Beyond the Hofmeister Series: Ion Specific Effects on Proteins and Their Biological Functions

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ABSTRACT

Ions differ in their ability to salt out proteins from solution as expressed in the lyotropic or Hofmeister series of cations and anions. Since its first formulation in 1888, this series has been invoked in a plethora of effects, going beyond the original salting-out/salting-in idea to include enzyme activities and the crystallization of proteins, as well as to processes not involving proteins like ion exchange, the surface tension of electrolytes, or bubble coalescence. Although it has been clear that the Hofmeister series is intimately connected to ion hydration in homogeneous and heterogeneous environments and to ion pairing, its molecular origin has not been fully understood. This situation could have been summarized as follows: Many chemists used the Hofmeister series as a mantra to put a label on ion specific behavior in various environments, rather than to reach a molecular level understanding and, consequently, an ability to predict a particular effect of a given salt ion on proteins in solutions. In this Feature Article we show that, the cationic and anionic Hofmeister series can now be rationalized primarily in terms of specific interactions of salt ions with the backbone and charged side chain groups at the protein surface in solution. At the same time, we demonstrate the limitations of separating Hofmeister effects into independent cationic and anionic contributions due to the electroneutrality condition, as well as specific ion pairing, leading to interactions of ions of opposite polarity. Finally, we outline the route beyond Hofmeister chemistry in the direction of understanding specific roles of ions in various biological functionalities, where generic Hofmeister-type interactions can be complemented or even overruled by particular steric arrangements in various ion binding sites.

Introduction

Some salts are good at precipitating proteins from aqueous solutions, while others are not. Why is this the case? What is it, beyond the charge of the salt ions (the absolute value of which is the same for all monovalent salts), that determines the protein salting out ability of a particular salt? Are the chemical details of the interactions of ions with water and with each other crucial? Or, is Hofmeister series chemistry more about the specific interactions of individual salt ions with the surfaces of aqueous proteins?

Here, we address the above questions, combining molecular level computer modeling and spectroscopic techniques as well as thermodynamic considerations in order to obtain a scale-bridging (from molecular to macroscopic) understanding of specific ion effects on proteins in aqueous solution. Achieving this goal allows us not only to address problems concerning the salting out of proteins, but also sheds light on other issues such as salt effects on protein stability and denaturation or enzymatic activity. Before getting into the technical details, it is important first to introduce the history of studies concerning ion specific effects on proteins, which started in the German part of the Charles University in Prague in the 1880s with Franz Hofmeister. Below, we build on previous reviews of this history[1-15] and walk the reader through the developments, which eventually led to today's molecular level understanding of the Hofmeister series (Figure 1).

Hofmeister and his collaborators summarized their investigations of ion specific effects in a series of seven articles published in the German literature between 1887 and 1898. The two most important ones, i.e., the second paper entitled "About regularities in the protein precipitating effects of salts and the relation of these effects with the physiological behavior of salts"[16] and the third publication entitled "About the water withdrawing effect of the salts"[17] were translated into

English about a dozen years ago. [18] The extensive studies of the salting out of proteins and other substances by Hofmeister were ingenious in several respects. He was the first person to quantify salting out effects systematically for a whole set of salts (later called the Hofmeister series, see Figures 1 and 2). Moreover, he employed several series of salts with a common cation (or anion), allowing for the construction of separate Hofmeister series for anions and cations, as we know it today (Figure 2). It is worth mentioning that his first studies on the subject appeared only a few years after Arrhenius came up with the idea that salts actually dissociate into ions in water.[19] Hofmeister aimed at categorizing the salts, but also the species being salted out, encompassing several proteins, as well as other species, such as gelatin, colloidal ferric oxide, and sodium oleate.[16, 17] Based on these studies, he proposed a varying "water withdrawing effect" of different salts, which he tried to link directly to their salting out ability.[16, 17]

Hofmeister's (over)ambitious goal to rationalize specific ion effects on general solutes in terms of the interactions of salt ions with water was subsequently adopted by proponents of the picture of "kosmotropes" and "chaotropes".[20, 21] According to this view, the former group of ions, such as fluoride or sulfate, bring order (kosmos) to the solution and can organize several layers of water molecules around themselves, effectively "stealing" water from the solute, thus being efficient for salting out. In contrast, the latter ions, like iodide, perchlorate, or thiocyanate, do not possess this ability and thus are not effective salting out agents. This explanation of the Hofmeister phenomena is appealing because of its simplicity; however, it brings in serious problems. First, a quick glance at the Hofmeister series (Figure 2) shows that while this rationalization might work for anions, it fails for cations. Indeed, it is the "chaotropic" cations like ammonium, which are on the salting out side of the series, and not the "kosmotropic" ones, like magnesium or calcium. Second, there is mounting experimental and computational evidence that even strongly hydrated

ions at physiological (and higher) ionic strengths do not significantly influence water beyond their immediate solvation shells.[22-24] Therefore, the whole concept of "kosmotropes" and "chaotropes" may need to be set aside. Finally, Nature itself provides direct evidence that salting out behavior cannot be explained by considering ions and water only and that the protein solute needs to be brought explicitly into the picture. The most notable example in this respect is lysozyme, which salts out of solution according to the Hofmeister series only at basic pH values and high ionic strength, but follows a *reversed* series under neutral and acidic conditions up to moderate salt concentrations.[25, 26, 27]

