Osmotic pressure of bovine serum albumin in the presence of calcium chloride with low ionic strength

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ABSTRACT

It is well known that the osmotic pressure of protein solutions deviates from ideal behavior at high concentrations. Our lab previously developed a free-solvent model for osmotic pressure that provides excellent prediction of the osmotic pressure for protein solutions at moderate ionic strength using only physically-realizable parameters. The model assumes that the hydrated proteins behave ideally in solution as the electrostatic interactions in the high ionic strength solutions are largely screened. In this study, we evaluate the predictability of the free-solvent model for protein solutions made up of CaCl2 solutions at low ionic strengths. In low ionic strength solutions, the Debye length can extend out to be on the order of the Stokes-Einstein radius of the protein; therefore electrostatic influences could induce protein-protein interactions. Using osmotic pressure data from solutions of bovine serum albumin (BSA) in 3 mM CaCl2 at pH 7.4, we estimated the parameters, hydration and ion-binding, of the free-solvent model using a non-linear least squares regression algorithm. The results generated an excellent fit to the data but produced a negative ion binding parameter, (-2.67 ± 0.241 mol CaCl2/mol BSA). Thus, the free-solvent model is highly sensitive to ion binding variations but results in an excellent fit to the highly-nonlinear monotonic data. These results suggest that the general concept of the free-solvent has validity, but that perhaps additional corrections of protein-protein or other interactions must be introduced to account for the observed osmotic pressure at low ionic strength solutions. Furthermore, HPLC results of BSA in 3 mM CaCl2 shows two peaks at 278 nm, suggesting that the BSA solution used has a monomer and a lower MW species; thus a two-protein model for this solution can also be considered.

Keywords: osmotic pressure, free-solvent model, bovine serum albumin, salt ion binding

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Overseeing the B2K Group (Biotransport and Bioreaction Kinetics), Professor Rodgers research interests combine experiemental work with mathematical analysis to address problems related to biotransport phenomena, bioreaction kinetics and thermodynamics in biomedical engineering and bioseparations.

A copy of this paper in its entirety can be found online at

www.ugr.ucr.edu in Volume VII.

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Danielle Nicole Ornelas is a graduating senior in Bioengineering. She presented her research at the UCR Symposium for the Undergraduate Research, Scholarship, and Creative Activity and will also be presenting at the upcoming UC Systemwide Bioengineering Conference at UC San Diego and the Biomedical Engineering Society Annual Conference in Seattle. Danielle joined the Biotransport and Bioreaction Kinetics (B2K) group in May 2012 and has been involved in osmotic pressure research that allows her to understand the behavior of osmotic pressure of concentrated protein solutions. She worked closely with the Loma Linda Pediatric Surgery department to help design and build an infant surgical simulator that facilitates the practice of duodenal atresia surgery. Danielle is eager to pursue a M.S. in bioengineering, followed by further graduate work in the medical field. She is an active member of the Health Sciences Partnership Mentor Program, works as a Student Success Counselor for the Bourns College of Engineering, and has dedicated seven years of her time volunteering at a local hospital. Danielle gives special acknowledgement and thanks to Noriko Uka Ozaki, Devin McBride, and Dr. Victor G. J. Rodgers for their enduring support and guidance, and for helping to make her the researcher she is today.

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Introduction

Proteins are an important component for living cells and they are involved in many physiological processes, including maintenance of the osmotic balance.[1] Osmotic pressure is a natural phenomenon that results from the flow of diffusible species across a semi-permeable membrane from one chamber to another in order to reach chemical potential equilibrium. Osmotic pressure is known to deviate from ideal behavior at high protein concentration.[2,3] In order to describe this non-ideal behavior, our lab previously developed the free-solvent model, which represents the proteins as single species hydrated macromolecules (Figure 1).[2,3] Hydrated macromolecules consist of a protein with bound ions and water molecules, essentially excluding bound solvent from the bulk. When considering hydrated macromolecules as the solutes, the free-solvent model becomes an ideal model given that there are no attractive forces between hydrated macromolecules. Therefore, the free-solvent model suggests that the behavior of osmotic pressure is primarily explained by the solute-solvent interactions rather than solute-solute interaction. The remaining ions and water that are not interacting with the protein are considered as the free solvent. The free-solvent model focuses on three physiologically significant parameters: the mole fraction of all non-attractive species, protein-ion binding, and protein hydration.

