Title

Interrogating the Osmotic Pressure of Self-Crowded Bovine Serum Albumin Solutions: Implications of Specific Monovalent Anion Effects Relative to the Hofmeister Series

Journal

The Journal of Physical Chemistry B, 122(33)

ISSN

1520-6106

Authors

Hale, Christopher S
Ornelas, Danielle N
Yang, Jennifer S
et al.

Publication Date

2018-08-23

DOI

10.1021/acs.jpcb.8b07000

Interrogating the Osmotic Pressure of Self-Crowded Bovine Serum Albumin Solutions: Implications of Specific Monovalent Anion Effects Relative to the Hofmeister Series

Christopher S. Hale, Danielle N. Ornelas, Jennifer S. Yang, Larry Chang, Kevin Vang, Ramsey N. Batarseh, Noriko Ozaki, Victor G. J. Rodgers*

Dept. of Bioengineering, B2K Group (Biotransport and Bioreaction Kinetics Group),

University of California, Riverside, CA 92521

*corresponding author (victor.rodgers@ucr.edu)

Abstract

The free solvent-based (FSB) model and osmotic pressure was used to probe the ion binding and protein hydration for self-crowded bovine serum albumin in 0.15 M NaF, NaCl, NaI and NaSCN solutions. All experiments were conducted with solutions at pH 7.4. The regressed results of the FSB model behavior to the measured osmotic pressure was excellent, albeit, the osmotic pressure data for NaSCN was noisy. The resulting ion binding and hydration were realistic values and the covariance of the two parameters was exceptionally low, providing substantial credibility to the FSB model. The results showed that the kosmotropic F^- and neutral Cl^- solutions generated significantly higher ion binding and protein hydration than the chaotropic solutions of I^- and SCN^-. Further, the ionic strength ratio and resulting hydration implied that the chaotropic solutions had substantially higher aggregation than the other salts investigated. Overall, the FSB model provides an additional, complementary tool to contribute to the analysis of crowded protein solutions relative to anions in the Hofmeister series as it can interrogate crowded solutions directly; something that is not possible with many measurement techniques.

Introduction

Background

Ion effects on protein solutions have been investigated for more than a century beginning with Hofmeister.[1] Hofmeister and colleges found that ions could be arranged based on their ability to crystallize protein solutions generating the famous Hofmeister series. Figure 1 illustrates the range of anions when in aqueous solutions for their ability to salt out egg white protein.[1-2]

$$HPO_4^{2-} > SO_4^{2-} > F^- > Cl^- > Br^- > NO_3^- > I^- > ClO_4^- > SCN^-$$

Figure 1: The Hofmeister series for anions ranged from left to right for their ability to salt out egg white proteins in solution.[2]

Subsequently, researchers, including Hofmeister, have searched for the reasoning behind this phenomenon.[3-6] Historically, the water/ion interaction were analyzed to give rise to the categorization of cations and anions as either chaotropes or kosmotropes based on potential of the ion to order water. Chaotropes are denoted as “water structure breaking” and kosmotropes are “water structure making”. The concept of water ordering implies that ions influence water well away from their solvation layer. Recently, researchers have argued that the water ordering

concept is overstated.[7-13] However, local interactions of ions with water, such as electrostriction, are generally accepted. Electrostriction is the reduction of volume of a solvent due to the presence of ions.[14] In fact, the rod-like shape of SCN^- naturally induces electrostriction of the local solvent.[14]

A more quantitative approach categorizes ions as chaotropes or kosmotropes based on their Jones-Dole viscosity B coefficient.[15-16] This relationship was based on original observations by Poiseuille that some salts increased or decreased the viscosity of water.[17] Chaotropes are large weakly hydrated monovalent ions of low charge density (e.g., SCN^-, I^-, NH_4^+, K^+). Chaotropes are denoted as "salting in" because they can result in a decrease in stability and protein crystallization, upon the addition of salt. Kosmotropes, on the other hand, are known for their ability to "salt out" protein solutions and are associated with increased stability and protein crystallization. This grouping consists of small strongly hydrated ions of high charge density (e.g., SO_4^{2-}, HPO_4^{2-}, F^-, Al^{3+}, Ca^{2+}, and Na^+).

Subsequently, the concepts of chaotropes and kosmotropes has morphed into new categorization for ions. Specifically, they are correlated with the size of the ion, electronegativity and charge density.[16, 18] Size of ions is assumed to affect the salt ion-binding. An ionic sphere of a large ion results in a steric hindrance when ion-binding sites on protein are not fully exposed or when they are in narrow cavities on the protein surface. Ionic charges are expected to have influence on salt ion-binding since ion-residues interactions are electrostatically-driven. Collins (2004) suggested that proteins with an excess of weakly hydrated positively charged surface amino acids readily crystallize with weakly hydrated ions such as chloride or thiocyanate, but have difficulty with strongly hydrated ions such as sulfate or phosphate.[19] Also, Zangi *et al*. (2006) reviewed the role of simplified hydrophobic surface interfaces in solution and determined charge density was the key to Hofmeister's ordering.[20] However, Waldron *et al*. (2003) used calorimetry to show that anion binding of their model protein-protein complex was independent of charge. [21] Electronegativity, the tendency of an atom to attract electrons, has been shown to have an impact on the binding affinity to some proteins.[22] Infrared studies have shown that kosmotropic salts can increase ion hydration over chaotropic salts; in addition, protein hydration decreases in the presence of kosmotropic salts when compared to chaotropic salts.[23] Majumdar *et al.* (2013) used size exclusion chromatography to determine the rate of protein aggregation between SO_4^2, Cl^-, and SCN^-. They found that NaSCN accelerated the protein aggregation rate compared to NaCl, while Na_2SO_4 had a small stabilizing effect on the proteins, reducing the rate of aggregation.[24] A comprehensive summary on the physical properties associated with the Hofmeister series can be found elsewhere.[8, 25-26] Table 1 provides a brief historical perspective of proposed justification for anion hierarchy in the Hofmeister series.

We now recognize that, although some generalizations can be made for the association of ions with proteins, no simple relationship captures all of protein/ion/water observed behavior. The classic example is lysozyme; at moderate pH and ionic strength it demonstrates a reverse Hofmeister effects.[27-28] Consequently, more recent work has abandoned the search for a unified global model for the observed Hofmeister effects and have examined the anion-specific surface/water/ion complex. Many have proposed that the anion-protein interaction occurs at the backbone of the protein and have examined this on a molecular scale using molecular dynamics and experimental methods for well-defined peptides.[26, 28-33] Algaer and van der Vegt

(2011) concluded that I^- interacted primarily with the hydrophobic parts of their model peptide.[31] However, Rembert *et al*. (2012) determined that SCN^- and I^- bind at hybrid amide nitrogen/α-carbon binding sites where Cl^- only weakly binds at the same locations.[32] They also concluded that hydrophobic sites do not contribute significantly to anion binding. More recently, Okur *et al*. (2017) showed that steric arrangements at ion binding sites may dominate the outcome of protein-ion interactions.[26]

Table 1: Historical Perspective of Anion Significance in the Hofmeister Series
[5-6, 8, 16, 34]

$HPO_4^{2-} > SO_4^{2-} > F^- > Cl^- > Br^- > NO_3^- > I^- > ClO_4^- > SCN^-$	
Kosmotropes	Chaotropes
Water structure making	Water structure breaking
Salting out	Salting in
Strongly hydrated	Weakly hydrated
Small size, high charge density	Large size, low charge density
Stronger interactions with water than self	Weaker interactions with water than self
Reduces protein aggregation rate	Increases protein aggregation rate

Thus, the complexity of protein behavior in aqueous salt solutions warrants continued detail analysis. Overall, however, a central element of interaction is associated with the specific contributions from protein-ion, protein-water and ion-water interactions. In this regard, information relative to ion binding and protein hydration in the presence of various salts provides important clues to the protein-ion-water systems.