The last point clearly demonstrates that not only the hydration properties of salt ions but also their interactions with protein surfaces needs to be understood in order to rationalize the Hofmeister series. This has been recognized since the 1960s and reductionist models of protein surface groups have been proposed for the interactions of salt ions in water probed by various thermodynamic and spectroscopic techniques.[28-31] The picture emerging from these studies, which focused primarily on the protein backbone, is that the amide group interacts favorably with weakly hydrated anions (e.g., bromide, iodide, perchlorate, or thiocyanate) and, to a much lesser extent, with strongly hydrated cations (like lithium, magnesium, or calcium). It follows from simple thermodynamic considerations that attractive ion-backbone interactions lead to salting in (and destabilization) of the protein. This implies a weaker salting out (and stabilization) ability for ion more strongly partitioned to the protein surface,[32] which puts the above results in accord with the Hofmeister series (Figure 2).

Recent work, for which the term "Renaissance for Hofmeister" has been coined,[33] builds on the above pioneering studies and turns attention to the specific groups presented at protein surfaces. As such, a quantitative view of ion-protein interactions in aqueous solutions is beginning

to take shape.[4, 9, 13, 32, 34-45] In this Feature Article, our goal is to summarize the current understanding of the molecular origins of Hofmeister ordering for ions at protein surfaces and to link it to macroscopic behavior. At the same time, we explore the limitations of classifying salt effects on proteins into separate anionic and cationic series and propose moving "Beyond Hofmeister",[13] in the direction of systematic investigations of specific ion effects on biological function.

Methodology

Molecular Dynamics Simulations

Molecular dynamics (MD) simulations can provide insight into ion-protein interactions in aqueous solutions with unprecedented spatial (and temporal) resolution that is otherwise extremely difficult to obtain by experiments alone. Indeed, MD simulations allow scientists to follow the motions of each individual atom of the system indetail.[46, 47] There are, however, two potential problems. The first one concerns obtaining statistically significant data. This is typically not a crucial issue in rather concentrated aqueous salt solutions (such as those of alkali cations corresponding to physiological conditions) where most ion-protein distributions converge at computationally accessible sub-microsecond timescales.[48] Moreover, a reductionist approach allows us in many instances to work with small molecules carrying the crucial functional groups as proxies to larger proteins, which further simplifies the calculations and speeds up convergence.[44] The situation may be more complicated for more strongly binding polyvalent ions, such as calcium or magnesium. In these cases, convergence of ion-protein functional group interactions can be enhanced by moving to concentrations that are higher than those at standard physiological

conditions and/or by employing dedicated free energy methods (such as umbrella sampling) rather than performing brute force direct simulations.[49]

The second issue concerns the accuracy of the interaction potentials employed for ions, water, and proteins when using common force fields. It is clear that the final result can only be as good as the underlying potential. Standard non-polarizable force fields often provide a satisfactory description of aqueous proteins and simple ions, such as sodium, potassium, or chloride. However, they tend to overestimate ion-protein and ion-peptide interactions for highly charged ions like divalent magnesium and calcium or trivalent lanthanides,[50] while underestimating interactions with proteins or their proxies for soft (polarizable) anions, e.g., thiocyanate.[44] Improvement can often be achieved by including electronic polarization effects either explicitly by employing a polarizable force field[51] or implicitly by scaling the ionic charges and adjusting the ionic radii.[52] In these more difficult cases, it is particularly important to benchmark the results for model systems against structural experiments (such as neutron or X-ray scattering) and/or ab initio MD simulations explicitly treating the electronic structure.[53] Luckily, it is now becoming computationally feasible to statistically converge interactions between biologically relevant ions and charged side chains or backbone groups in water by using density functional theory methods.[54]

Experimental Techniques

From the experimental point of view, a multi instrumental approach (described in detail below) has been adopted to probe the three main components of the macromolecular interfaces - the water molecules, the macromolecules, and the ions in solution. Macromolecular hydration and the specific changes caused by ion absorption were explored via vibrational sum frequency spectroscopy (VSFS), along with ATR-FTIR and NMR techniques. The systems were tuned by

systematically varying specific functional groups. Furthermore, the lower critical solution temperature (LCST) of numerous thermoresponsive polymers and polypeptides was also investigated in order to understand the effects of salts on macroscopic behavior. Thermodynamic information about the polymer transition process near the LCST was obtained by using differential scanning calorimetry[55-57] and isothermal titration calorimetry.[58]

Probing Macromolecular Hydration: Vibrational Sum Frequency Spectroscopy (VSFS) Measurements

