Scientific Paper

Osmometry and Small-Angle X-Ray Scattering of Human Serum Albumin in Buffer Solutions†

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† Dedicated to the memory of Prof. Dr. Davorin Dolar

Abstract

Osmotic pressure measurements of human serum albumin (HSA) dissolved in 0.15M NaCl and in 0.1 M phosphate buffer at three values of pH are reported as a function of protein concentration. Experimentally obtained osmotic coefficients were well below one, indicating large deviations from ideality even for dilute protein solutions. This effect could be ascribed to formation of dimers or even higher aggregates in protein solution. The membrane equilibria measurements of osmotic pressures were therefore complemented by the small-angle X-ray scattering (SAXS) results that show no evidence of protein aggregates in solutions, leaving the question why is the osmotic coefficient so small unanswered.

Key words: human serum albumin, osmotic coefficient, small-angle X-ray scattering, indirect Fourier transformation

1. Introduction

Understanding the thermodynamics and structure of aqueous solutions of proteins is of substantial importance for the biological sciences as well as for related technologies. Separation of proteins from mixtures and purification are among the most important techniques which require a detailed knowledge of protein behavior in aqueous solutions.

Aqueous protein solutions are multi-component systems that contain protein molecules, low molecular weight electrolyte and water, and display a complex behavior, which is still far from being completely understood. Equilibrium thermodynamic properties indicate strong deviations from the ideal behavior at various conditions including pH, concentration and type of added electrolyte.[1,2] Understanding of protein behavior in aqueous systems requires knowledge of the principal forces acting between the protein molecules;[1,3,4] these forces are modulated by the presence of a multi-component solvent. The interaction between protein molecules can be investigated by X-ray and neutron scattering, by hydrodynamic methods or by studying colligative properties of the solutions. Among the latter, Donnan pressure measurements are traditionally an important source of information about protein-protein interactions.[5-7]

2. Experimental and Methods

2.1. Materials

NaCl, KH_2PO_4 and K_2HPO_4 were purchased from Kemika (Zagreb, Croatia). Human serum albumin (HSA) was purchased from Sigma Chemical Co., (St. Louis, MO, USA) product number A8763, lot 111K7612. HSA was dried at T = 40 °C for 24 hours in presence of P_2O_5. Water content in original samples was found to vary from 3.7 – 5.4%. NaCl, KH_2PO_4 and K_2HPO_4 were dried for two hours at 120.5°C. 0.1M phosphate buffer solutions and 0.15M NaCl solutions (pH=4.0, 5.4, and 8.0) were prepared by adding a weighted amount of solvent to weighted amount of HSA. All solutions were prepared gravimetrically and the densities of solutions needed to calculate molar concentrations were determined by DMA 602 digital density meter (Anton Paar, Graz, Austria).

2.2. Osmotic Pressure Measurements

Equilibrium between the solution containing protein molecules and the same buffer solution without the protein is established across a semi-permeable cellulose-acetate membrane which prevents protein molecules to diffuse into the compartment with protein-free solution. This situation is also known as a Donnan equilibrium. A pressure difference was measured utilizing a Knauer membrane osmometer

(Model 73101). The electro-chemical potential μ of all charged ionic species but protein must be the same in both compartments α and β, as well as the chemical potential of solvent μ_w:

$$\tilde{\mu}_i \equiv \mu_i^{0,(\alpha)} + kT \ln \rho_i^{(\alpha)} + z_i e_0 \Psi^{(\alpha)} = \\ = \mu_i^{0,(\beta)} + kT \ln \rho_i^{(\beta)} + z_i e_0 \Psi^{(\beta)} \\ \mu_w^{(\alpha)} = \mu_w^{(\beta)} \quad (1)$$

Proteins are polyampholytes, being able to possess both positive and negative charges simultaneously, and can be treated as weak polyelectrolytes. There has been several attempts to treat such systems theoretically.[8-10] Taking into account the ability of small ions to cross the semi-permeable membrane, the Donnan pressure Π of a protein solution is related to its molality m_p and the net charge Z_{net} by the following equation:[11]

$$\Pi = RT\phi(m_p + \frac{(Z_{net} m_p)^2}{4m_{salt}})\rho \quad (2)$$

where T is the absolute temperature, R the gas constant, m_{salt} molality of buffer, ϕ osmotic coefficient, and ρ density of water. However, analysis of osmotic pressure experimental data is not always straightforward and often requires additional assumptions.

