Osmotic pressures of aqueous bovine serum albumin solutions at high ionic strength

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Received 14 July 1998; accepted 2 October 1998

Abstract

Using osmometers similar to those described by Vilker et al. [V.L. Vilker, C.K. Colton, K.A. Smith, J. Colloid and Interface Sci. 79 (1981) 548–566] and Amos et al. [D.A. Amos, C.J. Radke, S. Lynn, J. Phys. Chem. B 102 (1998) 2739–2753], osmotic pressures of aqueous bovine serum albumin (BSA) solutions were measured at three pH (4.5, 5.4, 7.4) and at different sodium chloride concentrations (from 1 to 5 M). Experimental data were compared with results calculated from two van der Waals-type models using a potential of mean force including hard-sphere and double-layer repulsions, and van der Waals attraction. In both models, the Carnahan–Starling equation of state represents the contribution of the hard-sphere repulsion to the osmotic pressure. Van der Waals attraction and double-layer repulsion are represented by their contributions to the osmotic second virial coefficient in the first model, or in the second, to the random-phase-approximation (RPA). Although both models give a semi-quantitative description of the osmotic pressures of BSA solutions at various conditions, the first model represents the data slightly better than the second, using the same number of adjustable parameters. Further improvement of modeling requires a more accurate potential of mean force for protein molecules in salt solution. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Osmotic pressure; Proteins; RPA theory

1. Introduction

Industrial-scale production of biological products often requires a selective, non-denaturing purification method. Salt-induced precipitation has been successfully applied for separating proteins in downstream processing [1,2]. For efficient design of a protein separation process, good understanding...
of the phase behavior of proteins in aqueous electrolyte solutions is helpful. A reliable molecular thermodynamic model for this purpose relies on an accurate potential of mean force between protein molecules at various solution conditions. However, current understanding is limited to how proteins interact in aqueous electrolyte solutions, not only because of the complicated molecular structure of proteins, but also because of the complex many-body effects of small ions and water molecules.

Interactions between proteins in an aqueous solution can be indirectly measured by light-scattering (low-angle laser light scattering and dynamic light scattering), by osmotic-pressure, and by hydrodynamic methods (diffusion, sedimentation and viscosity) [3–8]. In this work, we are interested in osmotic-pressure measurement. Osmotic-pressure data at low protein concentrations can be used to determine the protein molecular weight and the second virial coefficient that is closely related to the potential of mean force; at high protein concentrations, osmotic-pressure data may provide valuable information to test the theoretical models for describing the properties of aqueous protein solutions. Two disadvantages of osmometry are poor accuracy at very low protein concentrations (where low-angle light scattering is better) and slow data acquisition.

This work discusses construction and use of two identical membrane osmometers similar to those reported by Vilker et al. [9], and by Amos et al. [10]. Our osmometers were tested by repeating some of Vilker’s osmotic-pressure results for aqueous Bovine Serum Albumin (BSA) solutions at low sodium chloride concentration. Osmotic pressures of BSA solutions at high sodium chloride concentrations were measured at three pH (4.5, 5.4 and 7.4). High salt-concentration conditions are of interest because they are used in industrial protein-separation processes. Previously published osmotic-pressure measurements of BSA solutions were restricted to low salt concentrations; the sodium chloride concentrations in this work range from 1.0 to 5.0 M. Sodium chloride is used because interactions between salt ions and protein molecules are relatively easy to interpret.

The experimental results were compared with those calculated from two van der Waals type models for protein solutions. In each model, the potential of mean force between protein molecules in an electrolyte solution contains contributions from hard-sphere repulsion, dispersion and electrostatic interactions. The last two contributions are approximated using the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory [11]. The Carnahan–Starling equation of state is used to calculate the contribution of hard-sphere repulsive interaction to the osmotic pressure [12]. In the first model, the DLVO potential is used through its contribution to the osmotic second virial coefficient. The second model uses the random-phase-approximation theory (RPA) as discussed, for example, by Vlachy et al. [13]. Both models provide a semi-quantitative description of the osmotic pressure of BSA solutions at low salt concentrations but not at high salt and protein concentrations. At these conditions, when electrostatic shielding is high, it appears that the potential of mean force should be more repulsive than that given by the DLVO theory. As a result, both models underpredict the osmotic pressure at high salt and protein concentrations.