With regards to ion-protein interactions, a number of researchers over the century have attempted to quantify ion binding. Linderstrøm-Lang (1924) accomplished the first theoretical treatment of ion binding in native proteins.[35] Linderstrøm-Lang applied the interionic attraction theory of Debye and Hückel[36] to the analysis of the influence of electrostatic forces on acid-base equilibria in proteins. Tanford (1950) used the theory of Linderstrøm-Lang to provide a computed titration curve for human serum albumin (HSA) and protons.[37] Scatchard *et al.* (1949, 1950) also used the methods of Linderstrøm-Lang to address chloride ion and thiocyanate ion binding to HSA from sodium salts in low concentrations using a membrane dialysis and an electromotive force method.[38-40] They found that thiocyanate ions bind at higher numbers than chloride ions at similar concentrations. Fox *et al.* (2015) used ITC and X-ray crystallography to examine the effect of anions on protein solvation in concavities.[41] Others argue that electrostatic contributions to protein-ion interaction are not enough in interpreting interactions and that dispersion interactions, which can be accessed from bulk properties, and ionic quantum fluctuation forces are critical.[2, 42-43]

The difficulty in understanding ion interactions with protein solutions is exacerbated in crowded environments. In biological systems, it is recognized that macromolecular crowding may be a significant factor in ion interactions in protein solutions.[44] Zimmerman and Trach showed that interactions of ions on proteins that were observed in dilute in-vitro solutions, did not exists in in-vivo crowded systems where the total protein concentration can be as high as 400 g L^{-1}. Reboiras *et al.* (1986) recognized the significance of solution crowding on ion binding to proteins and extended the work of Scatchard (1950), by studying potassium salt binding to isoionic bovine

serum albumin (BSA) at high concentrations (up to 268 g L^{-1}), using the EMF method with ion-exchange membrane electrodes.[39, 45] They showed a protein concentration dependency in ion binding.

Relevance of Osmotic Pressure in the Interpretation of Anion Effects

The FSB Model Directly Relates Osmotic Pressure in Crowded Solutions to Protein Hydration and Ion Binding

Osmotic pressure does not give direct crystallization information. Nevertheless, a number of researchers have used osmotic pressure to address observed phenomena of proteins in ionic aqueous solutions via protein-protein interactions.[46-51] However, we have shown that, via our FSB model, the bulk property, osmotic pressure, of self-crowded proteins and crowded binary globular protein solutions can directly provide ion binding and hydration properties of proteins, specifically in the highly crowded regions where the rate of change of osmotic pressure to protein concentration is highly non-linear.[52-63] Because the FSB model focuses on protein hydration and ion-binding, it can provide a fortuitous approach in interrogating anion effects on crowded protein solutions.

Theoretical Development of the FSB Model

The concept of a free solvent model dates back to the twentieth century when Frazer and collaborators addressed non-idealities in sucrose solutions by considering hydration.[64-65] Scatchard (1946) also proposed a 'free-solvent' model that addressed the interaction of diffusible species with the non-diffusible species in solution.[66] Our FSM model considers the physically realizable parameters, salt ion binding and hydration, as dominant in determining non-ideality and, recognizing the importance of the concentration variable in a Maxwell-Boltzmann distribution[67], uses the mole fraction as its basis.[53] Our FSB model is remarkably successful in predicting the osmotic pressure of albumin in solutions when using the ion binding parameters determined by Scatchard.[39, 53] Not only does the predicted ion binding match that of Scatchard, but the hydration, associated with the water deviating from the bulk chemical potential, was associated with a monolayer. Analysis of the FSB model results has also shown that the non-idealities in self-crowded proteins solutions is dominated by monolayer hydration and ion binding for many globular proteins solutions at moderate ionic strength across a decade of differences in molecular mass.[55] As a result, the FSB model, under the assumption that only a monolayer of water interacts with each protein, provides a means for determining the solvent accessible surface area (SASA) of proteins when protein structure is unavailable.[60]

Our original FSB osmotic pressure model is fully developed elsewhere.[52-53] In summary, the FSB model treats the hydrated protein as a separate macromolecule and all water and salt ions in its 'influence' are absorbed in its definition. The model assumes that counterions (Na^+) to binding anions, are also within the influence of the macromolecule. Scatchard *et al*. (1950) showed that Na^+ do not bind to albumin in dilute solutions.[39] This assumption has been discussed elsewhere.[68] If this assumption is not made, then electroneutrality must be considered.[39-40, 69]

With 'influenced' water and ions associated with the protein macromolecule, the FSB model recalculates the mole fraction of the remaining 'free solvent' (water and ions) that have the propensity to diffuse across the semi-permeable membrane. The subsequent predicted osmotic pressure, based on the modified mole fraction of the free solvent, rests on the ideal solution framework where additional macromolecule 'free-solvent' interactions are ignored. Figure 2

illustrates this general concept. A more detailed structural orientation of water about anions can be found elsewhere.[70]

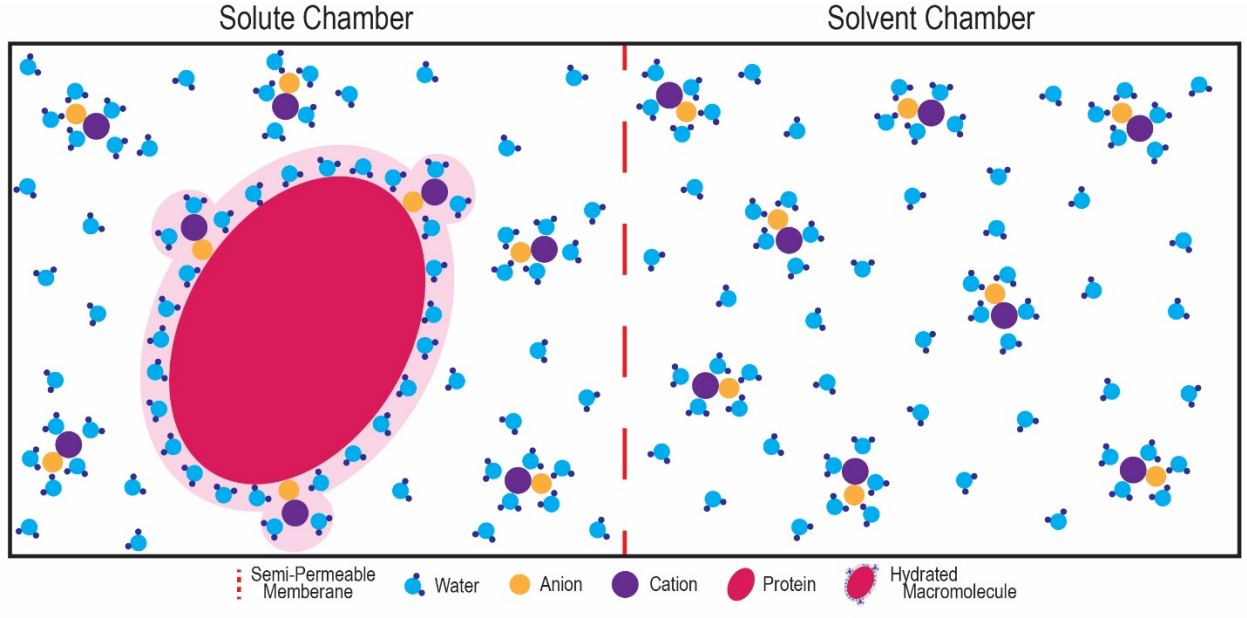

Figure 2: Illustration of relationship of species in the FSB model. Water and ions influenced by the presence of the protein are collapsed into the single 'hydrated macromolecule' species. Thus the hydrated water and bound ions are no longer considered part of the continuum. The resulting mole fraction of solvent or water is calculated based on the remaining diffusible species in both chambers. In this study, hydration of the ions is also taken into account. The regressed ion-binding and hydration parameters for self-crowded BSA solutions are determined for 0.15 M sodium salt solutions with the monovalent anions, Cl^-, F^-, I^- and SCN^- and interpreted with respect to their position in the Hofmeister series.