A detailed description of the VSFS system, data fitting and analysis protocols can be found elsewhere.[59-62] Briefly, a 1064nm Nd:YAG laser was employed as the fundamental beam with an output power of 50 mJ with a 17 ps pulse duration. This fundamental beam past through an optical path including an optical parametric generator/amplifier (OPG/OPA) stage in which a 532 nm visible and a tunable infrared (2000 cm^{-1} – 4000 cm^{-1}) beam were generated. Based on the dipole approximation, the spatially and temporarily overlapped beams generated a sum frequency signal that was surface specific.[63] Over the last three decades, this method has been used to probe the vibrational spectrum of various interfaces including the air (or substrate or oil)/water[64-66] and air/macromolecule/water interfaces.[45] The latter have been specifically exploited to probe Hofmeister effects. Measurements were made as a function of salt identity and concentration to achieve molecular level insights into ion - macromolecule interactions. In a typical experiment, model macromolecules were dissolved in aqueous solution at the desired salt concentration and introduced into a Langmuir trough. The hydrophobic moieties of the macromolecules partitioned to the air/water interface to form a Gibbs monolayer and the VSFS spectrum of the air/macromolecule/water interface was measured in the 2800 – 3800 cm^{-1} spectral window including the C-H, O-H, and N-H stretch modes using the ssp polarization combination (s- sum

frequency, s-visible, and p- infrared). Such a polarization combination provides signal contributions for vibrational modes that oscillate parallel to the surface normal. Namely, the C-H modes pointing towards the air and the aligned interfacial macromolecular hydration water molecules were the main components of each spectrum. Such hydration layer-specific spectra are rich in information, as shown in the Results and Discussion section. Moreover, additional spectroscopic techniques like NMR were utilized to obtain site-specific information.

Probing Specific Moieties on Macromolecules: NMR and Attenuated Total Reflection (ATR)-FTIR

Two techniques have been used to probe specific chemical moieties on polypeptides/acrylamide polymers. First, proton (H)-NMR measurements as a function of salt concentrations helped to elucidate ion specific chemical shifts for C-H and N-H residues on macromolecules. The details of these measurements can be found elsewhere.[44] Briefly, all spectra were acquired on a 400 MHz NMR spectrometer equipped with a 5 mm TXI probe at a temperature below the lower critical solution temperature (LCST) of the thermoresponsive macromolecules. The chemical shift assignments of the desired macromolecules, [1H,1H]-NOESY and [1H,1H]-TOCSY, were employed. The H-NMR spectra were acquired using Watergate for water suppression[67] for all experiments. It was also verified that there were no measurable peak shifts as a result of this suppression profile. Furthermore, sample solutions were externally referenced to sodium 2,2-dimethyl-2-silapentane-5-sulfonate in pure D_2O. The chemical shift of each proton on the macromolecule was monitored as a function of both salt identity and concentration. This provided site specific information on ion-macromolecule interactions. The change in the chemical shift with increasing salt concentration was fit to an empirical equation in the form of a linear term and a term containing a Langmuir binding isotherm.

In order to probe the amide oxygen for specific cation interactions, attenuated total reflection (ATR)-FTIR technique was employed. The details of our ATR-FTIR system can be found elsewhere.[68] In this case, a Nicolet 470 FTIR spectrometer was used, which was equipped with a Pike Miracle ATR attachment containing a single-bounce ZnSe crystal. A liquid nitrogen cooled MCT detector was utilized to measure the infrared signal. A sample spectrum was collected at 2 cm^{-1} resolution over a window from 1000 to 4000 cm^{-1}. An otherwise identical salt solution was employed without the model amide molecule, butyramide, was used to obtain background spectra.

Protein/Polymer Solubility Measurements

Solubility measurements were performed in order to probe the macroscopic behavior of biomacromolecules, i.e., polymers/polypeptides, and proteins. The LCST values of thermoresponsive polypeptides and proteins were measured as a function of salt identity and concentration to explore ion specific effects. Poly (*N*-isopropyl acrylamide) (PNIPAM)[69-71] and poly (*N, N* diethyl acrylamide) (PDEA),[72] along with neutral,[44, 73] positively and negatively[74] charged elastin-like polypeptides (ELPs) and lysozyme[26] were utilized as model biomacromolecules. The salt specific LCST curves were modeled as a function of salt concentration by using the following empirical equations:

$$T = T_0 + c[M] + \frac{B_{max}[M]}{K_D + [M]} \quad (1)$$

$$T = T_0 + c[M] + \frac{B_{max}[M]e^{-b[M]^k}}{K_D + [M]e^{-b[M]^k}} \quad (2)$$

The first equation models ion interactions with neutral biomacromolecules, while the second model also includes electrostatic charge neutralization interactions. These equations have a linear

term and a Langmuir binding isotherm where T_0 is the phase transition temperature for the macromolecules in the absence of any added salts and [M] is the molar salt concentration. The constants B_{max} and c have units of temperature (ºC) and ºC/[M], respectively. The B_{max} constant denotes the maximum change in the LCST value upon ion binding and c refers to the linear portion of the change in the phase transition temperature. In the second equation, the constant b has units of inverse molarity and is related to the strength of the electrostatic interaction between the charged macromolecules and the ions. It has been observed phenomenologically that the constant, k, has a value of 1 for anions binding to positively charged macromolecules and 2 for cation binding to negatively charged ones. These two empirical equations have been shown to describe ion-macromolecule interactions rather well. [26, 74] The thermodynamic origins of these models are discussed below.