2. Membrane osmometer

Fig. 1 shows schematically the experimental apparatus for osmotic-pressure measurements. Except for some slight differences in the osmometer cells (size, o-ring structure, and discharge channels), this apparatus is almost the same as that used by Amos et al. [10]. The osmometer cell consists of two
Fig. 1. Schematic diagram of the osmometer system.

Fig. 2. Osmotic pressure of BSA aqueous saline solution at pH = 7.4 and 0.15 M sodium chloride concentration.
chambers separated by a membrane permeable to solvent molecules and small ions but impermeable to proteins. One chamber contains a protein solution and the other contains a protein-free solvent, i.e., a solution of salt and buffer ions. External pressure is applied to the protein side such that, at equilibrium, no net mass transfer occurs between the two chambers. The applied pressure is controlled by one of the two precision pressure regulators, one for low-pressures (< 1000 mm H₂O) and the other for higher pressures. The osmotic pressure, equal to the applied external pressure at equilibrium, is read either from a home-made water manometer or from a high-precision pressure gauge. Resolution of the volume change in the capillaries is about 0.002 ml. The osmometer is kept isothermal by immersion in a water bath controlled at 25 ± 0.1°C. External pressure was supplied by a high-pressure nitrogen tank. In Appendix A, Figs. 10–12 describe the osmometer and its accessories. Attainment of equilibrium may require several days for high protein concentrations. To accelerate data acquisition, two identical osmometer systems were constructed.

The reliability of both osmometers was tested by measuring some osmotic pressure of BSA solutions at pH = 7.4 and 0.15 M sodium chloride concentration. The same solution condition has been used by Vilker et al. [9] to measure the osmotic pressure of BSA solutions. Because their measurements were in the relatively high concentration range, Vilker’s empirical correlation was used to test our measurements. Fig. 2 shows the comparison between our measured results and those calculated from Vilker’s empirical correlation at the same solution conditions. The agreement is good for both osmometers.

3. Experimental procedure

3.1. Materials

Bovine Serum Albumin (BSA) (> 98% albumin, heat-shock fraction) was purchased from Sigma (St. Louis, MO) and stored at about 5°C. Analytical-grade monobasic and dibasic sodium phosphates, sodium hydroxide pellets, hydrochloric acid (0.1 N) and crystal sodium chloride were purchased from Aldrich (Milwaukee, WI). Sodium azide was from Eastman Kodak (Rochester, NY). All chemicals were used as received and all solutions were prepared using deionized water. Cellulose membranes with 10,000 molecular-weight cut-off were purchased from Millipore (Bedford, MA). The molecular weight of BSA is 66,000.

3.2. Procedures

Sodium chloride aqueous solutions of 1.0, 3.0 and 5.0 M in Na⁺ at pH = 7.4, 5.4 and 4.5 were prepared using 0.1 N mono-basic/dibasic sodium phosphate buffer solution and crystal sodium chloride. All solutions contained sodium azide (0.020 g/l) as an anti-bacterial agent. High-concentration BSA-stock solutions (about 100 g/l) were prepared by dissolving albumin crystals in a given sodium chloride solution. Each stock solution was then diluted to about 20, 40, 60 and 80% of its original protein concentration with the corresponding sodium chloride-buffer solution. pH was measured using a Sargent–Welch Model 8400 Ion/pH meter with a Fisher Scientific Model SN 13-620-286 electrode. The final solution pH was adjusted to the desired value by addition of non-buffered aqueous solution of 0.1 N sodium hydroxide or hydrochloric acid. Vigorous vortex
mixing was applied during acid/base addition process to prevent local protein denaturation. The slight difference in Na\(^+\) concentration induced by pH adjustment was neglected. All solutions were kept at 5°C overnight before osmotic-pressure measurement.