Thus, the FSB model renders the solution ideal with respect to the remaining, diffusible solvent species. The modified mole fraction of the free water, $(x_1)_{FW}$, considers the hydrated macromolecule as the impermeable solute. The free-solvent model with the mole fraction of the free water, $(x_1)_{FW}$, as the composition variable is

$$\pi = -\frac{RT}{\overline{V}_1} ln\left(\frac{(x_1^{II})_{FW}}{(x_1^{I})_{FW}}\right) \tag{1}$$

where R is the ideal gas constant, T is absolute temperature, $\overline{V}_1$ is the specific volume of species 1 and superscripts I and II represent the solvent and solution chambers. The mole fraction of free water is determined by the remaining moles of water that are not bound to the protein. Assuming the solution is made up of *n* distinct species and *p* proteins, and letting species 1 be the solvent, species 2 through $(p+1)$ be the proteins, and species $(p+2)$ through *n* be the remaining diffusible species, the initial total moles of the solution in compartment II is $N^{\mathrm{II}} = \sum_{i=1}^{n} N_i^{\mathrm{II}}$, where i denotes each species. The final total moles of free-solvent in chamber II, after protein-solvent interactions, is $N_*^{\mathrm{II}} = N^{\mathrm{II}} - \sum_{i=1;i\neq 2\to p+1}^{n} \sum_{j=2}^{p+1} \nu_{ij} N_j^{\mathrm{II}} - \sum_{j=2}^{p+1} N_j^{\mathrm{II}}$, where N_j^{II} denotes the

moles of protein j in solution and ν_{ij} is the number of moles of species i interacting with protein j to make the hydrated protein. Then, the mole fraction of free-solvent in chamber II is

$$(x_1^{II})_{FW} = \frac{N_1^{II} - \sum_{j=2}^{p+1} \nu_{1j} N_j^{II}}{N_*^{II} + \sum_{j=2}^{p+1} N_j^{II}} \tag{2}$$

while in chamber I, the mole fraction of free-solvent is[52]

$$(x_1^{I})_{FW} = \frac{N_1^{I}}{\sum_{i=1, i\neq 2\to p+1}^{n} N_i^{I}}. \tag{3}$$

In this work, we also consider ion hydration and introduce the ion-hydration parameter, ν_{32}. Then, for a single protein species in a monovalent salt aqueous solution, the free-solvent mole fraction in the solvent side of the osmometer, we write

$$(x_1^{I})_{\mathrm{FW}} = \frac{N_1^{I} - \nu_{13} N_3^{I}}{N_1^{I} - \nu_{13} N_3^{I} + N_3^{I}}, \tag{4}$$

and for the solution chamber,

$$(x_1^{II})_{\mathrm{FW}} = \frac{N_1^{II} - \nu_{12} N_2^{II} - \nu_{13} N_3^{II}}{N_1^{II} - \nu_{12} N_2^{II} - \nu_{13} N_3^{II} + N_2^{II} + N_3^{II}(1 - \nu_{32} N_2^{II})} \tag{5}$$

to obtain

$$\pi = \frac{RT}{\overline{V}_1} \frac{\left(N_1^{I} - \nu_{13} N_3^{I}\right)\left(N_1^{II} - \nu_{12} N_2^{II} - \nu_{13} N_3^{II} + N_2^{II} + N_3^{II}(1 - \nu_{32} N_2^{II})\right)}{\left(N_1^{I} + N_3^{I}\,(1 - \nu_{13})\right)\left(N_1^{II} - \nu_{12} N_2^{II} - \nu_{13} N_3^{II}\right)}. \tag{6}$$

The Calculated Hydration and Ion Binding Determined from the FSB Model are Independent

Regression of the protein hydration and protein-ion binding through the FSM model, results in solutions with very low covariance with respect to these two parameters. This independence of parameters is critical and provides additional credibility to model relevance and the validity of the calculated hydration and ion-binding parameters. Moreover, the estimated parameters are dominated by the highest concentration results where deviation of the osmotic pressure from ideality is most prevalent. This is indicative of the strongly non-ideal behavior of the osmotic pressure profile, with respect to concentration, that is characterized by the FSB model.[53]

The FSB Model May Indicate Protein Interaction via Reduction in Hydration

Non-interactive proteins with a prescribed hydration and ion-binding can provide a signature change in the osmotic pressure profile.[55] However, interactive proteins, via aggregation, generate a reduction in hydration or solvation in the FSB model results. Thus, analysis of the FSB model results may also provide indication that specific anions result in significant protein-protein interactions which could be due to aggregation, denaturation or unfolding.[54]

The FSB Model Provides Explanation for Negative Second Virial Coefficient for Non-Attractive Proteins

The FSB model was also used to explain the observed negative second virial coefficient for non-attractive lysozyme solutions. In this work, under the assumptions of the FSB model, that negative second virial coefficients correspond with a higher local ionic strength around the protein than that in the bulk.[61]

The FSB Model Provides a Theoretical Saturation Limit

Because the FSB model provides a relationship of protein hydration, it also provides a theoretical prediction of the protein saturation concentration. Previously we showed that the predicted saturation concentration for bovine immune-gamma globulin in phosphate-buffered solution of 0.13 M salt (0.12 M NaCl, 0.0027 M KCl and 0.01 M phosphate buffers) at 7.4 pH was 546.7 g/L, which compared well with experimental observation.[52]

Goal of this Work

In this study we address the monovalent anion-specific effects on self-crowded BSA solutions via the calculated hydration and ion binding parameters generated from regression of osmotic pressure data via the FSB model (Eqn (6)). Specifically, we determine the osmotic pressure of BSA in NaF, NaI and NaSCN solutions up to 538g/L and compared the results to our previously reported results for BSA in NaCl [53]. All studies are done with a 0.15 M salt concentration and at pH 7.4. Using the FSB model, we determined the hydration and ion-binding for each specific monovalent salt solution. Because the free solvent model is predictive at the highest concentrations, these results represent values for protein concentrations at the highest range. The results of this work are interpreted relative to the Hofmeister series for these anions.

Materials & Methods

Measurement of Osmotic Pressure

The osmotic pressure experiments are conducted for protein concentrations (No. A30075, BSA, Research Products International, Mt Prospect, IL), up to near-saturation, in 0.15 M sodium salt solutions (sodium fluoride (No. S6776, Sigma-Aldrich, St. Louis, MO); sodium chloride (No. S9888, Sigma-Aldrich, St. Louis, MO); sodium iodine (No. 409286, Sigma-Aldrich, St. Louis, MO); sodium thiocyanate (No. 251410, Sigma-Aldrich, St. Louis, MO)) at pH 7.4 and 25°C.

Osmotic pressure is obtained from an osmometer described elsewhere.[53] The protein chamber is filled with protein solution, at the desired concentration, until a meniscus is formed above the chamber walls. Any air bubbles present are removed from the solution. A membrane (NADIR® PM UP010, 10k Da MWCO, Polysulfone, Microdyn Nadir, Germany, Wiesbaden), is soaked in ultrapure water (EASYpure RoDi D13321, Thermo Scientific Barnstead Water System, Thermo Fisher Scientific, Waltham, MA) for at least one hour and is then placed on top of the protein chamber, ensuring no air pocket forms. The protein chamber is sealed and excess solution is expelled. Next, a membrane support is placed on the opposite side of the membrane to prevent bowing deformation caused by the increased osmotic pressure. A rubber gasket is then placed on the other side of the membrane housing to seal the solvent chamber from leaks. The osmometer assembly is then screwed together and connected to a beaker of solvent, open to atmosphere. Solvent is then circulated through the solvent chamber using a peristaltic pump (Model EW-07524-50, Master Flex L/S, Cole Palmer, Vernon Hills, IL). Pressure reading are obtained using a pressure transducer (Model 7356-51 Cole-Palmer, Vernon Hills, IL) that is

digitally recorded through data acquisition (Model NI SCC-68, National Instruments, Austin, TX). Pressure reading stabilize after 5-6 hours.

Solvent solutions are prepared by dissolving the proper amount of sodium salt in one liter of nanopure water to produce a 0.15 M solution. To prepare a BSA solution, this solvent is then used to dissolve a weighed amount of Bovine Serum Albumin, using a stir bar to facilitate mixing. The solution pH is then measured by a pH Meter (Model 13-641-253, ThermoScientific Orion 720A+, Thermo Fisher Scientific, Waltham, MA) and adjusted using 1 M HCl (No. HX0603, Millipore Sigma, Burlington, MA) and 1 M NaOH (No. S318, Thermo Fisher Scientific, Waltham, MA) while undergoing stirring to prevent local denaturation. The amount of acid and base used to adjust pH is considered part of the solvent and is taken into account when determining concentration. Before a solution is used, the pH is checked to be within 0.05 pH of their desired value. The concentration of solutions are determined by dividing the amount of protein or salt by the volume of solvent used to make the solution. The volume of solvent includes volume of protein or salt in the solution using the specific volume of the protein or the density of the salt.

