 50 ps for water adjacent to non-polar groups to approximately 100 ps for water adjacent to polar residues on the protein surface [21]. Longer values would of course be expected for any water located in clefts, internalised within the protein, or in any other location with hindered diffusion.

How can we relate these findings to the long-observed hydrodynamic properties of proteins, and indeed of other macromolecular solutes? We wish to suggest a novel way of looking at the issue, which enables us to give a description, both qualitative and quantitative of what we mean when we say that water molecules are 'bound' to protein: it being accepted that all such water molecules are in rapid exchange with water in bulk solution, with in many cases having a rather short dwell time, as illustrated above.

Our basic proposition is a simple one. For a given water molecule, let the mean dwell time (in apposition to some site on the protein surface) be td_w. Recalling that all sites on the surface of a protein are in continuous rotational movement, we can define a mean rotational dwell time for the site, td_s, being the average time which that site spends apposed to a locus equivalent in size to a water molecule. We can then say that if

$$td_w \gg td_s \tag{1}$$

then the water molecule effectively 'rotates' with the protein molecule, and can be said to be 'bound', whilst in the opposite case

$$td_s \gg td_w \tag{2}$$

the water molecule will detach before significant rotational movement has occurred, and will not contribute to any effects arising from a 'bound layer'. If the two dwell times are of similar magnitude, then the water molecule will make a fractional contribution to the hydration layer.

This very simple formalism enables us to define the concept of water molecules being bound into a hydration layer in a way which is amenable to quantitative analysis: since values for td_w can be known (see above) and values for td_s can be estimated for our model from a knowledge of the rotational hydrodynamics of spherical particles. We are not, however, aware of any explicit calculations of this latter parameter, and so have made estimates which can be compared with the empirical estimates for td_w to verify whether the conditions specified in our formalism [Eqs. (1) and (2)] are met.

There is unfortunately no simple way in which we can compute precise values of this (average) site dwell time. The basic information which we have concerning particle motion is the rotational relaxation time, and this is defined in terms of the decay of the average $\cos(\phi)$ with respect to time of an assembly of particles originally oriented with respect to a space axis. However, if we can treat the time of initial decay from an initial perfect orientation with respect to a space axis through a small angle $\delta\phi$ as an approximation to the angular relaxation time, then since a surface area on a sphere defines solid angle we have a way of computing an approximate value for a 'site relaxation time' and hence — by further approximation — the site dwell time. The assumptions made and the approach adopted are summarised below.

2.3. Assumptions

1. The dwell time of a water binding site is defined as the average time during which that site remains apposed to a fixed locus in the surrounding continuum, i.e. its centre does not move during that time by more than a distance D (diameter of site).
2. The information we have available is the relaxation time of the protein (τ).
3. We can approximate the site dwell time (td_s) by an estimate for the site relaxation time (td_r): this is defined as the time for a population of spheres initially perfectly oriented along a fixed axis ($\phi = 0$, $\cos(\phi_0) = 1$) to relax through an angle ($\delta\phi$) equal to the angle subtended by the site. Following custom, we fix the average orientation at infinite time as $\cos(\phi) = 0$.
4. It is the rotational motion of the protein which defines the td_r: translational motion is viewed simply as a statistical distribution in space superimposed on a set of particles in Brownian motion.
5. The angular contribution (steradians) of a single water-binding site can be approximated by 5 Å^2 divided by the protein surface area. For a sphere, this angular contribution is taken to be the same for any single plane.

Whilst for the sake of simplicity we make no allowance for the oligomeric nature of water, it clearly possible that oligomers of water would bind via a single constituent monomer.

Calculation: having computed (τ) in the usual way for spherical proteins of given diameter, vbar $= 0.73$ ml/g, we can find $(t_{r,s})$ from the usual equation

$$\cos(\phi_0 - \delta\phi)/\cos(\phi_0) = \exp[-t/\tau]$$

For $T = 293$ K, we obtain estimates for $(t_{r,s})$ of the order of 2.6 ps (5 nm diameter sphere — approx. $M = 54$ kDa) to 20 ps (10 nm diameter sphere — approx. $M = 430$ kDa). We note that this range of values is appreciably lower than the estimates given (above) for the experimentally determined and computed (MD) dwell times of water molecules in the vicinity of a protein surface. It is of some interest to note that the experimental (NMR) values do actually refer to crowded state, for basic methodological reasons, and hence are relevant to close approach of protein monomers to each other.