The regenerated cellulose membrane was soaked in deionized water for 1–2 h and then in the desired sodium chloride-buffer solution overnight before use. The osmometer cell was assembled by sandwiching the pre-prepared membrane between two Plexiglas chambers. The solvent (i.e., aqueous sodium chloride and buffer solution) was injected into both chambers using syringes, and the osmometer was set in the water bath overnight before osmotic-pressure measurement. Cell leakage and zero-point error were prevented by checking the solvent level at each capillary.

Before each measurement, both solution and solvent chambers of the osmometer were flushed by injecting and removing excess amount of desired solvent; the solution chamber was rinsed two or three times with the sample solution. The protein solution and the solvent were then simultaneously injected into the corresponding chambers to the liquid levels in both capillaries reaching about 2/3 of

Table 1

<table>
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<th></th>
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<th>pH 5.4</th>
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<td>( \Pi ) (mm H(_2)O)</td>
<td>( \Pi ) (mm H(_2)O)</td>
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<tr>
<td>( C_\text{s} )</td>
<td>( C_\text{p} ) (g/l)</td>
<td>( C_\text{p} ) (g/l)</td>
<td>( C_\text{p} ) (g/l)</td>
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<tr>
<td>19.5</td>
<td>85</td>
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<td>224</td>
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<tr>
<td>150</td>
<td>892</td>
<td>99.1</td>
<td>430</td>
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\( C_\text{s} \) = molar concentration of salt (sodium chloride).
\( C_\text{p} \) = mass concentration of protein (BSA).
1 mm H\(_2\)O = 9.80 Pa.
their full length. Pressure was applied gradually to the solution side such that there was no net mass flow between the two chambers. Equilibrium was attained when there was no liquid-level change in both capillaries for about 3 h at a given applied pressure. At the conclusion of each measurement, solvent and solution samples were taken simultaneously using different syringes while the solution was still under pressure. The protein concentrations in both chambers were measured using a Shimadzu Model UV-160 spectrophotometer. Concentration measurement on the solvent side was conducted to monitor the working status of the membrane osmometer because leakage could occasionally occur due to inappropriate assembling of the osmometer. The extinction coefficient for BSA at 278 nm is 0.66 in aqueous solutions, that is assumed to be independent of pH and salt concentration [8].

4. Experimental results

Table 1 shows the measured osmotic pressures of BSA solutions at different pH and salt concentrations. To display the trend of measured osmotic pressure as a function of pH, Fig. 3 shows reduced osmotic pressure (osmotic pressure/protein concentration) versus concentration for pH = 7.4 and 4.5 in 1 M sodium chloride solution. Because of higher protein charge as discussed in the next paragraph, the osmotic pressure at pH = 7.4 is much higher than that at pH = 4.5 at protein concentrations less than 60 g/L.

According to Vilker et al. [9], the BSA charge numbers in 0.15 M sodium chloride aqueous solution are −20.4, −9.1 and +4.5 at pH 7.5, 5.4 and 4.5, respectively. We realize that the protein charge may depend on salt concentration. Because this dependence can only be found by potentiometric titration, we assume that protein charge numbers are independent of salt concentration in this work. This assumption will not change the qualitative feature of our results. At pH = 4.5, attractive
forces between proteins dominate over repulsion interactions; the former leads to negative second virial coefficient. A negative second virial coefficient reduces osmotic pressure relative to that given by the van’t Hoff equation. Association between BSA molecules has been reported by Kanal et al. [14] in the range $4 < \text{pH} < 6$. Fig. 4 shows the effect of salt concentration on the osmotic pressure of BSA solution at pH 7.4. In general, the osmotic pressure decreases as salt concentration rises because of increased screening of the electrostatic repulsion between protein molecules. Fig. 5 shows the osmotic pressure of BSA aqueous solutions at high salt concentrations at pH 5.4. At these conditions, the osmotic second virial coefficients are negative, indicating that the overall force between BSA
Table 2
Molecular parameters obtained from data regression

<table>
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<th>Model</th>
<th>( H ) / ( kT )</th>
<th>( \delta ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B model</td>
<td>0.31</td>
<td>0.012</td>
</tr>
<tr>
<td>RPA model</td>
<td>2.48</td>
<td>0.005</td>
</tr>
</tbody>
</table>

\( H \) = Hamaker constant.
\( \delta \) = empirical parameter reflecting minimum surface-to-surface distance.

molecules is attractive. As expected, because screening declines with falling ionic strength, the interaction between proteins is more repulsive at lower salt concentrations.