Solution Theory and Thermodynamic Models

A complete understanding of the effects of salts on proteins not only requires molecular insights, but also scale-bridging models that allow one to connect microscopic interactions and structures to measurable macroscopic observables, such as unfolding (melting) temperatures or LCST values as well as solvation or association free energies. To this end, the fluctuation theory of solutions can be employed as a starting point.[75-78] From this link between statistical mechanics and thermodynamics, the average solution structure in terms of the radial distribution function can be integrated via excess adsorption or preferential binding coefficients, $\Gamma_{ps} = \left(\frac{\partial m_s}{\partial m_p}\right)_{T,\mu_w,\mu_s} = -\left(\frac{\partial \mu_p}{\partial \mu_s}\right)_{T,\mu_w,m_p}$. This makes it possible to determine thermodynamic properties, such as changes in the chemical potentials of individual species (w - water, p – protein/polymer/solute, and s – salt;

m represents molality units). In particular, for proteins in a mixed solvent, these correspond to changes in the relative thermodynamic stabilities of their respective equilibrium states upon the addition of salt. The central outcome[77] of this theory connects the transition free energy $\Delta G(T, c_s)$ at a temperature *T* and a salt concentration c_s of two states (e.g., monomer vs. dimer, $2M \leftrightarrow D$, or the folded vs. the unfolded state of the protein, $F \leftrightarrow U$) to the change in the preferential binding coefficient $\Delta\Gamma = \Gamma_{dimer} - \Gamma_{monomer}$, or $\Delta\Gamma = \Gamma_{unfolded} - \Gamma_{folded}$ via

$$\frac{\partial \Delta G(T, c_s)}{\partial c_s} = -k_B T \frac{\Delta \Gamma(c_s)}{c_s} a_{ss}(c_s). \quad (3)$$

Here, Γ_i characterizes the excess adsorption of a salt over that of water at the protein surface in the respective protein state *i* (dimer/monomer or unfolded/folded) and is in principle directly accessible from molecular simulations. Bulk thermodynamics comes in as the solution non-ideality via $a_{ss} = \left(\frac{\partial ln a_s}{\partial ln c_s}\right)_{T,p}$, where a_s represents the salt activity. Eq. (3) bridges microscopic and thermodynamic behavior and thus enables insights to be obtained from atomistic computer simulations and macroscopic experiments.

It has been shown recently that the connection to experiments can be made in a direct way if the response of the two-state transition free energy $\Delta G(T)$ to the perturbation by salt is evaluated in more detail close to the transition temperature of the pure water reference state.[75, 79] T_0 is a constant defined as the temperature at which the populations of the two states are equal to one another in the absence of salt, such that $\Delta G(T_0) = \Delta H_0 - T_0 \Delta S_0 = 0$, where ΔS_0 is the transition entropy. A Taylor expansion of $\Delta G(T, c_s)$ about this reference state (i.e., temperature T_0 and c_s = *0 M*) in the variables c_s and *T* leads to an explicit expression for the change in the transition temperature[75]

$$\Delta T(c_s) = -\frac{mc_s + \frac{1}{2}m'c_s{}^2}{\Delta S_0 + \Delta S'_0\, c_s}. \quad (4)$$

This change is a function of salt concentration and thermodynamic coefficients $m = -\left(\frac{\partial \Delta G}{\partial c_s}\right)_{T=T_0}, m', \ \Delta S_0 = -\left(\frac{\partial \Delta G}{\partial T}\right)_{c_s=0}$, and $\Delta S'_0$, where the primes denote derivatives with respect to c_s. Of particular importance is the parameter m which is related to the well-known 'm-value' that has been traditionally used to describe linear cosolute effects on protein folding, $\Delta G^{F\leftrightarrow U}(c_s) = \Delta G^{F\leftrightarrow U}_{H_2O} - mc_s$.[80-83] The m-value is known to have a negative value for stabilizing/salting out (i.e., from the protein surface excluded) salts and a positive value for destabilizing/salting in (i.e., protein-attracted) salts.[80] Within our picture, *m* describes linear changes for small c_s while the parameter m' accounts for higher-order nonlinear effects of salts. Importantly, through eq.(3), both parameters are directly related to the simulation-accessible preferential binding coefficient $\Delta\Gamma$.[75] $\Delta S'_0$ describes the effect of the salt on the transition entropy. Due to the symmetry of mixed derivatives in the Taylor-expansion, the latter is the same as the temperature derivative of the parameter *m*.

We note that eqn (4) is mathematically equivalent to eqn (1), which empirically combines a linear part with a Langmuir-type binding isotherm.[70, 84] Eqn (4) can also be extended to approximately include electrostatic interactions between the ions and charged macromolecules in the limit of small charges and high screening, [79] resulting in a different form than eqn (2).

The significance of eqn (4) is that we can now determine the leading order thermodynamic coefficients of salt-induced changes by fitting to experimental $\Delta T(c_s)$ curves, e.g., via LCST data and directly linking them through the preferential binding parameter to microscopic ion-protein adsorption structures, which are accessible through computer simulations. Hence, this gets us

closer to achieving a multiscale picture of salt-induced effects on macromolecular solubility and stability, connecting microscopic structures to macroscopic phase behavior.

Results and Discussion

Recent studies have demonstrated that the interactions of anions and cations with chemically diverse protein surface groups need to be individually considered and understood in order to rationalize the Hofmeister series.[13] Such microscopic information is accessible by computer simulations based on physically reasonable and sufficiently accurate force field parameters, allowing for the experimental data to be rationalized consistently and with high information content. Moreover, although cationic and anionic effects are often discussed separately, electroneutrality requires that the two types of ions have inseparable behavior on a global scale. Similarly, only the effects for the whole protein (i.e., not individual surface patches) are measured in thermodynamic experiments. Nevertheless, such observations result from an interplay of individual local interactions, which calls for a detailed molecular level understanding of the dominant players.[11, 15, 39] In the following discussion we thus dissect the protein surface into its major building blocks that are relevant for salt-protein interactions. Namely, we consider the protein backbone, the negatively and positively charged side chains, as well as the hydrophobic and polar side chains.