A semi-empirical way of analyzing experimental results for the osmotic pressure has been proposed by Fullerton and Zimmerman.[12] Their approach is based on observations that hydration of proteins depends on parameters such as pH, temperature, and the concentration of added electrolyte. In the analysis of Fullerton and coworkers[12] it is assumed that a fixed amount of water is bound to a protein and therefore not accessible to other molecules. Non-ideal behavior is assumed to be caused by this inaccessible water, $m_w - m_w^c$, which is proportional to the mass of the protein present in solution, m_p. The corrected solvent mass to be included in the equation for osmotic pressure is, $m_w^c = m_w - I\, m_p$, where I is an empirical interaction parameter. At low solute concentration it is correct to use molality instead of molar concentrations[5], thus having the expression $\Pi = mRT$ instead of the van't Hoff's one. With this assumption the corrected osmotic pressure equation reads:[12]

$$\Pi = \frac{m_p RT\rho}{m_w^c M_p} \quad (3)$$

and can be rewritten in the form:

$$\frac{m_w}{m_w^c} = \frac{RT\rho}{M_p \Pi} + I \quad (4)$$

The equations above contain two empirical parameters, i.e. M_p and I which can be obtained by fitting the experimental data. By plotting the ratio m_w/m_p vs. Π, we obtain a straight line with an intercept I and the slope equal to $RT/\rho M_p$. Note that M_p is the "molar mass" of a protein as seen by this particular method, and that the value of M_p reflects various interactions. For an ideal system (an absence of interactions) the interaction parameter I vanishes, while the value of M_p should coincide with the true molar mass (66.7 kDa in this case). Note that I, defined as a mass of water bound per mass of protein, is an empirical parameter which is obtained by fitting the experimental data.

2.3. Small-Angle X-Ray Scattering Measurements

Small-Angle X-Ray Scattering spectra were measured with a so-called "Kratky compact camera" (Anton Paar KG, Graz, Austria),[13] which was attached to a conventional X-ray generator Krislalloflex 760 (Bruker AXS GmbH, Karlsruhe, Germany) equipped with a sealed X-ray tube (CuK_α X-rays with a wavelength $\lambda = 0.154$ nm) operating at 35 kV and 35 mA. The samples were measured in a standard quartz capillary with an outer diameter of 1 mm and wall thickness of 10 μ m. The scattered X-ray intensities were detected with the position sensitive detector PSD ASA (M. Braun GmbH, Garching, Germany) in the small-angle regime of scattering vectors $0.1 < q < 5$ nm^{-1} where $q = 4\pi/\lambda \cdot \sin(\vartheta/2)$, ϑ being the scattering angle. The measuring times of 20 hours yielded sufficient measuring statistic. Scattering data were first corrected for the absorption of the X-rays in the sample, further corrected for the empty capillary and solvent scattering and finally put on absolute scale using water as a secondary standard[14] (program PDH; PCG software, Institute of Chemistry, Graz, Austria). The scattering intensities obtained in this way were still experimentally smeared because of the finite dimensions of the primary beam.[15]