Determining Hydration and Ion Binding

The parameters for hydration, ν_{12}, and ion binding, ν_{32} are determined by nonlinear regression of Eq. 6 (TableCurve 2D, Systat Software, San Jose, CA) to best fit the osmotic pressure versus concentration profiles for each anion solution in the study. The ion hydration parameter, ν_{13}, was estimated from the literature (Table 2). Table 2 also includes the reported crystal radius, apparent dynamic hydration number (ADHN) and kosmotrophicity for each ion. The crystal radius is inversely related to surface charge density.[16] The ADHN for each ion is considered to be a primary factor in ion binding to proteins.[71] Kosmotrophicity is a categorization of whether an ion behaves as a kosmotrope (k) or a chaotrope (c). This evaluation is based on a number of factors including the viscosity B coefficients[34]

Results and Discussion

Osmotic Pressure Results

Measured osmotic pressure profiles for protein concentrations up to near saturation for BSA in NaF, NaI, and NaSCN are shown in Table 3 and plotted in Figure 3 with the previous NaCl data from Yousef *et al.* (1998).[53] All solutions show classic non-linear monotonic increase in rate of change in osmotic pressure versus increased concentration, albeit the data associated with NaSCN is relatively noisy compared to the other solutions. The error in reproducibility found in the NaSCN solutions was significantly higher than other solutions studied and this may have additional importance in understanding the effect of SCN^- on the overall solution properties. All proteins remained in solution for all measured protein concentrations.

The osmotic pressure data demonstrates a distinct separation in osmotic pressure relative to anion in solution at protein concentrations above approximately 300 g/L. At a representative concentration of about 448 g/L, the osmotic pressure for NaCl is highest, followed by that of NaSCN and NaI, with that from the NaF solution being the lowest. The osmotic pressure for NaF appears to increase in its rate of change relative to NaSCN and NaI at the highest concentrations.

Table 2. Properties of Ions in Study

Anion	Bound Water Molecules [72]	Crystal Radius (Å) [73]	Apparent Dynamic Hydration Number [71, 74]	Kosmotrophicity [34]
F^-	7.2	1.33	5	k
Cl^-	5.5	1.81	0	k/c
I^-	6.6	2.20	0	c
SCN^-	8.8	2.13	-	c

k = kosmotrope, c= chaotrope

The FSB Model Provides Excellent Fit with Regressed Ion Binding and Hydration Values

As can be seen from Figure 3, the two parameter FSB model successfully captures the physics of the osmotic pressure behavior for increasing protein concentrations for NaCl, NaI, and NaF solutions. The model also well represents the data for NaSCN, given that the data is relatively noisy. The regressed ion binding and hydration values determined for the best fit of the FSB model to each of the data sets are summarized in Table 4. One note, the correction for ion hydration did not show significant changes for the NaCl regressed parameters[53]. This is due to large relative hydration of protein that is also absorbed in the macromolecule assumption.

Table 3. Osmotic Pressure of BSA in 0.15M NaF, NaCl, NaI, and NaSCN, pH 7.4, 25°C

NaF		NaCl[a]		NaI		NaSCN	
[BSA] (g/L Soln)	Osmotic Pressure (kPa)	[BSA] (g/L Soln)	Osmotic Pressure (kPa)	[BSA] (g/L Soln)	Osmotic Pressure (kPa)	[BSA] (g/L Soln)	Osmotic Pressure (kPa)
297	67.6	84	6.4	296	93.8	299	69.2
314	62.7	91	7.9	343	183.4	299	69.7
343	115.1	211	44.3	397	259.2	322	71.4
372	123.4	211	44.5	421	283.4	346	94.3
396	172.4	289	113.0	447	316.5	397	114.5
422	202.0	325	133.0	471	347.5	343	173.4
446	253.0	354	190.0	481	409.5	394	267.7
470	293.0	357	218.0	487	413.7	423	293.0
480	346.1	413	349.0	507	474.4	468	403.6
		428	374.0	522	513.0	538	484.7
		448	485.0			515	504.0

a. NaCl data from Yousef et al.[53]

The ion binding and hydration are well within range of values independently determined by others. Studies using water-^{17}O magnetic resonance showed that the hydration for globular proteins is on the order of 1 g H_2O/g protein.[75] The regressed values for hydration in this study

range from 0.64 g H_2O/g BSA to 1.11 g H_2O/g BSA; well within physically realistic values. In addition, as mentioned earlier, Scatchard reported that ion binding for dilute NaCl solutions of human serum albumin (HSA) was 8 mol NaCl/mol HSA, the same as the regressed ion binding using the FSB model for this neutral monovalent salt. Thus, given that the fitted FSB model captures the physics of the osmotic pressure curve for concentration and the hydration and ion binding parameters are physically meaningful, this FSB modeling approach has significant credibility and can be used to glean protein hydration and ion binding relationships for crowded solutions prepared within the approximations for the model.[54]

Two distinct behaviors are seen in Figure 3. NaCl and NaF both show an initial gradual change in osmotic pressure with increasing concentration followed by a more rapid change in osmotic pressure at higher concentrations. NaI and NaSCN have a less aggressive increase in osmotic pressure at the higher concentrations relative to the initial rates of change in lower concentrations. Perhaps due to the noisy NaSCN data, but there was no discernable difference in the osmotic pressure rate of change with respect to concentration for NaI and NaSCN.

Table 4: Regressed Ion Binding and Hydration Parameters from Osmotic Pressure Data

0.15 M Salt, pH 7.4	Ion Binding $\left(\frac{\text{mol Salt}}{\text{mol BSA}}\right)$ ν_{32}	Scatchard Ion Binding $\left(\frac{\text{mol Salt}}{\text{mol HSA}}\right)$ [39]	Hydration $\left(\frac{\text{mol } H_2O}{\text{mol BSA}}\right)$ ν_{12}	Hydration $\left(\frac{\text{g } H_2O}{\text{g BSA}}\right)$	Covariance
NaF	7.4 ± 0.59	N/A	3546 ± 129	0.962 ± 0.035	5.77 x10^{-6}
NaCl	7.7 ± 0.57	8	4102 ± 111	1.113 ± 0.030	6.72 x10^{-6}
NaI	0.8 ± 0.97	11	2407 ± 177	0.643 ± 0.048	2.71 x10^{-5}
NaSCN	2.6 ± 2.11	15	2554 ± 394	0.693 ± 0.107	1.47 x10^{-3}

HSA: human serum albumin

Confidence intervals and parameter sensitivity for each solution can be found in the Supplement.

Ion Binding

While the ion binding for the neutral salt NaCl was consistent with the work of Scatchard and colleagues, the ion binding for NaI and NaSCN were not. Among the three salts NaCl, NaI and NaSCN, Scatchard *et al.* (1949, 1950) showed, using EMF and titration, that HSA has higher binding with thiocyanate ions, followed by iodide with chloride having the lowest ion binding.[39-40, 76] The FSB model shows that for BSA in self-crowded concentrations, the binding is highest for chloride with no appreciable binding for NaI and NaSCN. This may be due to any number of factors or combination thereof including size of hydrated ions or surface charge density. This may also be a result of variation in ion binding at very high concentrations. Scatchard *et al.* (1949, 1950) used protein concentrations up to approximately 300 g/L, much lower than those used in this study. Nevertheless, this observation is consistent with models that consider the hydrated ions as a relatively large sphere that results in a steric hindrance or reduced surface charge

density where ion-binding sites on protein are not fully exposed or when they are in narrow cavities on the protein surface or where the electrostatic interactions are relatively weak.[16, 77]

It should be noted that regression analysis to fit the FSB model to the osmotic pressure data using the ion binding values from Scatchard *et al.* (1950)[39-40] for NaI and NaSCN did not converge. This limitation gives further credibility to the independence of ion binding and hydration in fitting the FSB model.