This reductionist approach to ion-protein interactions builds upon earlier studies of ions at more homogeneous aqueous interfaces. After investigations of ions at the water/vapor and water/oil interfaces,[85, 86] more complex model surfaces were considered. In particular, considerable insight and generic rules were obtained via MD simulation studies of salt interactions with functionalized monolayers.[11, 15, 87] Varying the surface charge and polarity, different rank orderings

have been found for cations and anions, providing a rationalization for the complexity of the Hofmeister series.[11, 15, 87] These studies prove that ion-specificity appears even at more uniform surfaces containing the same functional groups present in proteins. In other words, Hofmeister ordering clearly persists beyond proteins, which were designed by natural selection over millions of years.

Ions at the protein backbone – direct Hofmeister series

Molecular Dynamics Simulations

The fact that proteins contain a wide range of sizes and shapes, and contain charged, polar, and hydrophobic side chain ratios has led many researchers to assume that the Hofmeister ordering of ions may be driven by some universal and ubiquitous feature of proteins. A natural candidate is the peptide bond at the protein backbone. Indeed, both cations and anions follow the Hofmeister series at the peptide bond, which has been documented by a number of MD simulation studies of proteins,[35, 40] short peptides (such as triglycine)[44, 88, 89] polymers/polypeptides PNIPAM, ELPs,[44] as well as by studies of small molecular systems such as *N*-methyl acetamide (NMA). [42, 43] Specifically, weakly hydrated anions and strongly hydrated cations are attracted to the protein backbone. In general, interactions of anions like iodide, thiocyanate, and perchlorate with the N-H end of the peptide bond, and the adjacent methylene groups are found to be stronger than those of cations such as sodium, lithium, or calcium with the adjacent C=O group.[44]

Experiments

Experimental techniques which work over a variety of length scales (both microscopic and macroscopic ones) are required to obtain a full molecular level picture of the influence of salt ions

on biomacromolecules.[44] First, the hydrocarbon protons on ELP $(VPGVG)_{120}$ were monitored at each site with proton NMR as a function of salt concentration. It was found that only the weakly hydrated anions (SCN^- and I^-) influenced the chemical shifts in a non-monotonic fashion and only for protons on carbons adjacent to electron withdrawing groups. The most favorable interactions on polypeptide backbones occurred for a hybrid binding site that consisted of the amide nitrogen and the adjacent α-carbon. The apparent dissociation constants (K_D), achieved from the nonlinear change in the chemical shifts, was shown to be as tight as 50 mM between a thiocyanate anion and this site. (see Figure 3A) The reason is that the CH moieties for these α-carbon positions maintained a partial positive charge, which led to the binding of weakly hydrated anions. By contrast, the hydrophobic side chains of isopropyl groups of the valine residues did not show any anion binding. In thermodynamic measurements, the K_D's values found with these polymers (e.g. ELPs, poly(*N*-isopropyl acrylamide), or poly *N, N* diethyl acrylamide) are typically hundreds of mM, which necessarily represents an average over the varying sites on the macromolecules.[44, 62, 69, 70, 72, 73, 82, 87] Interestingly, the presence of a partially positively charged hydrogen from the amide N-H groups are not required for this binding to occur, although they may slightly contribute when present.[72]

Ion specific effects at the protein backbone were also examined at the air/biomacromolecule/water interface via vibrational sum frequency spectroscopy. In these studies, ELP $(VPGVG)_{120}$ and poly(*N*-isopropyl acrylamide), see insets of Figure 3A and Figure 3B, were employed as model macromolecules and the vibrational resonances of the interfacial water molecules were probed. Figure 3B plots the vibrational spectra of a Gibbs monolayer for PNIPAM in the present of 1M sodium salts. Significantly, a substantial enhancement in the oscillator strength of the water OH stretch bands (3200 cm^{-1} and 3400 cm^{-1})

in the VSFS experiments was observed upon the introduction of weakly hydrated anions. This increase followed a direct anionic Hofmeister series for the air/PNIPAM/water interface. This signal is direct evidence of anionic absorption to the macromolecules over the sodium counter cations, which leads to an alignment of interfacial water molecules with respect to the surface normal and, in turn, goes a rise in the VSFS signal.[59, 61] The change in the VSFS OH stretch spectrum upon the addition of strongly hydrated anions was much smaller, which is evidence that these anions are not preferentially partitioned to the interface. The ion specific trends obtained from the interfacial water signal are in agreement with the idea that weakly hydrated anions partition to an interface containing an amide moiety, much like the backbone of proteins.