2.4. Indirect Fourier Transformation of SAXS data

Some of the measured small-angle X-ray scattering spectra were evaluated utilizing the Indirect Fourier Transformation method IFT.[16,17] IFT is a completely model free method that can only be successfully applied for the systems where interparticle interactions can be neglected. This procedure yields the pair-distance distribution function $p(r)$ that serves as a tool for the determination of the scattering particles' geometry. In this approximation the scattering intensity from one scattering particle $I_1(q)$ can be written as the Fourier transformation of the $p(r)$ function:[16,17]

$$I_1(q) = 4\pi \int_0^\infty p(r) \frac{\sin(qr)}{qr} dr \quad (5)$$

where r is the distance between two scattering centers within the particle. In the case of homogeneous particles the $p(r)$ function directly represents a histogram of distances inside the scattering particle.[18,19] At the distances r bigger than the maximum dimension of particle the $p(r)$ function acquires the value of zero. This provides a useful tool for the determination of

particle's maximum dimension. Furthermore, from the functional form of this function the type of scattering particles' symmetry can be deduced.

3. Results and Discussion

In our previous study it was already shown that the HSA without the fatty acids (HSA1) exists as solvatized dimers in water solutions, but the HSA with the fatty acids (HSA2) was found as monomers in water solutions.[2] In the present study the HSA2 phosphate buffer solutions were investigated into more details. The measurements of the osmotic pressure yielded surprising results. Namely, the obtained osmotic coefficients of the HSA2 phosphate buffer solutions were found to be unexpectedly low. Trying to investigate this phenomenon further the SAXS measurements of the HSA2 samples in a concentration and pH series were performed and analyzed.

3.1. Osmotic Pressure

Deviations from the ideal behavior are often described in terms of an osmotic coefficient ϕ ($\phi = \Pi/\Pi_{id}$, where Π_{id} is the ideal osmotic pressure). In Figure 1, the osmotic pressure of solutions of HSA2 in 0.15 M NaCl are shown at three different pH values. Osmotic pressure data were fitted using the Eq. 2. to obtain protein's net charge Z_{net} and the osmotic coefficient of the solution ϕ. At pH=4.0, the net charge is 11.8 and the osmotic coefficient 0.75, at pH=5.4 Z_{net} =15.4 and ϕ = 0.80, and at pH=8.0 Z_{net} =18.9 and ϕ = 0.77. Net charge values are in a reasonably good agreement with those found in Ref. 3. The model used here to analyze the experimental data assumes i) that protein molecules do not form dimers or even higher aggregates, ii) activity coefficients of all species are unity, and iii) Z_{net} is constant at all conditions studied. In addition, the Eq. 2. is only valid for 1:1 electrolyte/buffer (NaCl in this case), while for the phosphate buffer (see Figure 2), where a unique mixture of mono-, di-, and even tri-valent ions are present in the solution at a given pH, it may give incorrect net charge and osmotic coefficient.

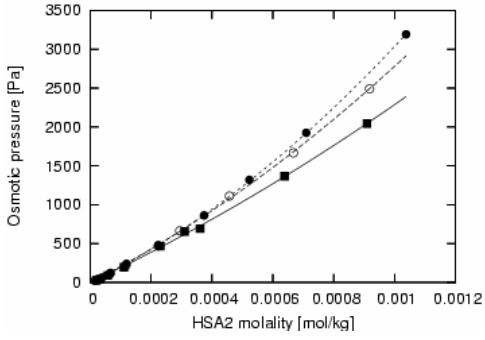

Figure 1. Osmotic pressure as a function of protein concentration for solutions with pH 4.0 (squares; full line), 5.4 (open circles; dashed line) and 8.0 (filled circles; dotted line). Concentration of the NaCl was 0.15M.