Protein Hydration

Kosmotropes Have a Significantly Higher Protein Hydration than Chaotropes

The ion binding for Cl^- and Fl^- are within experimental error with data associated with Cl^- having a significantly higher hydration. The hydration for NaCl was 1.113 ± 0.030 g H_2O/g BSA while that for NaF was 0.962 ± 0.035 g H_2O/g BSA. The 12% reduction in hydration from solutions of NaCl to NaF had a substantial shift in the osmotic pressure for the highly self-crowded solution; at 446-448g/L BSA, the osmotic pressure was reduced by nearly half in NaF as compared to NaCl.

Kosmotropes Increase Rate of Change in Osmotic Pressure via Increased Hydration

The NaI and NaSCN results are not significantly different between each other but have significantly lower hydration than NaCl and NaF solution results. However, because of the nearly unobserved ion binding, the osmotic pressure of NaI and NaSCN was over 50 kPa higher than that of NaF. We showed previously that at high self-crowded concentrations, the FSB model predicts that increases in ion binding can deaccelerate the increase in osmotic pressure with respect to increasing concentration.[63]

Hydration Changes Due to Anion Effects May Correlate with SASA Changes

We have previously reported that a monolayer of water on BSA is approximately 1g H_2O/1g BSA when in moderate NaCl at physiological pH.[55] This relationship allows one to approximate the solvent accessible surface area (SASA) for other globular proteins.[60] The regressed values of 0.64 g H_2O/1g BSA and 0.69 g H_2O/1g BSA for NaI and NaSCN, respectively, may imply that the protein reduced in volume or aggregation may be taking place.

Assuming the radius is that of equivalent spheres and there is a monolayer of water in each case, the radius reduction from 1.111 g H_2O/1g BSA to 0.64 g H_2O/1g BSA corresponds to about 24%. Tanford *et al.* (1955) reported a similar increase in BSA radius due to shifts in pH below its isoelectric point. However, in this case, BSA is assumed to be in a compact form at physiological pH that undergoes reversible expansion at low pH.[78] The observed change may more likely be coupled to protein aggregation.[79-81]

It is generally accepted that proteins at high concentrations partially unfold resulting in non-polar interactions that induce aggregation.[80] Aggregation would reduce the SASA and this would be reflected in the regressed hydration number from the FSB model.

We investigate the amount of SASA reduction that would be associated with BSA aggregation using the rigid-body docking tool, ZDOCK.[82] This algorithm considers electrostatic, statistical potential and shape complementary in determining the change in SASA for protein–protein interactions, $\Delta SASA$. The BSA crystal structure was obtained from the Protein Data Bank (PDB ID:4F5S, modified to contain only 1 BSA molecule).[83-84] Initially ZDock was used to predict the docking of two monomers. The top reported results were analyzed by using Chimera (UCSF)

software to determine the SASA for the multimer.[85] A MatLab script (MathWorks, Natick, MA) summed the UCSF Chimera analysis to determine the multimers total available SASA. The monomer and/or a representative sample of the 10 outputs, determined by average available SASA, was used in determination of larger multimers. Table 5 summarizes these results.

ZDock was able solve the SASA for BSA multimers up to octamers. At the octamer level, this corresponds to a 17% decrease in SASA when comparing eight monomers. Thus, the hydration results from regression of the FSB model remain plausible in reference to potential aggregation of self-crowded BSA in these chaotropic solutions.

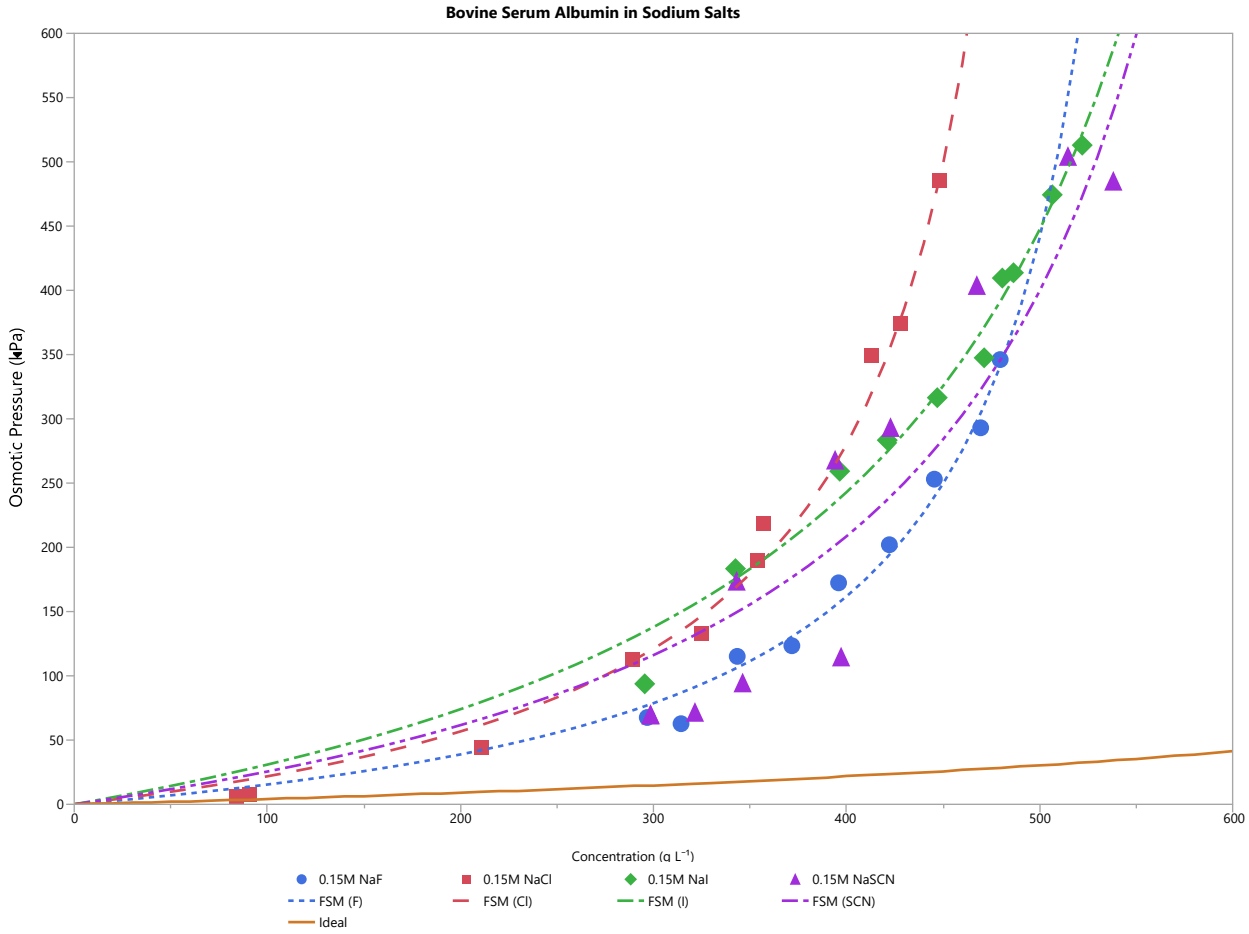

Figure 3: Measured osmotic pressure vs. BSA protein concentration in NaF, NaCl, NaI and NaSCN. The dashed lines present the best-fit FSB model from regressed hydration and ion binding for each of the monovalent sodium salts investigated. As can be seen, the FSB model has an excellent fit for NaF, NaI and NaCl and is acceptable for the relatively noisy NaSCN data. The solution for the ideal model for BSA (no hydration or ion binding) is also shown in the solid line as a reference. Data for the NaCl case is from.[53]

Relevance of Hydration and Ion Binding to the Second Virial Coefficient

The second virial coefficient for osmotic pressure have been traditionally associated with solute-solute interactions and have been found to be dependent of solute concentration.[46] The values of the second virial coefficient do not provide direct insight to the phenomena of protein-protein interaction, however, it is well accepted that, larger second virial coefficients account for larger

aggregation formation (higher multimers).[86] Previously, we showed the relationship between the second virial coefficient and the ionic strength ratio which is determined from the FSB model parameters.[61] The ionic strength ratio, α, is

$$\alpha = \frac{\nu_{32}/\nu_{12}}{M} \tag{7}$$

where *M* is the bulk ionic strength of the solution. Our results showed that the decrease in α corresponds to an increased second virial coefficient, implying increased protein aggregation. Since the values for α for both SCN^- and I^- are significantly lower than those for Cl^- and F^-, this may imply that the chaotropic solutions have higher aggregation.