Cation interactions with amide moieties were also explored employing the small molecule of butyramide (inset in Figure 3C). A series of aqueous metal chloride salt solutions were employed in combination with ATR-FTIR to monitor the contact pair formation between the metal cations and the amide oxygen. Figure 3C shows ATR-FTIR spectra of butyramide in the amide I region, in the absence and presence of 5 M salt concentrations. No apparent change in the amide I band (1620 cm^{-1}) could be seen in the presence of weakly hydrated cations even with 5 M concentration of their chloride salts (Figure 3C i and ii compare no salt with 5 M NaCl). In sharp contrast, molar concentrations of more strongly hydrated cations (Ca^{2+}, Mg^{2+}, and Li^{+}) gave rise to a new peak at 1645 cm^{-1}, assigned to metal cation- contact-pair bound amides, (data with 5 M $CaCl_2$ in Figure 3C iii). Note that this binding is relatively weak and that only 30% of the binding sites are occupied in salt solutions even at 5 M $CaCl_2$, which is near the salt solubility limit. As such, the apparent equilibrium dissociation constants should be no tighter than ~8.5 M. [45]

In a complementary set of experiments, the interactions of cations with amide moieties were investigated at the air/butyramide/water interface via VSFS. Such experiments were sensitive

to the interfacial cation partitioning for not only contact pair formation, but also solvent shared and solvent separated pairs. Figure 3D displays VSFS spectra in the CH and OH stretch regions for interfacial butyramide molecules and their adjacent water structure in the presence of various metal chloride salts in the subphases. The sharp vibrational resonances in the 2800 – 3000 cm^{-1} region are from the CH stretch bands of the butyramide molecule, while the broader bands in the higher frequency region come from NH and OH stretches. Specifically, the water OH stretch peak (3200 cm^{-1}) was strongly enhanced by the preferential binding of strongly hydrated cations. Whereas, this same peak remained essentially unchanged for the chloride salts of weakly hydrated cations. This data suggested that only strongly hydrated cations have preferential absorption over their respective counter anion. [45] Thus, the hydration data is in good agreement with a direct cationic Hofmeister series. The data from Figure 3B and 3D, for anions and cations respectively, demonstrate that weakly hydrated anions bind tighter than strongly hydrated cations to interfaces with amide moieties. Interestingly, this is not necessarily the case for small molecules, such as NMA or triglycine, where cation binding is often found to be stronger. [88, 90, 91]

Although cationic affinities to the peptide bond were found to be relatively weak compared to anion binding, they may play a role in affecting the stability of secondary structure elements of proteins, such as α-helices or β-sheets, where the ions and water molecules compete with intra-chain backbone hydrogen bonds. Structural stabilities of oligopeptides of various polarity, ranging from hydrophobic,[92] over neutral but polar, to highly charged,[93, 94] and of complex compositions (AK[95, 96], AE[97], EK[95, 98], polyGLU[93, 94, 96]) were studied in different salt solutions. This allowed ion-peptide interaction motifs and binding kinetics (retention times, etc.)[96, 99] to be related to macroscopic observables, such as folding times and helical stabilities. Computational predictions for Hofmeister ordering of ion effects on the structural stability of oligopeptides were also

supported by direct spectroscopic evidence from circular-dichroism (CD) and Förster resonance energy transfer (FRET) measurements.[100]

Thermodynamic modeling

As illustrated above, several model compounds can be used as proxies for the protein backbone. The thermoresponsive polymer PNIPAM is one of the most widely used examples. Eqn (4) was employed to analyze the thermodynamic equilibrium and the preferential binding of salts, with the focus on cations, to PNIPAM. [43, 57, 70, 71, 75] Cations, typically as chloride salts, are mostly found to decrease the LCST in a linearly fashion as a function of salt concentration. The corresponding thermodynamic analysis shows the lowering of the transition free energy with salt concentration, in quantitative accord with calorimetry experiments, [55-57] together with a stronger salt depletion from the swollen state.[75] It is generally observed that strongly hydrated cations are preferentially excluded from the PNIPAM surface.[29, 41, 43, 70] As seen from the radial distribution function between the PNIPAM monomer and the ions from Na_2SO_4 (see Figure 4), this is reflected by an ion-depleted zone close to the monomer. Analogous results were found for strongly hydrated ions near the methyl group of PNIPAM and NMA [42, 44].

The existence of a depletion zone for salt ions next to the polymer enables the building of a simplified semi-quantitative thermodynamic model. Starting with a salt inaccessible (but water accessible) volume $\Delta V(c_s, T)$, the accompanying free energy change paid for the transfer of ions from water to the salt solution can be expressed as $\Delta\Delta G \cong k_B T c_s \Delta V$.[75] Using an approximate surface area for the *N*-isopropylacrylamide monomer of 100 Å^2 and a depletion layer thickness of 1 Å, the model predicts a negative coefficient $m = -k_B T \Delta V$ on the order of −100 kJmol^{-1}m^3, which

is consistent with literature *m*-values (or, in older notation, transfer free energies) for strongly hydrated ions. [9, 28, 29, 101]

The affinity patterns of anions for the peptide bond are more complex compared to those of cations. Unlike cationic case, the *m'* parameter for weakly hydrated anions (i.e., their sodium salts), which denotes nonlinear effects, is non-vanishing.[70, 75] Although the LCST curves start at the origin in a linear fashion, the deviation from linearity already shows up at very low salt concentrations ($c_s \simeq 50$ mM).[75] For the most weakly hydrated anions, the initial slope is even positive (that is, yielding a positive parameter *m* before the LCST turns over at a maximum to a negative slope $\Delta T'(c) < 0$), implying a stronger adsorption (or lesser exclusion) to the extended versus the collapsed states at small salt concentrations. [75] The turnover effect is the strongest for $NaClO_4$ where the maximum of the LCST curve was found at about $c \simeq 50$ mM. Thus, a more complex model is needed to fit the data for the anion series, compared to the simple excluded volume approach applicable for cations. Thermodynamic analysis of PNIPAM shows that the parameter m' is always negative, which can be interpreted as a weakened attraction of anions for the PNIPAM surface with increasing salt concentration. On a microscopic level, this may be attributed to slow but gradual charging of the PNIPAM surface (and/or its vicinity) due to the excess partitioning of anions (NaSCN in Figure 4). In this way, the buildup of repulsive electrostatic interactions causes the binding of anions to become anti-cooperative at higher concentrations.