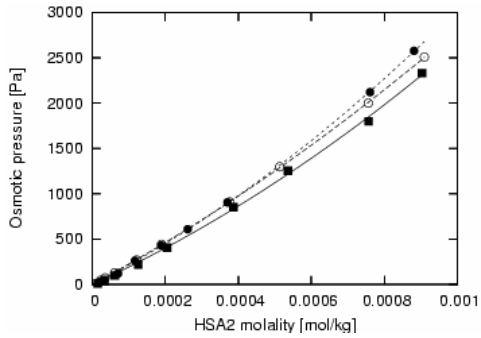

Figure 2. Osmotic pressure as a function of protein concentration for solutions with pH 4.0 (squares; full line), 5.4 (open circles; dashed line) and 8.0 (filled circles; dotted line). Concentration of the phosphate buffer was 0.10 M.

Values of the osmotic coefficients obtained by fitting Eq. 2. to the experimental data are around 0.75, which is well below unity throughout the whole concentration range. For strong linear polyelectrolytes the osmotic coefficients are reported elsewhere[20] to be even smaller – around 0.2. Such small values of the osmotic coefficient are in these cases successfully explained in terms of Manning's ion condensation theory.[21] But for proteins, which are weak polyelectrolytes, this approach cannot be used. The values of net charge are namely different from protein's bare charge, which means that a fraction of counterions (or other ions present in a solution) are strongly bonded to the protein's binding sites and thus contribute to its apparent molar mass as described below.

Following the Fullerton's semi-empirical analysis, the apparent molar mass of the protein and the interaction parameter I were calculated. The m_w/m_p ratios of the HSA2 solution at three different values of pH in 0.1 M phosphate buffer and in 0.15 M NaCl are shown in Figure 3 and Figure 4, respectively. The symbols represent measurements and the lines are the best fits provided by equation (4). The values of M_p, obtained on the basis of results shown in Figure 3 are 108, 91, and 77.3 kDa, for pH values of 4.0, 5.4, and 8.0, respectively. Note that these values are considerably higher than the monomer value (66.7 kDa). The values of I obtained in the same calculation are 10, 9, and 14.7, for pH values of 4.0, 5.4, and 8.0, respectively. Results for m_w/m_p and I in 0.15 M NaCl are given in Table 1.

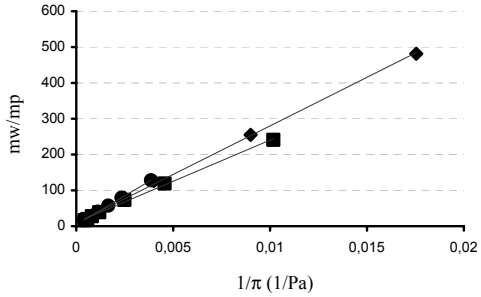

Figure 3. Ratio m_w/m_p as a function of the reciprocal of the osmotic pressure for HSA2 solutions in 0.1 M phosphate buffer at pH 4.0 (circles), 5.4 (diamonds) and 8.0 (triangles).

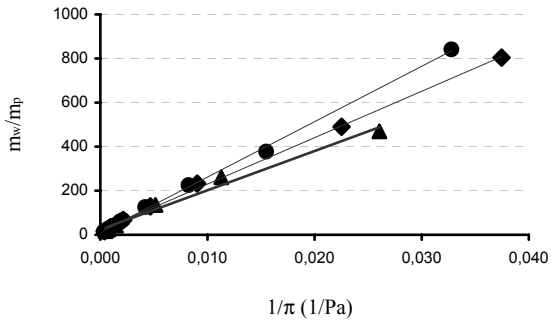

Figure 4. Ratio m_w/m_p as a function of the reciprocal of the osmotic pressure for HSA2 solutions in 0.15 M NaCl. Notation as for Fig.3.

Table 1. Values of the ratio m_w/m_p and I for HSA2 in two different solvents at three pH values.