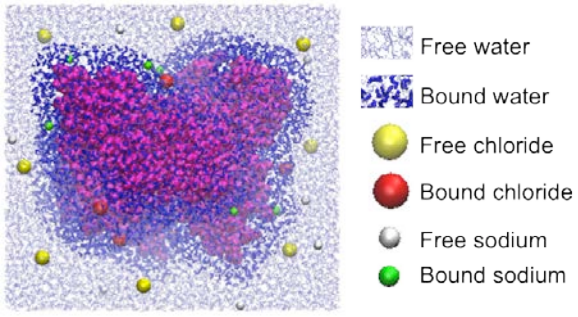


Figure 1 – A hydrated macromolecule (BSA) illustrating bound water and bound ions. Bound water and bound ions are excluded from the bulk solution, reducing the mole fraction of water and ions.

The detailed development of the free-solvent model is described elsewhere by Yousef *et al.* (1998).[4] This model subtracts bound solvent from the total solvent mole fraction.[4] As discussed above, it is assumed that protein-bound salt ions and water molecules are within the macromolecule definition (Figure 1).[4] The free-solvent model, where chamber I contains the solvent and chamber II contains solvent and the protein, can be described as

$$\pi = \frac{RT}{\bar{V}_1}\ln\left(\frac{N_1^{\mathrm{I}}\left(\sum_{i=2}^{n}N_i^{\mathrm{II}} - \sum_{i=1,i\neq 2\to p+1}^{n}\sum_{j=2}^{p+1}\nu_{ij}N_j^{\mathrm{II}}\right)}{\sum_{i=1,i\neq 2\to p+1}^{n}N_i^{\mathrm{I}}\left(N_1^{\mathrm{II}} - \sum_{j=2}^{p+1}\nu_{1j}N_j^{\mathrm{II}}\right)}\right) \quad (1)$$

where n is the total number of species, p is the number of proteins, N_i^k is the moles of species i in chamber k, and ν_{ij} is the number of species i interacting with protein j.[5] For a single protein in a monovalent salt solution, Equation (1) can be reduced to

$$\pi \approx \frac{RT}{\bar{V}_1}\ln\left(\frac{\left(N_1^{\mathrm{II}} + \left(1-\nu_{12}-\nu_{32}\right)N_2^{\mathrm{II}} + N_3^{\mathrm{II}}\right)N_1^{\mathrm{I}}}{\left(N_1^{\mathrm{II}} - \nu_{12}N_2^{\mathrm{II}}\right)N^{\mathrm{I}}}\right) \quad (2)$$

where subscripts 1, 2, and 3 represent water, protein, and salt, respectively.[5] Two biologically significant parameters can be extracted from the free-solvent model: hydration $\left(\nu_{12}\right)$ and salt ion binding $\left(\nu_{32}\right)$.

Previous studies show that the osmotic pressure is highly dependent on pH, especially at high protein concentrations, and is very sensitive to small differences in salt ion binding.[4,6] Based on the osmotic pressure, bovine serum albumin (BSA) in 0.15 M NaCl has a salt ion binding difference of 2.8 mol NaCl/mol BSA for pH 5.4 versus 7.4, while the hydration remains relatively constant. This subtle difference in ion binding, however, results in an osmotic pressure difference of 36 psi between the two solutions at 420 g BSA/L solution.[4] This indicates that the subtle difference in salt ion binding is coupled to a significant difference in osmotic pressure.