5. Theoretical analysis of osmotic-pressure data

5.1. Potential of mean force between proteins in aqueous electrolyte solutions

To represent the osmotic pressure of BSA solutions at different pH and salt concentrations, we assume that proteins in solution can be represented by charged hard spheres of diameter \( \sigma \) immersed in an aqueous solvent containing salt ions. The total potential of mean force between protein molecules includes contributions from hard-sphere repulsion \( W_{hs}(r) \), from dispersion attraction \( W_{dis}(r) \), and from double-layer electrostatic repulsive interaction, \( W_{dl}(r) \) [11].

\[
W_{\text{total}}(r) = W_{hs}(r) + W_{\text{dis}}(r) + W_{\text{dl}}(r)
\]

where \( r \) is center-to-center distance between two protein molecules. The last two terms are known as the Derjaguin–Landau–Verwey–Overbeek (DLVO) potential, \( W_{\text{DLVO}}(r) \).

![Fig. 6. Calculated and measured osmotic pressures of BSA solutions at \( C_s = 5 \text{ M} \). Solid lines are calculated from the B model; the dashed lines are from RPA theory.](image-url)
The dispersion potential between two spherical molecules is given by [15]

\[
W_{\text{dis}}(r) = -\frac{H}{12} \left( \frac{\sigma^2}{r^2 - \sigma^2} + \frac{\sigma^2}{r^2} \frac{2 \ln \frac{r^2 - \sigma^2}{r^2}}{r^2} \right) \quad r > \sigma,
\]

where \( H \) is the Hamaker constant of proteins in water. Because Eq. (2) diverges when two protein molecules are in contact, we assume that there is a minimum distance between two protein surfaces, denoted as \( \delta \). A possible interpretation of \( \delta \) is to regard it as the average diameter of atoms on the protein surface. However, because \( \delta \) is obtained by adjusting experimental osmotic-pressure data to our calculations, it is essentially an empirical fitting parameter. The strength of dispersion interaction is sensitive to the minimum surface-to-surface distance.

The double-layer repulsive interaction \( W_{\text{dl}}(r) \) is approximated by

\[
W_{\text{dl}}(r) = \frac{z_p e^2}{4\pi \varepsilon_0 \varepsilon r} \frac{e^{-\kappa(r-\sigma)}}{\left(1 + \kappa \sigma / 2\right)^2},
\]

where \( z_p \) is protein charge, \( e \) is the charge of an electron, \( \varepsilon \) is the dielectric constant of water and \( \varepsilon_0 \) is the permittivity constant. \( \kappa \) is the Debye screening parameter related to the small-ion concentration \( \rho_i \), valence \( z_i \) and temperature \( T \) by

\[
\kappa^2 = \sum_i \frac{\rho_i e^2 z_i^2}{\varepsilon_0 \varepsilon k T},
\]

where \( k \) is the Boltzmann constant. Here subscript \( i \) refers to all small ions. Eq. (3) is derived using the linear approximation of the Poisson–Boltzmann equation [11].