Solvent	pH	M_p (kDa)	I (mL/g)
0.1M phosphate buffer	5.4	91 (1 ± 0.011)	9 (1 ± 0.24)
	4.0	108 (1 ± 0.026)	10 (1 ± 0.24)
	8.0	77.3 (1 ± 0.002)	14.7 (1 ± 0.011)
0.15 M NaCl	5.4	118 (1 ± 0.015)	20 (1 ± 0.24)
	4.0	139 (1 ± 0.064)	24 (1 ± 0.56)
	8.0	99 (1 ± 0.013)	11 (1 ± 0.39)

3.2. Small-Angle X-Ray Scattering Intensities

The results of the osmotic pressure measurements on the HSA with the fatty acids (HSA2) were complemented with the small-angle X-ray scattering measurements. SAXS measurements of the HSA2 solutions in a phosphate buffer were first performed in a concentration series at constant pH of 4.0, 5.4 and 8.0. The resulting SAXS spectra on the absolute scale are shown in Figure 5a, 5b and 5c, respectively. One can observe that the samples with the lowest concentrations exert somewhat worse measuring statistics, which is due to the small concentration of the scattering particles that enables only a weak excess scattering. Interestingly, when the spectra are normalized to the concentration as depicted in Figure 6 the curves nicely coincide and as such indicate that the geometry of the scattering particles does not change with the concentration considerably. This feature is confirmed with the IFT evaluation results shown in Figure 7 that were obtained for the samples at pH of 5.4. The latter pH value was chosen because these samples are considered to be at the iso-electric point; meaning that the net charge on the HSA2 molecules is zero. In this case the interparticle interactions are minimized and the use of IFT procedure that neglects the interparticle correlations is therefore justified. The resulting pair-distance distribution functions $p(r)$ are shown in Figure 7. Their characteristic functional form indicates that the scattering particles are more or less homogeneous and adopt the ellipsoidal shape. The maximum dimensions of these HSA2 scattering particles

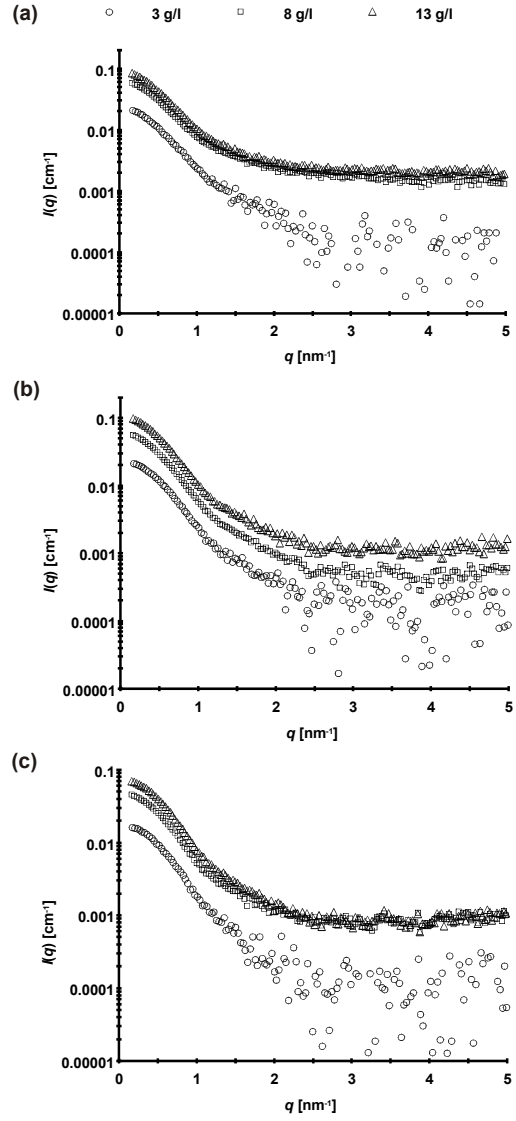

Figure 5. Experimental SAXS intensities of the HSA2 solutions on absolute scale at 25°C and pH value of (a) 4.0, (b) 5.4 (iso-electric point) and (c) 8.0. The legend indicates concentration of HSA2 in g/L.