Furthermore, it has been shown that the binding of ions with BSA is dependent on the ionic strength of the solution; [7,8,9] however, the effects of salt ion binding on osmotic pressure in low ionic strength solutions have received limited analysis.[5] Therefore, the investigation of the effects of ionic strength on ion binding and its resulting osmotic pressure will be the focus of this study. This research attempts to define the dependence of ion binding and osmotic pressure on low ionic strength by comparing our results to published osmotic pressure data with higher ionic strength. The outcome of this research is expected to contribute to further understanding of protein-ion interactions, which have implications on various physiological processes.

Material and Methods

Research Design: BSA was chosen as a model protein for this investigation because of the abundance of existing experimental osmotic pressure data,[4,10] as well as the fact that data is available pertaining to its interactions with ions.[7,8,9] The osmotic pressure of BSA (A30075, Research Products International) up to near-saturation concentrations in the presence of 3 mM $CaCl_2$ (C3306-100G, Sigma) at pH 7.4 at room temperature was measured as previously described.[4]

Here, a constant flow of salt solution into the salt chamber was maintained in order to ensure that an infinite source of ions and water was available. The pH was adjusted using aliquots of 1M NaOH and 1M HCl. The addition of the acid and base resulted in the increase of bulk ionic strength; however, these additional ions were essentially removed from the BSA solution by the use of an infinite source of 3 mM $CaCl_2$ solution connected to the solvent chamber.

Device Setup: Osmotic pressure was measured using an osmometer previously developed in our lab.[5] The osmometer (Figure 2) is composed of two chambers, a solvent chamber and protein chamber, separated by a 3 kDa molecular weight cut-off semi-permeable membrane (regenerated cellulose, Spectrum). The membrane was chosen such that the transport of proteins across the membrane was negligible.

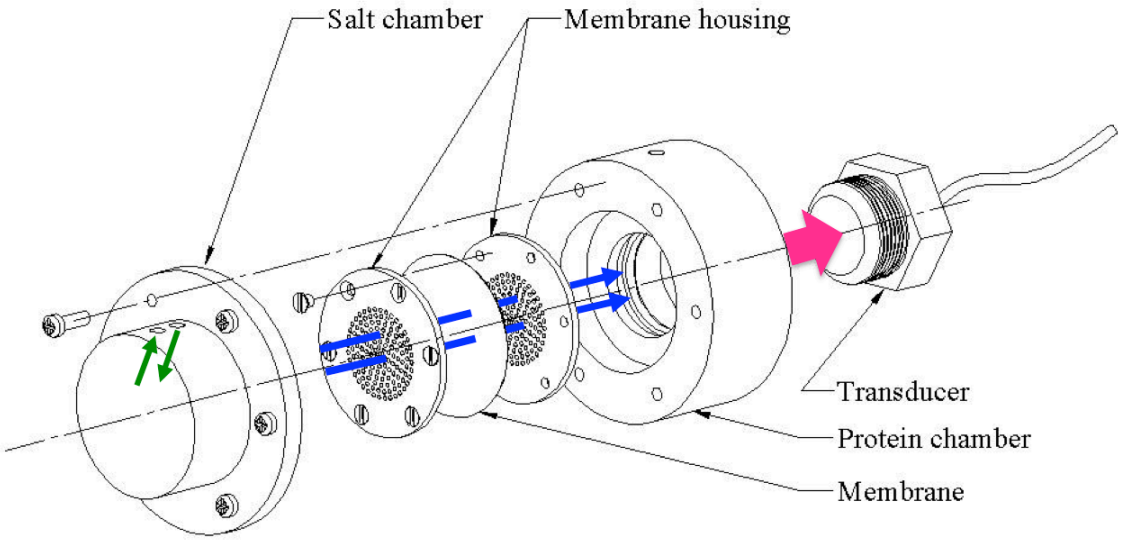


Figure 2 –Schematic diagram of the osmometer used in this study. The movement of diffusible species (blue arrows) across the semi permeable membrane induces a pressure build-up (pink arrow). The infinite sink is connected to the inlet and outlet of the salt chamber (green arrows).