Hydration Shifts May Be a Consequence of Excluded Volume Effects

Tadeo *et al. (*2007) examined the influence of Hofmeister anions on protein stability using the B1 domain of protein L as their model.[87] They determined that effects of anions did not impact the SASA of the protein domain. Assuming the validity of the regressed hydration values, our results may also be a quantitative representation of an excluded volume effect.[47, 51, 88-92] Nevertheless, these results will be useful as researchers continue to elucidate the complex mechanisms of anions on protein hydration in crowded solutions.

Table 5: Evaluation of $\Delta SASA$ of BSA Multimers

COMPARISON (Δ)	$SASA$ (Å²)	$-\Delta SASA$ (Å²)	$(-\Delta\%)$
Monomer Only	28075	-	-
2 Monomer → Dimer	52519	3631	6.47
4 Monomer → Tetramer	75266	12798	11.40
8 Monomer → Octamer	79096	34260	15.25
8 Monomer → Octamer (2 Tetramers)	99502	34260	17.22

Theoretical Saturation

Because the FSB model provides the hydration of proteins in crowded solutions it can be used to determine a theoretical saturation limit. This theoretical saturation limit is based on the assumptions of the FSB model including negligible variation in hydration with respect to protein concentration. We showed previously that despite the low osmotic pressure profile for lysozyme, it salted out well below the predicted theoretical saturation limit.[55] In any case, regardless of its actual significance, it can be used to qualitatively evaluate anion effects on crowded protein solutions.

The theoretical saturation limit for BSA in each of the monovalent sodium salts was determined using regressed hydration and are presented in Table 6. As can be seen, the theoretical saturation limit follows in the order of $Cl^- < F^- < I^-$ and SCN^-, which is not in the traditional Hofmeister order. The theoretical saturation values for NaF solutions are determined by the highest concentration measured in this study and, from reviewing Figure 3, its value may be substantially lower with osmotic pressure results from higher concentration levels. Nevertheless, it is clear that Cl^- will have the lowest saturation limit which is inconsistent with a Hofmeister order. The model also predicts that NaSCN and NaI solutions have the highest theoretical saturation which implies that

these chaotropes keep proteins in solution longest among the anions studied. However, in this study, actual saturation values were not determined. These results are consistent with the work of Rembert *et al.* (2012) who indicate that SCN^- and I^- keep proteins in solution.[32] However, we have not extrapolated onto BSA their observation (based on their model polypeptide) that these anions interact with a hybrid binding site between amide nitrogen and adjacent α −carbons. Table 6 summarizes the ionic strength ratio for each solution.

Table 6: Calculated Properties from Osmotic Pressure Data

0.15 M Salt, pH 7.4	Local Molarity (M)	Ionic Strength Ratio α	Theoretical Saturation Limit (g/L)
NaF	0.116 ± 0.013	0.772 ± 0.086	589 ± 12
NaCl	0.103 ± 0.010	0.690 ± 0.068	543 ± 8
NaI	0.020 ± 0.022	0.131 ± 0.149	725 ± 25
NaSCN	0.056 ± 0.047	0.372 ± 0.313	700 ± 49

Conclusion

In this work we investigated the FSB osmotic pressure model as a tool to interrogate the anion effects on self-crowded BSA solutions of sodium salts at moderate ionic strength and pH 7.4. The FSB model assumes a Maxwell–Boltzmann distributed ideal relationship between free-solvent and hydrated macromolecules that encompass protein and influenced water and ions. Solutions of NaF, NaCl, NaI and NaSCN were investigated. The results indicate that the NaF and NaCl result in both higher ion binding and hydration than the chaotropic solutions of NaI and NaSCN. The values of hydration for NaI and NaSCN solutions were substantially reduced which may imply increased aggregation or excluded volume. The ion binding for NaI and NaSCN was determined to be negligible compared to solutions of NaF and NaCl. The FSB model continues to require validation. However, it is an effective and complementary tool for probing the effects of anions in crowded protein solutions in terms of the physically realizable parameters, ion binding and protein hydration, especially near saturated conditions.

Acknowledgements

This research was funded by an endowment from Jacques S. Yeager, Sr.

References

1. Hofmeister, F., Zur Lehre von der Wirkung der Salze. *Arch Exp Pathol Phar* **1888,** *24* (4), 247-260.
2. Medda, L.; Barse, B.; Cugia, F.; Boström, M.; Parsons, D. F.; Ninham, B. W.; Monduzzi, M.; Salis, A., Hofmeister Challenges: Ion Binding and Charge of the BSA Protein as Explicit Examples. *Langmuir* **2012,** *28* (47), 16355-16363.
3. Hofmeister, F., Zur Lehre von der Wirkung der Salze. *Arch Exp Pathol Phar* **1888,** *25* (1), 1-30.
4. Traube, J., The Attraction Pressure. *The Journal of Physical Chemistry* **1909,** *14* (5), 452-470.
5. Chaplin, M., Water Structure and Science. 2000.
6. Zhang, Y.; Cremer, P. S., Chemistry of Hofmeister anions and osmolytes. *Annu Rev Phys Chem* **2010,** *61*, 63-83.
7. Omta, A. W.; Kropman, M. F.; Woutersen, S.; Bakker, H. J., Negligible effect of ions on the hydrogen-bond structure in liquid water. *Science* **2003,** *301* (5631), 347-9.
8. Zhang, Y.; Cremer, P. S., Interactions between macromolecules and ions: The Hofmeister series. *Curr Opin Chem Biol* **2006,** *10* (6), 658-63.
9. Smith, J. D.; Saykally, R. J.; Geissler, P. L., The Effects of Dissolved Halide Anions on Hydrogen Bonding in Liquid Water. *J Am Chem Soc* **2007,** *129* (45), 13847-13856.
10. Mancinelli, R.; Botti, A.; Bruni, F.; Ricci, M. A.; Soper, A. K., Hydration of Sodium, Potassium, and Chloride Ions in Solution and the Concept of Structure Maker/Breaker. *The Journal of Physical Chemistry B* **2007,** *111* (48), 13570-13577.
11. Tobias, D. J.; Hemminger, J. C., Chemistry - Getting specific about specific ion effects. *Science* **2008,** *319* (5867), 1197-1198.
12. Zangi, R., Can Salting-In/Salting-Out Ions be Classified as Chaotropes/Kosmotropes? *J Phys Chem B* **2010,** *114* (1), 643-650.
13. Funkner, S.; Niehues, G.; Schmidt, D. A.; Heyden, M.; Schwaab, G.; Callahan, K. M.; Tobias, D. J.; Havenith, M., Watching the Low-Frequency Motions in Aqueous Salt Solutions: The Terahertz Vibrational Signatures of Hydrated Ions. *J Am Chem Soc* **2012,** *134* (2), 1030-1035.
14. Marcus, Y., Electrostriction in Electrolyte Solutions. *Chem Rev* **2011,** *111* (4), 2761-2783.
15. Jones, G.; Dole, M., THE VISCOSITY OF AQUEOUS SOLUTIONS OF STRONG ELECTROLYTES WITH SPECIAL REFERENCE TO BARIUM CHLORIDE. *J Am Chem Soc* **1929,** *51* (10), 2950-2964.
16. Collins, K. D., Charge density-dependent strength of hydration and biological structure. *Biophys J* **1997,** *72* (1), 65-76.
17. Poiseuille, J. L. M., Sur le mouvement des liquides de nature différente dans les tubes de trèspetits diamètres. *Annales de chimie et de physique* **1847**, 76-110.
18. Collins, K. D.; Washabaugh, M. W., The Hofmeister Effect and the Behavior of Water at Interfaces. *Q Rev Biophys* **1985,** *18* (4), 323-422.
19. Collins, K. D., Ions from the Hofmeister series and osmolytes: effects on proteins in solution and in the crystallization process. *Methods* **2004,** *34* (3), 300-311.
20. Zangi, R.; Hagen, M.; Berne, B. J., Effect of ions on the hydrophobic interaction between two plates. *J Am Chem Soc* **2007,** *129* (15), 4678-4686.