are around 16 nm, which is in good agreement with the value obtained for HSA2 in water (~15 nm).[2] These results therefore confirm that HSA2 exists in monomer form also in the phosphate buffer solutions that were the topic of the present study. Furthermore, when the $p(r)$ functions from Figure 7a are normalized to the unit concentration (see Figure 7b) they practically coincide and therefore confirm that the scattering particles indeed do not change with concentration. Similarly, the desmeared scattering curves that represent the actual scattering curves on an absolute scale nicely coincide (see the inset in the Figure 7). This confirmation of the HSA2 monomers in the studied buffer solutions leaves the cause for the corresponding relatively low osmotic coefficients reported in the previous chapter still unresolved.

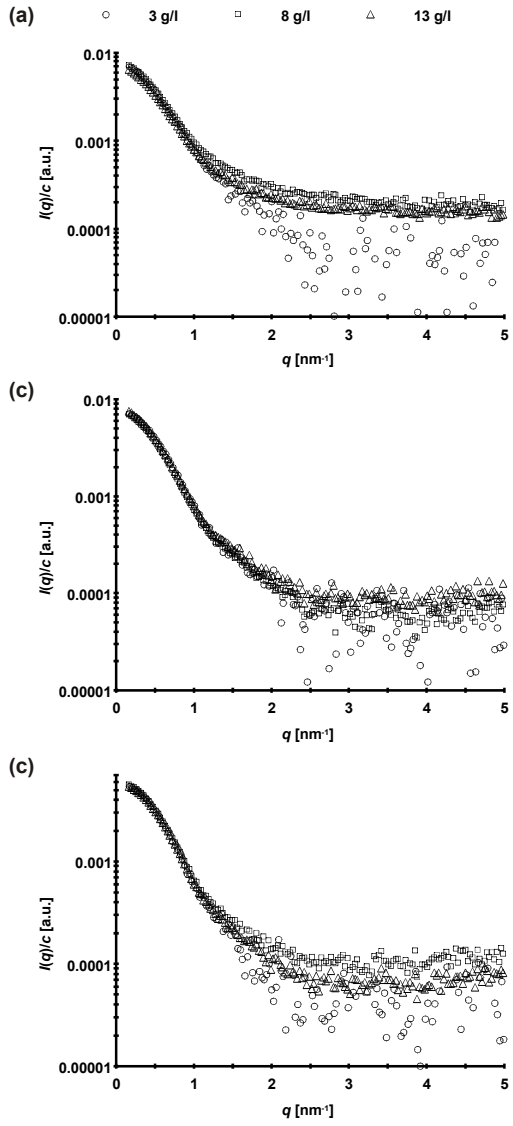

Figure 6. Experimental SAXS intensities of the HSA2 solutions normalized to the concentration at 25°C and pH value of (a) 4.0, (b) 5.4 (iso-electric point) and (c) 8.0. The legend indicates concentration of HSA2 in g/L.