Therefore, only ions and water molecules are capable of transport through the membrane. A pressure transducer with the appropriate pressure range (PX-726 (0-25 psi) or PX-102 (0-100 psi), Omega Engineering), connected to the protein chamber, was used to measure the pressure build-up created by the influx of diffusible species into the protein chamber. The osmotic pressure data was collected continuously until steady state was reached. The data from the transducer was collected using data acquisition set-up and recorded using LabView (National Instruments, Austin, TX). No more than 20 hours of continuous data collection was performed due to the possibility of BSA denaturation at further extended times.

Data Analysis: The obtained pressure data was plotted as pressure versus time. Osmotic pressure oscillates overtime and eventually reaches steady state. After reaching steady state, the average osmotic pressure and standard deviation were calculated from peak to peak. The acquired osmotic pressure measurements of BSA in 3 mM $CaCl_2$ were compared to previous measurements of BSA in the presence of 150 mM NaCl at pH 7.4 at room temperature.

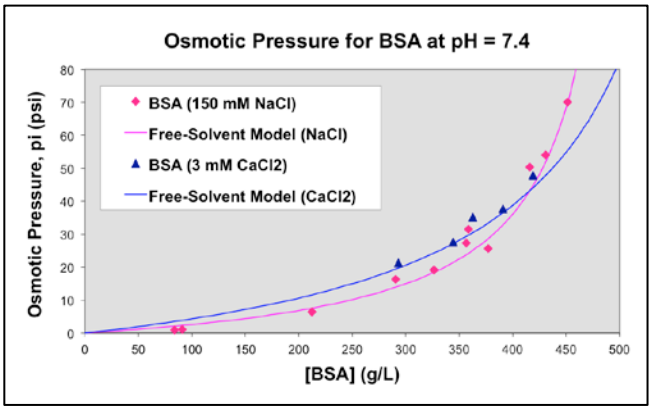
When the complete osmotic pressure-concentration profile was obtained, the osmotic pressure data was fit to the free-solvent model (Equation 2) using non-linear least squares regression (TableCurve 2D (Systat Software, San Jose, CA, USA)) to obtain the salt ion binding and hydration values. In addition, the data was regressed only on salt ion binding with hydration fixed to 1.177 g H_2O/g BSA in order to understand how the difference in salt ion binding is coupled to the difference in osmotic pressure.[4] It is expected that the hydration value remains relatively constant for a globular protein unless a conformation change, such as denaturation, occurs.

Results

The osmotic pressure was measured for five different concentrations of BSA in the presence of 3 mM $CaCl_2$ at pH 7.4 at room temperature. The pressure of each protein solution was plotted with respect to time and the osmotic pressure was calculated using the average pressure along with its standard deviation after reaching steady state. The osmotic pressure of various BSA concentrations in 150 mM NaCl at pH 7.4 was also plotted in order to visually compare our obtained data with previously published data.[3]

The data was initially regressed on two parameters, salt ion binding and hydration. The obtained salt ion binding is -5.60 ± 0.460 mol $CaCl_2$/mol BSA and the hydration is 0.821 ± 0.0579 g H_2O/g BSA. The free-solvent model curve predicting the osmotic pressure of a range of BSA concentrations can be seen below in Figure 3.

Figure 3 – Osmotic pressure versus BSA concentration



in 3 mM $CaCl_2$ and 150 mM NaCl at pH 7.4. The solid curves represent the free-solvent model with respect to both 3 mM $CaCl_2$ and 150 mM NaCl. The data for BSA in 150 mM NaCl was taken from the published work of Yousef *et al.* (1998).[4]