21. Waldron, T. T.; Modestou, M. A.; Murphy, K. P., Anion binding to a protein-protein complex lacks dependence on net charge. *Protein Sci* **2003,** *12* (4), 871-874.
22. Ajroud, K.; Sugimori, T.; Goldmann, W. H.; Fathallah, D. M.; Xiong, J. P.; Arnaout, M. A., Binding affinity of metal ions to the CD11b A-domain is regulated by integrin activation and ligands. *Journal of Biological Chemistry* **2004,** *279* (24), 25483-25488.
23. Light, T. P.; Corbett, K. M.; Metrick, M. A.; MacDonald, G., Hofmeister Ion-Induced Changes in Water Structure Correlate with Changes in Solvation of an Aggregated Protein Complex. *Langmuir* **2016,** *32* (5), 1360-1369.
24. Majumdar, R.; Manikwar, P.; Hickey, J. M.; Samra, H. S.; Sathish, H. A.; Bishop, S. M.; Middaugh, C. R.; Volkin, D. B.; Weis, D. D., Effects of Salts from the Hofmeister Series on the Conformational Stability, Aggregation Propensity, and Local Flexibility of an IgG1 Monoclonal Antibody. *Biochemistry* **2013,** *52* (19), 3376-3389.
25. Lo Nostro, P.; Ninham, B. W., Hofmeister phenomena: an update on ion specificity in biology. *Chem Rev* **2012,** *112* (4), 2286-322.
26. Okur, H. I.; Hladilkova, J.; Rembert, K. B.; Cho, Y.; Heyda, J.; Dzubiella, J.; Cremer, P. S.; Jungwirth, P., Beyond the Hofmeister Series: Ion-Specific Effects on Proteins and Their Biological Functions. *J Phys Chem B* **2017,** *121* (9), 1997-2014.
27. Ries-Kautt, M. M.; Ducruix, A. F., Relative effectiveness of various ions on the solubility and crystal growth of lysozyme. *Journal of Biological Chemistry* **1989,** *264* (2), 745-748.
28. Paterova, J.; Rembert, K. B.; Heyda, J.; Kurra, Y.; Okur, H. I.; Liu, W. S. R.; Hilty, C.; Cremer, P. S.; Jungwirth, P., Reversal of the Hofmeister Series: Specific Ion Effects on Peptides. *J Phys Chem B* **2013,** *117* (27), 8150-8158.
29. Nandi, P. K.; Robinson, D. R., Effects of Salts on Free-Energy of Peptide Group. *J Am Chem Soc* **1972,** *94* (4), 1299-&.
30. Nandi, P. K.; Robinson, D. R., Effects of Salts on Free-Energies of Nonpolar Groups in Model Peptides. *J Am Chem Soc* **1972,** *94* (4), 1308-&.
31. Algaer, E. A.; van der Vegt, N. F. A., Hofmeister Ion Interactions with Model Amide Compounds. *J Phys Chem B* **2011,** *115* (46), 13781-13787.
32. Rembert, K. B.; Paterova, J.; Heyda, J.; Hilty, C.; Jungwirth, P.; Cremer, P. S., Molecular mechanisms of ion-specific effects on proteins. *J Am Chem Soc* **2012,** *134* (24), 10039-46.
33. Jungwirth, P.; Cremer, P. S., Beyond Hofmeister. *Nat Chem* **2014,** *6* (4), 261-3.
34. Zhao, H., Are ionic liquids kosmotropic or chaotropic? An evaluation of available thermodynamic parameters for quantifying the ion kosmotropicity of ionic liquids. *J Chem Technol Biot* **2006,** *81* (6), 877-891.
35. Linderstrøm-Lang, K., On the Ionization of Proteins. *Compt. Rend. Trav. Lab. Carlsberg* **1924,** *15* (7), 1-29.
36. Debye, P.; Hückel, E., De La Theorie Des Electrolytes. I. Abaissement Du Point De Congelation et Phenomenes Associes. *Phys Z* **1923,** *24* (9), 185-206.
37. Tanford, C., Preparation and Properties of Serum and Plasma Proteins. XXIII. Hydrogen Ion Equilibria in Native and Modified Human Serum Albumin. *J Am Chem Soc* **1950,** *72* (1), 441-451.
38. Scatchard, G., The Attractions of Proteins for Small Molecules and Ions. *Annals of the New York Academy of Science* **1949,** *51*, 660-672.

39. Scatchard, G.; Scheinberg, I. H.; Armstrong, S. H., Physical Chemistry of Protein Solutions .4. The Combination of Human Serum Albumin with Chloride Ion. *J Am Chem Soc* **1950,** *72* (1), 535-540.
40. Scatchard, G.; Scheinberg, I. H.; Armstrong, S. H., Physical Chemistry of Protein Solutions .5. The Combination of Human Serum Albumin with Thiocyanate Ion. *J Am Chem Soc* **1950,** *72* (1), 540-546.
41. Fox, J. M.; Kang, K.; Sherman, W.; Héroux, A.; Sastry, G. M.; Baghbanzadeh, M.; Lockett, M. R.; Whitesides, G. M., Interactions between Hofmeister Anions and the Binding Pocket of a Protein. *J Am Chem Soc* **2015,** *137* (11), 3859-3866.
42. Ninham, B. W.; Yaminsky, V., Ion Binding and Ion Specificity: The Hofmeister Effect and Onsager and Lifshitz Theories. *Langmuir* **1997,** *13* (7), 2097-2108.
43. Parsons, D. F.; Bostrom, M.; Lo Nostro, P.; Ninham, B. W., Hofmeister effects: interplay of hydration, nonelectrostatic potentials, and ion size. *Phys Chem Chem Phys* **2011,** *13* (27), 12352-67.
44. Zimmerman, S. B.; Trach, S. O., Estimation of macromolecule concentrations and excluded volume effects for the cytoplasm of Escherichia coli. *J Mol Biol* **1991,** *222* (3), 599-620.
45. Reboiras, M. D.; Pfister, H.; Pauly, H., Activity-Coefficients of Salts in Highly Concentrated Protein Solutions .2. Potassium-Salts in Isoionic Bovine Serum-Albumin Solutions. *Biophys Chem* **1986,** *24* (3), 249-257.
46. Vilker, V. L.; Colton, C. K.; Smith, K. A., The osmotic pressure of concentrated protein solutions: Effect of concentration and ph in saline solutions of bovine serum albumin. *J Colloid Interf Sci* **1981,** *79* (2), 548-566.
47. Curtis, R. A.; Prausnitz, J. M.; Blanch, H. W., Protein-protein and protein-salt interactions in aqueous protein solutions containing concentrated electrolytes. *Biotechnol Bioeng* **1998,** *57* (1), 11-21.
48. Wu, J. Z.; Prausnitz, J. M., Osmotic pressures of aqueous bovine serum albumin solutions at high ionic strength. *Fluid Phase Equilibr* **1999,** *155* (1), 139-154.
49. Moon, Y. U.; Curtis, R. A.; Anderson, C. O.; Blanch, H. W.; Prausnitz, J. M., Protein—Protein Interactions in Aqueous Ammonium Sulfate Solutions. Lysozyme and Bovine Serum Albumin (BSA). *J Solution Chem* **2000,** *29* (8), 699-718.
50. Lima, E. R. A.; Biscaia, E. C.; Boström, M.; Tavares, F. W.; Prausnitz, J. M., Osmotic Second Virial Coefficients and Phase Diagrams for Aqueous Proteins from a Much-Improved Poisson−Boltzmann Equation. *The Journal of Physical Chemistry C* **2007,** *111* (43), 16055-16059.
51. Zhou, H.-X.; Rivas, G.; Minton, A. P., Macromolecular Crowding and Confinement: Biochemical, Biophysical, and Potential Physiological Consequences. *Annual Review of Biophysics* **2008,** *37* (1), 375-397.
52. Yousef, M. A.; Datta, R.; Rodgers, V. G. J., Free-solvent model of osmotic pressure revisited: Application to concentrated IgG solution under physiological conditions. *J Colloid Interf Sci* **1998,** *197* (1), 108-118.
53. Yousef, M. A.; Datta, R.; Rodgers, V. G. J., Understanding nonidealities of the osmotic pressure of concentrated bovine serum albumin. *J Colloid Interf Sci* **1998,** *207* (2), 273-282.