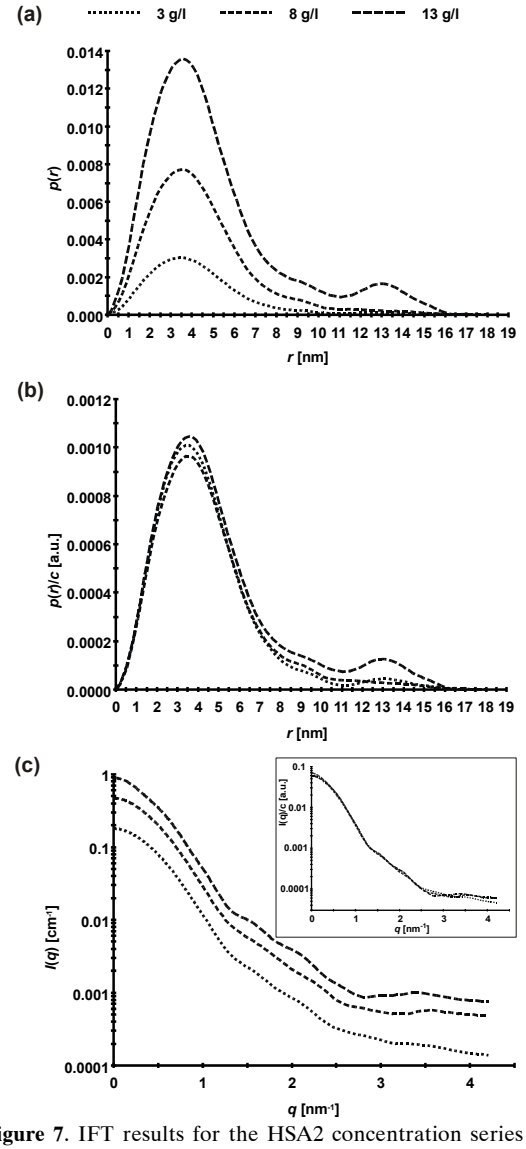

Figure 7. IFT results for the HSA2 concentration series at pH =5.4 from the Figure 5b and Figure 6b: (a) $p(r)$ functions, (b) $p(r)$ functions normalized to the concentration and (c) the resulting desmeared small-angle X-ray scattering curves. Inset: desmeared scattering curves normalized to the concentration.

It is interesting to interpret some of these SAXS data also in a pH series at constant concentration of HSA2 (e.g. 8 g/l). These scattering curves are shown in Figure 8 and are on an arbitrary scale in order to easier observe the onset of the interparticle interactions with changing the pH value from the pH of the iso-electric point. Namely, when the pH moves away from the iso-electric point the net positive or negative charge is exerted on the HSA2 molecule causing more pronounced interparticle interactions. These increasing repulsive liquid-type interactions are expressed in a decreased forward scattering at very small values of the scattering vector q.

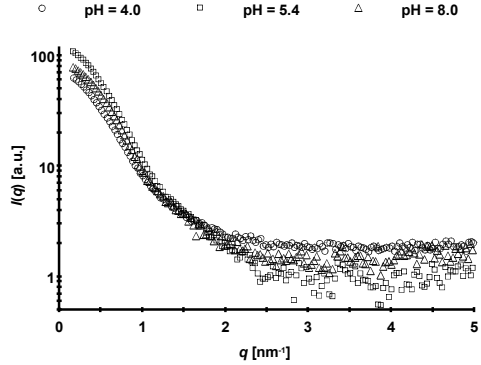

Figure 8. Experimental SAXS intensities of the HSA2 solutions (8 g/l) at 25°C and different pH values indicated in a legend. The curves are shown on an arbitrary scale in order to easier observe the onset of the interparticle interactions.

4. Conclusions

Thermodynamic properties of aqueous protein solutions are governed by complex interactions among all species in the solution. Both non-electrostatic and electrostatic forces contribute to the non-ideality of these solutions. The experimental data for HSA2 suggest strong deviations from the ideal behavior; the values of the osmotic coefficient for dilute solutions being considerably below unity. This result indicates presence of strong attractive forces between the protein molecules, small ions and solvent. Protein's apparent molar mass obtained by Fullerton's semi-empirical analysis, namely suggests strong hydration. Adsorbed ions can also give rise to the protein's apparent molar mass. As a check for other mechanisms like protein aggregation which can lower the osmotic coefficients, SAXS measurements were performed. However, SAXS results suggest that there are no dimers or even higher aggregates present in solutions of HSA2 under the conditions studied in the present work. The latter results are in agreement with the findings reported previously.[2]