Next, the data was regressed only on salt ion binding. It has been well established that the protein hydration has been shown to be approximately 1 g of H_2O per 1 g of protein based on ^{17}O magnetic resonance of globular proteins.[11,12] The hydration for BSA in 150 mM NaCl at pH 7.4 is 1.177 g H_2O/g BSA and utilizing this value yields a much different curve profile.[4] The salt ion binding value obtained was -2.67 ± 0.241 mol $CaCl_2$/mol BSA, the curve of which fell directly on top of the free-solvent model curve of BSA in 150 mM NaCl at pH 7.4 (ion binding value of 8.81 mol NaCl/mol BSA). By fixing the hydration to 1.177 g

H_2O/g BSA and varying the ion binding, we observed a variation in the curve profile of BSA in 3 mM $CaCl_2$ at pH 7.4. This implies that osmotic pressure is sensitive to small differences in ion binding, as can be seen below in Figure 4.

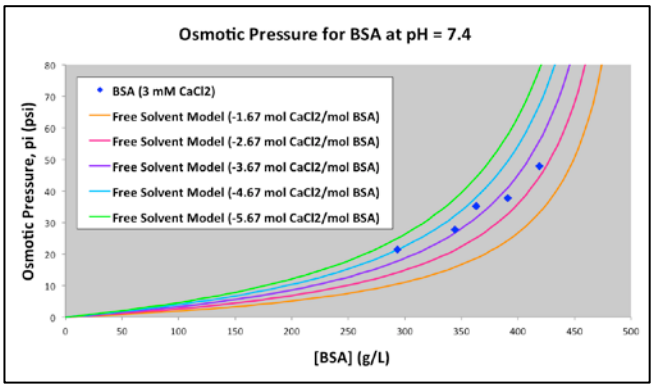


Figure 4 – Osmotic pressure profiles for BSA in 3 mM $CaCl_2$ at pH 7.4. The various curves show different osmotic pressure profiles that result from changing the salt ion binding values. This explains how osmotic pressure is sensitive to differences in ion binding.

Discussion

As seen in Figure 3, we expect simple monotonic behavior of osmotic pressure, a trend observed with 3 mM $CaCl_2$. Additional experimental data for protein concentrations above 420 g/L are necessary to fully capture the signature profile of BSA in 3 mM $CaCl_2$ since the uniqueness of the osmotic pressure is exhibited at concentrations near saturation. The saturation limit given by the hydration value is expected to be 522 g/L solution; however, the osmotic pressure is expected to approach infinity at the saturation limit.

As shown in previously published work, the osmotic pressure is sensitive to the salt ion binding, which is expected to be a function of the ionic strength.[5] Essentially, our experimental setup allows for the elimination of additional ions added to adjust pH via an infinite sink of salt solution. Particularly, studies conducted by Carr *et al.* (1952) have shown that sodium ions do not bind to serum albumin.[7,8,9]

The free-solvent model describing the non-ideal behavior of BSA in 150 mM NaCl at pH 7.4 is represented as a curve shown in Figure 3. The osmotic pressure of various BSA concentrations in 150 mM NaCl and 3 mM $CaCl_2$ at pH 7.4 was plotted to see how comparable the obtained data is with previously published data.[3] As seen above, the osmotic pressure profile of the two data sets seems to be very similar; however, the salt ion binding values are significantly different, 8.81 mol NaCl/mol BSA for 0.15 M NaCl versus -5.60 mol $CaCl_2$/mol BSA for 3 mM $CaCl_2$, which further verifies the robustness of the free-solvent model with respect to the salt ion binding. Additionally, when the hydration is set to 1.177 g H_2O/g BSA, both curves exhibit overlapping profiles despite the vast difference in salt ion binding values. This is because the osmotic pressure is determined for the protein solution in relationship to the solvent solution alone.

Intuitively, the ion binding value should have a minimum value of 0, in the case where no ions are interacting with the protein. The negative ion binding value calls for further investigation into how we can interpret its value with respect to the free-solvent model. As seen in Figure 4, the reduced ion binding in the presence of protein increases the steepness of the osmotic pressure curve, illustrating the sensitivity of the model to ion binding as modified by salt concentration. Thus, our data shows that ion binding has the potential to become a more sensitive parameter within the free-solvent model for low ionic strength solutions as the concentration of the protein in solutions increases.