54. Yousef, M. A.; Datta, R.; Rodgers, V. G. J., Confirmation of free solvent model assumptions in predicting the osmotic pressure of concentrated globular proteins. *J Colloid Interf Sci* **2001,** *243* (2), 321-325.
55. Yousef, M. A.; Datta, R.; Rodgers, V. G. J., Monolayer hydration governs nonideality in osmotic pressure of protein solutions. *Aiche J* **2002,** *48* (6), 1301-1308.
56. Yousef, M. A.; Datta, R.; Rodgers, V. G. J., Model of osmotic pressure for high concentrated binary protein solutions. *Aiche J* **2002,** *48* (4), 913-917.
57. Wang, Y.; Rodgers, V. G. J., Free-solvent model shows osmotic pressure is the dominant factor in limiting flux during protein ultrafiltration. *J Membrane Sci* **2008,** *320* (1-2), 335-343.
58. Wang, Y. H.; Rodgers, V. G. J., Determining Fouling-Independent Component of Critical Flux in Protein Ultrafiltration Using the Free-Solvent-Based (FSB) Model. *Aiche J* **2010,** *56* (10), 2756-2759.
59. Wang, Y. H.; Rodgers, V. G. J., Electrostatic contributions to permeate flux behavior in single bovine serum albumin ultrafiltration. *J Membrane Sci* **2011,** *366* (1-2), 184-191.
60. McBride, D. W.; Rodgers, V. G. J., Obtaining protein solvent accessible surface area when structural data is unavailable using osmotic pressure. *Aiche J* **2012,** *58* (4), 1012-1017.
61. McBride, D. W.; Rodgers, V. G. J., Interpretation of negative second virial coefficients from non-attractive protein solution osmotic pressure data: An alternate perspective. *Biophys Chem* **2013,** *184*, 79-86.
62. McBride, D. W.; Rodgers, V. G. J., Predicting the Activity Coefficients of Free-Solvent for Concentrated Globular Protein Solutions Using Independently Determined Physical Parameters. *Plos One* **2013,** *8* (12).
63. McBride, D. W.; Rodgers, V. G. J., A generalized free-solvent model for the osmotic pressure of multi-component solutions containing protein-protein interactions. *Math Biosci* **2014,** *253*, 72-87.
64. Frazer, J. C. W.; Myrick, R. T., The osmotic pressure of sucrose solutions at 30 degrees. *J Am Chem Soc* **1916,** *38*, 1907-1922.
65. Lotz, P.; Frazer, J. C. W., The osmotic pressures of concentrated solutions of sucrose as determined by the water interferometer. *J Am Chem Soc* **1921,** *43*, 2501-2507.
66. Scatchard, G., Physical Chemistry of Protein Solutions .1. Derivation of the Equations for the Osmotic Pressure. *J Am Chem Soc* **1946,** *68* (11), 2315-2319.
67. Glasstone, S., *Textbook of Physical Chemistry*. D. Van Nostrand Co., Inc.: Toronto, 1946.
68. Eagland, D., Nucleic Acids, Peptides, and Proteins. In *Water A Comprehensive Treatise: Aqueous Solutions of Amphiphiles and Macromolecules*, Franks, F., Ed. Springer US: Boston, MA, 1975; pp 305-518.
69. Tombs, M. P.; Newsom, B. G.; Wilding, P., Protein Solubility - Phase Separation in Arachin-Salt-Water Systems. *Int J Pept Prot Res* **1974,** *6* (4), 253-277.
70. Ohtaki, H.; Radnai, T., Structure and dynamics of hydrated ions. *Chem Rev* **1993,** *93* (3), 1157-1204.
71. Kiriukhin, M. Y.; Collins, K. D., Dynamic hydration numbers for biologically important ions. *Biophys Chem* **2002,** *99* (2), 155-168.

72. Lin, S.; Jordan, P. C., Structures and Energetics of Mono-Valent Ion Water Microclusters .2. Thermal Phenomena. *J Chem Phys* **1988,** *89* (12), 7492-7501.
73. Marcus, Y., Prediction of salting-out and salting-in constants. *J Mol Liq* **2013,** *177*, 7-10.
74. Collins, K. D., Why continuum electrostatics theories cannot explain biological structure, polyelectrolytes or ionic strength effects in ion-protein interactions. *Biophys Chem* **2012,** *167*, 43-59.
75. Rupley, J. A.; Careri, G., Protein hydration and function. *Adv Protein Chem* **1991,** *41*, 37-172.
76. Scatchard, G.; Black, E. S., The Effect of Salts on the Isoionic and Isoelectric Points of Proteins. *J Phys Colloid Chem* **1949,** *53* (1), 88-99.
77. Rembert, K. B.; Okur, H. I.; Hilty, C.; Cremer, P. S., An NH moiety is not required for anion binding to amides in aqueous solution. *Langmuir* **2015,** *31* (11), 3459-64.
78. Tanford, C.; Buzzell, J. G.; Rands, D. G.; Swanson, S. A., The Reversible Expansion of Bovine Serum Albumin in Acid Solutions1. *J Am Chem Soc* **1955,** *77* (24), 6421-6428.
79. Morris, A. M.; Watzky, M. A.; Finke, R. G., Protein aggregation kinetics, mechanism, and curve-fitting: A review of the literature. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **2009,** *1794* (3), 375-397.
80. Chan, P.; Curtis, R. A.; Warwicker, J., Soluble expression of proteins correlates with a lack of positively-charged surface. *Sci Rep-Uk* **2013,** *3*.
81. Kastelic, M.; Kalyuzhnyi, Y. V.; Hribar-Lee, B.; Dill, K. A.; Vlachy, V., Protein aggregation in salt solutions. *Proceedings of the National Academy of Sciences* **2015,** *112* (21), 6766.
82. Pierce, B. G.; Wiehe, K.; Hwang, H.; Kim, B.-H.; Vreven, T.; Weng, Z., ZDOCK server: interactive docking prediction of protein–protein complexes and symmetric multimers. *Bioinformatics* **2014,** *30* (12), 1771-1773.
83. Berman, H. M.; Westbrook, J.; Feng, Z.; Gilliland, G.; Bhat, T. N.; Weissig, H.; Shindyalov, I. N.; Bourne, P. E., The Protein Data Bank. *Nucleic Acids Res* **2000,** *28* (1), 235-242.
84. Bujacz, A., Structures of bovine, equine and leporine serum albumin. *Acta crystallographica. Section D, Biological crystallography* **2012,** *68* (Pt 10), 1278-89.
85. Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E., UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* **2004,** *25* (13), 1605-12.
86. Alford, J. R.; Kendrick, B. S.; Carpenter, J. F.; Randolph, T. W., Measurement of the second osmotic virial coefficient for protein solutions exhibiting monomer-dimer equilibrium. *Anal Biochem* **2008,** *377* (2), 128-33.
87. Tadeo, X.; Pons, M.; Millet, O., Influence of the Hofmeister Anions on Protein Stability As Studied by Thermal Denaturation and Chemical Shift Perturbation. *Biochemistry* **2007,** *46* (3), 917-923.
88. Eggers, D. K.; Valentine, J. S., Crowding and hydration effects on protein conformation: A study with sol-gel encapsulated proteins. *J Mol Biol* **2001,** *314* (4), 911-922.
89. Zhou, H. X., Protein folding and binding in confined spaces and in crowded solutions. *J Mol Recognit* **2004,** *17* (5), 368-375.

90. Imai, T.; Harano, Y.; Kinoshita, M.; Kovalenko, A.; Hirata, F., A theoretical analysis on hydration thermodynamics of proteins. *J Chem Phys* **2006,** *125* (2).
91. Jiménez, M.; Rivas, G.; Minton, A. P., Quantitative Characterization of Weak Self-Association in Concentrated Solutions of Immunoglobulin G via the Measurement of Sedimentation Equilibrium and Osmotic Pressure. *Biochemistry* **2007,** *46* (28), 8373-8378.
92. Scherer, T. M., Cosolute Effects on the Chemical Potential and Interactions of an IgG1 Monoclonal Antibody at High Concentrations. *J Phys Chem B* **2013,** *117* (8), 2254-2266.