Cations at negatively charged side chains – direct Hofmeister series

Molecular Dynamics Simulations

Our early simulation study on interactions of sodium and potassium ions with soluble

proteins showed that cations follow the Hofmeister series both at the backbone and at negatively charged side chains of glutamates and aspartates.[35] These calculations also demonstrated that cationic interactions with the anionic side chain groups at the protein surface are stronger than with the backbone. Subsequent studies, which reduced the problem of cationic affinities to acidic side chains of proteins to interactions of ions with glutamate and aspartate amino acids or even with the carboxylic group of model compounds like acetate or formate, further systematized and experimentally verified the early computational predictions. [38, 40, 42, 95, 96, 102] In a nutshell, cations order according to the Hofmeister series at the aqueous COO^- group with strongly hydrated ions such as sodium, lithium, or calcium forming stronger ion pairs than weakly hydrated ions like potassium, ammonium, or cesium. Pairing of divalent and trivalent cations with the carboxylic group is sufficiently strong that it can lead to overcharging of short oligoaspartates in aqueous solutions, as demonstrated by MD simulations and electrophoretic measurements.[50]

Experiments

Specific cation effects were also probed at net negatively charged polypeptides by monitoring the phase transition temperature of ELPs containing aspartic acid residues that were deprotonated under the conditions of the experiments.[74] Figure 5 A and C plots the LCST of the polypeptide as a function of concentration for chloride salts of monovalent and divalent cations. The data were fit to an empirical equation that consists of a linear term and a modified Langmuir binding isotherm which accounts for electrostatic interactions (eq. 2). Divalent cations (Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, and Zn^{2+}) resulted in a sharp decay in the LCST due to electrostatic screening and ion pairing between the negatively charged aspartate groups and the divalent cations. Such interactions resulted in apparent K_D's values that were found in the low mM range for all tested divalent cations. The effects of monovalent cations (Li^+, Na^+, K^+, Rb^+, Cs^+, NH_4^+ , NMe_4^+) were

approximately two orders of magnitude weaker with shallower decay trends observed (Figure 5A). Furthermore, these cation binding affinities to the macromolecules were in agreement with a direct cationic Hofmeister series.[74]

Thermodynamic modeling

Above, we described a model that accounts for cation-specificity at the neutral protein backbone, where salting out could be attributed to cation-specific depleted volume effects. Proteins are weakly charged macromolecules with charge fraction (i.e., ratio of total charge vs. number of amino acids or monomers) typically below 10%.[103] For such systems, the two-state thermodynamic model Eq.(4) of the folded/unfolded equilibrium has been extended to the case where the polymer is weakly charged.[79] Here, we include nonspecific electrostatic effects via a Donnan potential, but explicitly account for the salt-specificity defined above. The model was tested for a weakly (negatively) charged PNIPAM-copolymer up to a charge fraction of 5% in NaBr solution. Moreover, our theory was further applied in this work to analyze the effects of the alkali-chloride salts on the LCST of the weakly charged elastin-like polypeptides (ELP) plotted in Figure 5B. Decomposing the total LCST change into two contributions, it was found that for monovalent cations the electrostatic contribution can be modelled as a combination of a nonspecific dominant response (responsible for ~35 K decrease of the LCST), and a 'softer' cation-specific effect contributing around ~10 K/M. Moreover, it was found that the cation-specific effects were virtually the same as in the neutral ELP reference, i.e., the cations follow a direct Hofmeister series. We note that more advanced electrostatic descriptions, as well as couplings with specific polymer shapes, are available in principle. [104] These, however, necessarily lead to mathematically much more complex expressions and would only provide smaller improvements.

Anions at positively charged side chains - reversed Hofmeister series

Molecular Dynamics Simulations

Besides the protein backbone, the obvious hotspots at the protein surface for interactions with anions are the positively charged side chains of arginine, lysine, and (doubly protonated) histidine. While Nature operates with only a single anionic side chain group (COO^-), there are instead three cationic side chain groups – guanidinium, ammonium, and imidazolium. MD simulations of aqueous proteins/peptides, as well as single amino acids or molecular ions carrying these cationic groups show that interactions with anions are governed by a *reversed* Hofmeister series (see Figure 6 for ordering of anions at the ammonium group at the N-terminal of triglycine).[41, 98] In other words, unlike at the protein backbone, it is the strongly hydrated anions like fluoride or sulfate which dominate at positively charged side chains over weakly hydrated anions like iodide, perchlorate, or thiocyanate. As discussed in more detail below, this anionic behavior at positively charged side chains makes the ion specificity of anions richer than that of cations and is responsible for the occurrence of a Hofmeister reversal as observed for some cationic proteins like lysozyme.[25, 26]