![Fig. 7. Calculated and measured osmotic pressures of BSA solutions at \( C_s = 3 \) M and pH = 7.4, 5.4. Notation is similar to that in Fig. 6.](image-url)
5.2. Van der Waals-type equations of state for protein solutions

We use two van der Waals type equations of state to correlate experimental osmotic-pressure data of BSA solutions measured in this work and those reported by Vilker et al. [9]. Both equations contain the Carnahan–Starling equation of state to take into account short-range repulsive interactions [12]. In
the first model, the DLVO potential is considered through its contribution to the perturbation part of the second virial coefficient, denoted by $B$. The osmotic compressibility factor $Z$ is given by,

$$Z = \frac{\Pi}{\rho_p kT} = \frac{2}{\rho_p} \left\{ \left[ \left( z_p \rho_p/2 + \rho_s^2 \right)^{1/2} - \rho_s \right] \left[ 1 + \eta + \eta^2 - \eta^3 \right] + B \rho_p \right\}$$

where $\Pi$ is osmotic pressure; $\rho_p$ is the number density of protein; $\eta$ is packing fraction, $\eta = (\pi/6) \rho_p \sigma^3$; $\rho_s$ is the number density of salt in the ‘solvent’ side. Because the volume of the solvent side is large compared to that of the protein side, $\rho_s$ is identical to the original number density of salt. The perturbation part of the second virial coefficient $B$ is given by

$$B = 2\pi \int_{\sigma + \delta}^{\infty} \left[ 1 - \exp \left( - \frac{W_{DLVO}(r)}{kT} \right) \right] r^2 \, dr$$

The first term in Eq. (5) comes from the ideal Donnan effect for a 1:1 electrolyte solution. It takes into account the uneven distribution of small ions between the two chambers of the osmometer [9]. When the McMillan–Mayer theory is applied to calculate the osmotic pressure of protein solutions or other solutions of charged solutes, consideration must be given to the difference in ‘solvents’; i.e., the

![Fig. 10. Solution chamber of the osmometer cell.](image-url)
solvent’ (in this case, a salt solution) on the protein side of the osmometer is slightly different from that on the other side because of Donnan equilibrium. For the solutions considered here, the ideal Donnan effect provides a simple first-order correction due to the difference in solvents. (Another approach to calculate the osmotic pressure of a protein solution containing electrolytes is to consider explicitly interactions between proteins and small ions, as discussed by Amos et al. [10]). For convenience, we call Eq. (5) the B model.

Another way to include the DLVO potential is to use the random-phase approximation (RPA) (see, for example, [13]) that gives the equation of state

\[ Z = \frac{\Pi}{\rho_p kT} = \frac{2}{\rho_p} \left( \left( z_p \rho_p / 2 \right)^2 + \rho_s^2 \right)^{1/2} - \frac{1 + \eta + \eta^2 - \eta^3}{(1 - \eta)^3} + \rho_p \cdot \frac{U_1}{2 kT} \]  

(7)

where \( U_1 = 4 \pi \int_{r_\delta}^{r_\infty} [W^{\text{disp}}(r) + W^{\text{dil}}(r)] r^2 \, dr \). The RPA is a perturbation theory that approximates the direct correlation function of the solution by that of the reference system. \( U_1 \) is analogous to constant \( a \) in the van der Waals equation of state with dimensions of energy \( \times \) volume.

Fig. 11. Solvent chamber of the osmometer cell.
At high salt concentrations or at low protein charge, the ideal Donnan-effect term in Eq. (5) or Eq. (7) is negligible because the ‘solvents’ in both chambers of the osmometer are essentially identical. However, this term can be significant for the osmotic pressure of highly charged proteins in dilute salt solutions. For example, at pH = 7.4 and \( C_s = 0.15 \) M, it accounts for about 20% of the total osmotic pressure for the protein concentrations studied here.

In both models, molecular parameters \( \delta \) and \( H \) are assumed independent of solution pH, protein concentration and salt concentration. These parameters are obtained by regression of experimental osmotic-pressure data measured in this work and those reported by Vilker et al. [9]. The BSA diameter (\( \sigma = 6.26 \) nm) and charge numbers (valence at various pH are also from Vilker et al. [9]. Table 2 gives the two adjustable parameters for both models. The Hamaker constants obtained from the two models do not agree due to different approximations introduced in each model.