Our formalism, which as far as we can ascertain is novel, therefore makes a simple prediction: providing that the dwell time of water molecules adjacent to a protein surface lies in a range greater than approximately 2–20 ps, then the water molecules concerned effectively move with the protein surface within the time scale which would be associated with the movement of protein surface itself. This latter must be determinative of protein–protein interaction, under conditions where this is (other than for the presence of water) energetically favoured. Hence in simple terms, these individual regions of the protein surface are not 'free' of adjacently located water for long enough for protein–protein interaction to occur. This simple treatment ignores, among other things, the kinetics of the events treated, but it does provide an explanation for known facts and suggests problems which need to be tackled. One interesting consequence of the range of site dwell times computed above is that as the latter are numerically larger for larger particles, then (all other factors being equal) it is predicted that solutions of larger particles will show less stability with respect to aggregation than solutions of smaller particles. This accords with experience.

3. Discussion

The calculations of the net DLVO potential between particles with a size, charge and Lifshitz–Hamaker constant typical of globular proteins shows that this potential can be of the order of kT only, for separations of approximately 0.5 nm and upwards. At lower separation distances a much more appreciable net overall attraction is predicted. These conclusions are robust with respect to location of OHP, distance-dependence of the solvent dielectric constant or (within modest margins) the precise size or surface potential of the protein particle. A rather similar conclusion — albeit for a very different system — can be reached by inspection of the data published by Müller et al. [17]. The net potential energy between protein particles cannot therefore be responsible in any direct sense for 'keeping protein molecules apart in solution'. The latter phenomenon must be a property of the presence of the hydration layer, an effect long known in colloid science, and rather variably referred to as the 'hydration force' or 'steric force'. Of course, some individual proteins will have a very large surface potential, either intrinsically or under specific conditions, such as the presence of bound multi-valent anions. A significant net DLVO potential will then be operative. But it is likely that for many globular proteins under physiological conditions of pH and ionic strength our conclusion holds.

As noted earlier, the conclusion is in fact implicit in much published work on proteins in solution. The concept of the 'hard-sphere' [22] model of proteins in crowded solutions, reviewed by Minton [4], relates closely to the point which is now being made, since a significant net DLVO potential energy at separation distances which actually exist is plainly incompatible with hard-sphere behaviour. Under lower concentration conditions, the ability of the excluded volume

term to explain the so-called 'non-ideality' term (2nd virial coefficient) in the c-dependence of the chemical potential (in osmotic pressure or sedimentation equilibrium) rests upon the same assumptions. Beyond the hydration layer, there is (again we stress, under physiological conditions and for many but not all proteins) very little net repulsive potential energy. Within separation distances below approximately 0.5−0.6 nm, it is the presence of 'bound' water, for most practical purposes alone, which keeps individual protein molecules apart, rather than allowing them to fall into a deep energy minimum (i.e. aggregating). We thus see that the use of DLVO theory enables us to understand a broad spectrum of the solution properties of proteins, provided that the presence of a hydration layer of approximately 0.5−0.6 nm in thickness is allowed for.

Yet, the question is often asked as to how water molecules, whose presence on the protein surface is so very transient, can behave in this manner? We believe that our newly developed formalism offers a simple explanation. Water molecules must dwell on a locus ('site') on the protein surface for significantly longer than it takes for that site to move to a new location in space, if they are to be considered as 'bound' in an effective sense. It is not necessary that the water molecules should dwell for times in any way comparable to the rotary relaxation time of the protein: only that they should dwell for longer than the site relaxation time. Experimental evidence suggests that such is the case.

Nonetheless, kinetic energy is associated with the approach of one protein molecule to another, and there will be a finite — and in principle calculable-activation energy associated with the displacement of water bound in our newly defined sense. Hence the stability conferred upon protein solutions by the hydration layer as defined in our new formalism must be finite. Eventually, at least for proteins lacking a very large intrinsic charge, aggregation will occur. We can predict that conditions which favour the presence of bound water will favour the stability of protein solutions, an issue of some importance in relation to protein formulation chemistry. In further work we have

developed these concepts both theoretically and experimentally, and we demonstrate that the level of hydration of a given protein, so often treated in experimental work, must be considered as a variable with respect to a range of ambient physical conditions [23,24].