The regressed salt ion binding value obtained was negative, possibly due to repulsive protein-protein interactions. At pH 7.4, the net charge of BSA has been shown to be ~-20 e.[10] In an environment with low ionic strength, repulsive forces between BSA molecules dominate due to low screening of electrostatic interactions and it is this high potential field that interferes with ion-protein interactions. Therefore, the ion concentration could be higher in the bulk solution than in the monolayer of water surrounding BSA. The low ionic strength solution causes the Debye length to

be large (~39 Å), allowing for possible interactions between molecules to exist. This could invalidate the assumption made in the development of the free-solvent model and imply that our ideal model may not be valid for describing low salt concentration solutions.

Additionally, HPLC (high performance liquid chromatography) data of BSA in 3 mM $CaCl_2$ was taken, as seen below in Figure 5. BSA shows only a single monomer peak at 218 nm, the wavelength at which disulfide bonds absorb. However, the same BSA sample shows two peaks at 278 nm, the wavelength at which proteins absorb, indicating a dual species. These two peaks could possibly represent a monomer and lower molecular weight species. This indicates further need to revise the free-solvent model to incorporate a two-protein species model.

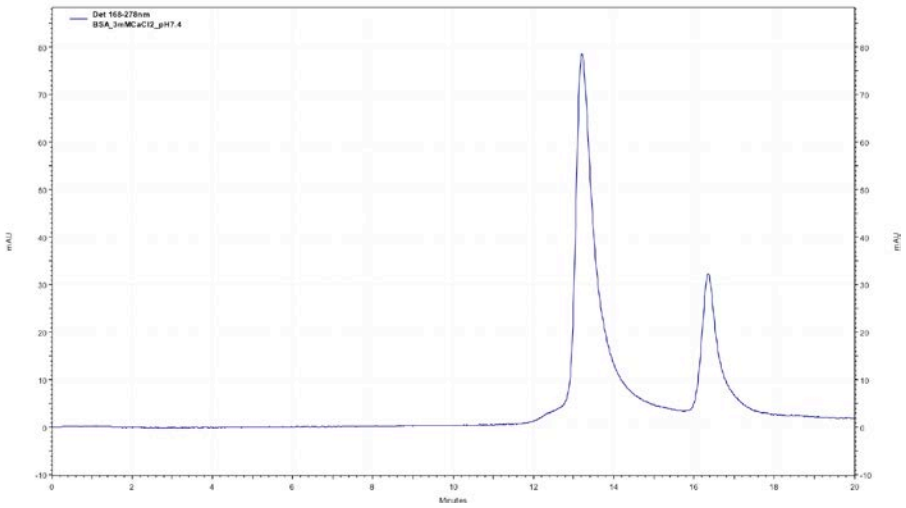


Figure 5 – HPLC results of BSA in 3 mM $CaCl_2$ shows two peaks at 278 nm, suggesting that the BSA solution used has a monomer a lower molecular weight species.

Conclusion

Previous studies have shown that the osmotic pressure is highly dependent on pH at high protein concentrations and has high sensitivity to small differences in protein salt ion binding.[3,5] Here we demonstrate the robustness of the free-solvent model with respect to regression on the salt ion binding. While the salt ion binding value we obtained is non-physiological, the non-ideal behavior of osmotic pressure data was still captured with the free-solvent model. With further investigation on the interpretation of the negative salt ion binding value, the free-solvent model may need to be revised by including the effect of charge interaction for low ionic strengths if physiologically realistic ion binding values are desired. This will be the study of future work. Additional data at higher BSA concentrations is also needed in order to better utilize the free-solvent model for both osmotic pressure prediction and study of ion-protein interactions since the free-solvent model can better capture osmotic pressure behavior at high concentrations.

Acknowledgements

We would like to thank the Biotransport and Bioreaction Kinetics (B2K) Group at UCR for their valuable discussion and insights.

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