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6. References and Notes

1. J. Wu, J. M. Prausnitz, *Fluid Phase Equilibria* **1999**, *155*, 139–154.
2. J. Reščič, V. Vlachy, A. Jamnik, O. Glatter, *J. Colloid Interf. Sci.* **2001**, *239*, 49–57.
3. Y. U. Moon, R. A. Curtis, A. Anderson, H. W. Blanch, J. M. Prausnitz, *J. Solution Chem.* **2000**, *29*, 699–717.
4. F. W. Tavares, D. Bratko, H. W. Blanch, J. M. Prausnitz, *J. Phys. Chem. B* **2004**, *108*, 9228–9235.
5. V. L. Vilker, C. K. Colton, K. A. Smith, *J. Colloid Interf. Sci.* **1981**, *79*, 548–566.
6. C. A. Haynes, K. A. Tamura, R. H. Körfer, H. W. Blanch, J. M. Prausnitz, *J. Phys. Chem.-US* **1992**, *96*, 905–912.
7. K. M. Kanal, G. D. Fullerton, I. L. Cameron, *Biophys. J.* **1994**, *66*, 153–160.
8. V. Vlachy, J. M. Prausnitz, *J. Phys. Chem.-US* **1992**, *96*, 6465–6469.
9. M. Druchok, Y. V. Kalyuzhnyi, J. Reščič, V. Vlachy, *J. Chem. Phys.* **2006**, *124*, 114902.
10. Jiménez-Ángeles, M. Lozada-Cassou, *J. Phys. Chem. B* **2004**, *108*, 1719–1730.
11. N. Fogh-Andersen, P. J. Bjerrum, O. Siggard-Andersen, *Clin. Chem.* **1993**, *39*, 48–52.
12. G. D. Fullerton, R. J. Zimmerman, C. Cantu, I. L. Cameron, *Biochem. Cell Biol.* **1992**, *70*, 1325–1331.
13. O. Kratky, H. Stabinger, *Colloid Polym. Sci.* **1984**, *262*, 345–360.
14. D. Orthaber, A. Bergmann, O. Glatter, *J. Appl. Crystallogr.* **2000**, *33*, 218–225.
15. O. Glatter, in: O. Glatter, O. Kratky (Eds.): Small Angle X-Ray Scattering, Academic Press Inc. London Ltd., London, **1983**, pp. 119–165.
16. O. Glatter, *Acta Phys. Austriaca* **1977**, *47*, 83–102.
17. O. Glatter, *J. Appl. Crystallogr.* **1977**, *10*, 415–421.
18. O. Glatter, in: O. Glatter, O. Kratky (Eds.): Small Angle X-Ray Scattering, Academic Press Inc. London Ltd., London, **1983**, pp. 167–196.
19. O. Glatter, in: P. Lindner, T. Zemb (Eds.): Neutron, X-rays and Light: Scattering Methods Applied to Soft Condensed Matter, Elsevier: North Holland, Amsterdam, **2002**.
20. H. Dautzenberg, W. Jaeger, J. Kötz, B. Philipp, C. Seidel, D. Stcsherbina *Polyelectrolites: Formation, Characterisation and Application*; Hanser: Munich, **1994**.
21. G. S. Manning, *J. Chem. Phys.* **1969**, *51*, 924.

Povzetek

V predloženem delu so zbrani rezultati merjenj osmoznega tlaka raztopin proteina človeškega serumskega ablmina v 0,15 M NaCl in v 0,1 M fosfatnem pufrupri treh različnih vrednostih pH, in sicer kot funkcija koncentracije proteina. Eksperimentalno določeni osmozni koeficienti so znatno nižji od 1, kar nakazuje na velika odstopanja od idealnosti tudi pri razredčenih raztopinah proteina. Eden izmed možnih vzrokov za tako velika odstopanja je nastanek dimerov ali celo večjih agregatov. To domnevo smo preverili z uporabo metode ozkokotnega sipanja rentgenske svetlobe na vzorcih raztopin proteina, vendar obstoja dimerov nismo dokazali. Vprašanje, zakaj je osmozni koeficient raztopin človeškega serumskega albumina nizek, tako ostaja nepojasnjeno.