References

[1] H. Blanch, 2000, http://wiffle.cchem.berkeley.edu/ ~ hwbgrp/research_interests1.htm

[2] C. Tanford, Phys. Chem. Macromol. 561-572 (1963) 457−525.

[3] K.S. Schmitz, An Introduction to Dynamic Light Scattering by Macromolecules, Academic Press, 1990, p. 264.

[4] A.P. Minton, Influence of excluded volume upon macromolecular structure and associations in 'crowded' media, Curr. Opin. Biotechnol. 8 (1997) 65−69.

[5] D. Grier, 2000, http://griergroup.uchicago.edu/ ~ grier/leshouches2/node6.html

[6] D.J. Shaw, Introduction to Colloid & Surface Chemistry, Butterworth & Co, 1980.

[7] R.J. Hunter, Foundations of Colloid Science, Clarendon Press, Oxford, 1987.

[8] B.A. Pethica, Adsorption equilibria in surface force balance studies, Colloids Surf. A 105 (1995) 257−264.

[9] D.G. Hall, B.A. Pethica, The thermodynamics of parallel flat plate condensers, Proc. R. Soc. Lond. A 364 (1978) 457−472.

[10] D.E. Kuehner, H.W. Blanch, J.M. Prausnitz, Salt-induced protein precipitation: Phase equilibria from an equation of state, Fluid Phase Equilibrium 116 (1996) 140−147.

[11] C.J. Coen, H.W. Blanch, M. Praunitz, Salting out of aqueous proteins: Phase equilibria and intermolecular potentials, AIChE J. 41 (1995) 996−1004.

[12] P.M. Williams, W.R. Bowen, The osmotic pressure of electrostatically stabilised colloidal dispersions, J. Colloid Int. Sci. 184 (1996) 241−250.

[13] W.R. Bowen, P.M. Williams, Dynamics ultrafiltration model for proteins: A colloid interaction approach, Biotech. Bioeng. 50 (1996) 125−135.

[14] W.R. Bowen, L-C. Pan, A.O. Sharif, Predicting equilibrium constants for ion exchange of proteins — a colloid science approach, Colloids Surf. 143 (1998) 117−131.

[15] J.A. Molina-Bolivar, F. Galisteo-González, R. Hidalgo-Alvarez, The role played by hydration forces in the stability of protein-coated particles: non-classical DLVO behaviour, Colloids & Surfaces B, Biointerfaces 14 (1999) 3−17.

[16] D.J. Müller, D. Fotiadis, S. Scheuring, S.A. Müller, A. Engel, Electrostatically balanced subnanometer imaging of biological specimens by atomic force microscope, Biophys. J. 76 (1999) 1101−1111.

[17] P.G. Squire, M. Himmel, Hydrodynamics and protein hydration, Arch. Biochem. Biophys. 196 (1979) 165−177.

[18] R. Kimmick, Self-diffusion and NMR relaxation in hydration shells of proteins, Proc. Ital. Phys. Soc. 43 (1993) 157−164.

[19] G. Otting, NMR studies of water bound to biological macromolecules, J. Prog. NMR Spectrosc. 31 (1997) 259−285.

[20] O. Bornet, M. Guerlesquin, Piotto, Evaluation of slaved pulses to study protein hydration, J. Magnetic Resonance 138 (1999), 107−114.

[21] M Levitt, R. Sharon, Accurate simulation of protein dynamics in solution, Proc. Natl. Acad. Sci. USA 85 (1988) 7557−7561.

[22] K.S. Schmitz, An Introduction to Dynamic Light Scattering by Macromolecules, Academic Press, 1990, pp. 192−195.

[23] N. Errington, A.K. Pancholi, K. Klage, A.J. Rowe, Volume-temperature dispersion (VTD) of proteins: a new approach combined with dynamic light scattering indicates that hydration is a temperature-related variable (2001) (submitted).

[24] N. Errington, P. Mistry, A.J. Rowe, Protein hydration varies with protein crowding and with applied pressure: a sedimentation velocity study, Prog. Colloid Polym. Sci. (2001) (submitted).