Figs. 6–9 compare experimental osmotic pressures for BSA solutions with calculated results using the van der Waals type equations of state. The experimental data for \( C_s = 0.15 \) M are from Vilker et al. [9]. In Figs. 6 and 7, the effect of charge on interaction between proteins is very small because of high ionic strength (5 and 3 M, respectively). The slight crossing of data for pH = 7.4 and 5.4 should be attributed to experimental uncertainties. While both models can semi-quantitatively describe the osmotic pressure of BSA solutions, the B model is slightly better than the RPA model. Both models predict osmotic pressures that are too small at high protein concentrations, perhaps because the DLVO theory is based on the linear approximation of the Poisson–Boltzmann equation; this approximation gives less repulsion when compared with the correct interaction potential between macroions at small separations [16]. At pH 4.5 and 5.4, deviations at low protein concentrations may be related to association of proteins.

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Fig. 12. Accessories of the osmometer cell.

Other accessories for the osmometer cell:
1. one 40 mm O-ring
2. one 30 mm O-ring
3. two NUPRO high pressure valves (SS-2P4T)
4. four SWAGELOK-316GSG connectors
5. two 5 mm fittings
6. four screws
To represent the data, we have tried using other, more sophisticated models including the Barker–Henderson perturbation theory with the macro-compressibility approximation [17] and the Percus–Yevick solution of the sticky hard-sphere model [18]. Regrettably, these models do not improve agreement with experiment. It appears that the main weakness of the two van der Waals models follows from the over-simplified potential of mean force used here for proteins in electrolyte solutions. Progress in understanding the properties of saline protein solutions is not likely to come from more sophisticated statistical mechanics but from an improved potential of mean force.

6. Conclusions

Two membrane-osmometer systems have been constructed for protein solutions at low and high protein concentrations. The reliability of these systems was tested by measuring the osmotic pressure of BSA solutions at pH = 7.4 and 0.15 M sodium chloride concentration; good agreement with Vilker’s data [9] was obtained. Although the osmometers described here are inexpensive and easy to operate, osmotic-pressure measurements are time consuming, especially at high protein concentrations.

New and previously published osmotic data show the effects of pH and salt concentration on the osmotic pressures of BSA solutions. The osmotic pressure falls as protein charge decreases or as salt concentration increases. The experimental data can be semi-quantitatively interpreted using either a second-virial-coefficient model, or a model based on RPA theory. However, neither model is successful at high salt concentrations. For more accurate description of the properties of protein solutions, we require a better potential of mean force. Toward meeting that requirement, molecular-simulation studies are in progress.

7. List of symbols

\[ a \quad \text{van der Waals’ parameter} \]
\[ B \quad \text{perturbation part of the osmotic second virial coefficient} \]
\[ C \quad \text{concentration} \]
\[ e \quad \text{electron charge} \]
\[ H \quad \text{Hamaker constant} \]
\[ k \quad \text{Boltzmann constant} \]
\[ r \quad \text{center-to-center distance} \]
\[ T \quad \text{temperature} \]
\[ U \quad \text{RPA energy parameter} \]
\[ W \quad \text{potential of mean force} \]
\[ Z \quad \text{osmotic compressibility factor} \]
\[ z \quad \text{protein charge} \]

**Greek letters**

\[ \Pi \quad \text{osmotic pressure} \]
\[ \delta \quad \text{minimum distance between two protein surfaces} \]
\( \varepsilon \)  
\( \varepsilon_0 \)  
\( \eta \)  
\( \kappa \)  
\( \rho \)  
\( \sigma \)  

Subscripts  
\( p \)  
\( s \)  

Superscripts  
\( \text{dis} \)  
\( \text{dl} \)  
\( \text{DLVO} \)  
\( \text{hs} \)  
\( \text{total} \)  

Acknowledgements

We are grateful to Ms. Amos for showing her apparatus, and to Ms. Lam for assistance with experimental measurements. This work was supported by the Director, Office of Energy Research, Office of Basic Energy Sciences, Chemical Sciences Division of the U.S. Department of Energy under Contract No. DE-AC03-76SF00098.

Appendix A. Detailed diagrams of a membrane osmometer

Details of the osmometer are given in Figs. 10–12.

References