Experiments

In analogy to the previous experimental section, the effect of Hofmeister anions on polypeptides with positively charged residues were experimentally investigated as a function of salt concentration by monitoring the LCST of an ELP containing 16 lysine residues. In this case, about 3% of the amino acids were positively charged. The other 97% consisted of valine, glycine, and proline residues. These data could also be modeled with eqn 2 and anions are expected to interact strongly with the polymer surface. At low salt concentrations, a sharp decay in the LCST

curves was observed that correspond to the electrostatic charge neutralization for all tested weakly hydrated anions. (Figure 5D) This relative effectiveness of anions to salting out the positively charged ELPs a reversed Hofmeister series ClO_4^- > SCN^- > I^- > NO_3^- > Br^- > Cl^-. Such an anion specific decrease in the LCST due to electrostatic charge neutralization showed a strong correlation with the partial molar volume of the Hofmeister anions (Figure 5D inset). Strikingly, at higher salt concentrations, the salt effect reverted to a direct Hofmeister series Cl^- > NO_3^- > ClO_4^- > Br^- > I^- > SCN^-. The ion specific trends at higher salt concentrations (> 0.2 M) correlated with the values of salt effect on the surface tension at the air/water interface. Namely, the positively charged ELPs salt-in with more weakly hydrated anions (i.e., NaSCN, and NaI), whereas the same ELPs salt-out when more strongly hydrated anions such as NaCl, $NaNO_3$, and NaBr were introduced. This higher salt concentration behavior mirrors our results with neutral ELPs and polymers. Moreover, the Hofmeister series reversal has been found in other systems as well. For instance the protein-protein aggregation behavior of lysozyme with salts of weakly hydrated anions were shown to demonstrate very similar behavior to positively charged ELPs,[26] with different mechanisms at low and high salt concentrations. More recently, other reversed Hofmeister series have also been reported, i.e., for the anion association to the *N*-terminus of uncapped triglycine oligo peptides.[88, 96]

Thermodynamic modeling

The electrostatic contribution can be introduced analogously as in the case of cations, i.e., at the nonspecific Donnan electrostatic potential level. To test this approach, we again employed the weakly charged elastin-like polypeptide (KV6, i.e., with low content of lysine residues), for which anion-specific effects on the experimental LCST were analyzed on data in Figure 5D. [74] The results of this analysis can be summarized that a dominant contribution originates from electrostatic screening. A smaller contribution, however, stems from strongly anion-specific

interactions with the ELP, which are generally more pronounced than those of the cations (as discussed above). Due to the small fraction of lysine residues, this anion-specific contribution does not cause the full reversal of the Hofmeister series at this level of description.

For peptides with surfaces of high charge density, such as polyARG, the Donnan description is no longer applicable. In this case, Bjerrum theory, [50, 105] which phenomenologically describes counter-ion complexation, or the more advanced Manning condensation model [102,106] can be employed for non-specific screening of highly charged surfaces by singly or multiply charged ions. The remaining effect is due to ion-specific interaction (analogous to the weakly charged ELP) with the highly charged surface created by the charged functional groups on the amino acid side chains (i.e., $-NH_3^+$, or $-Gnd^+$). Here, the reversal of the Hofmeister series is recovered for anions of polyARG.

Effects of cations and anions at hydrophobic and polar surface groups

The effect of Hofmeister anions at hydrophobic surfaces were elucidated in several different contexts including at the air/water,[85] oil/water,[107-109] and related hydrophobic surfaces.[91, 110] In these reports, the most weakly hydrated anions were found to absorb to a variety of liquid interfaces and alter the adjacent water structure under most conditions. Measurements on negatively charged, hydrophobic surfaces were made with VSFS on negatively charged silica surfaces covered with a monolayer of octadecyl trichlorosilane (OTS) molecules.[111] Such an interface can serve as a model system for hydrophobic patches located in the vicinity of anionic residues. As can be seen in the Figure 7A, the VSFS spectra are dominated by the CH_3 symmetric stretch (2875 cm^{-1}), and Fermi resonant (2940 cm^{-1}) bands of the OTS monolayer along with the hydration water signal (3200 cm^{-1} and 3400 cm^{-1}). The sodium salts of more weakly hydrated

anions (NaSCN or $NaClO_4$) enhanced the water ordering due to anion adsorption. Salts of less weakly hydrated anions ($NaNO_3$, NaBr, or NaCl), in contrast, suppress the water signal via a screening effect. Indeed, one would expect better sodium partitioning to the negatively charged surface compared to a generic anion. However, Na^+ is relatively well hydrated and cannot interact as strongly with the negatively charged surface as SCN^- and ClO_4^-, which can more easily shed their hydration shells at the interface, as illustrated in Figure 7B. As a consequence, the surface potential actually becomes more negative when low concentrations of weakly hydrated anions are introduced. Overall, a direct Hofmeister series is obeyed in terms of the attenuation of the water structure as: $Cl^- > Br^- > NO_3^- > ClO_4^- > SCN^-$.

In MD simulations of short-alanine based helix forming peptides it has been observed that I^- has a considerable affinity to the nonpolar alanine.[95, 98] This is in line with the recent observations of a large propensity of I^- to adsorb to simple hydrophobes and thereby appeared to "assist" Na^+ in its destabilizing action of the helical structure.[95, 98] The larger ions ClO_4^- and Gnd^+ were also found to have a propensity to favorably interact with the hydrophobic methyl group of the ALA side chain.[100] The I^- affinity to alanine was enhanced for the positively charged $(AK)_6$. However, the helix destabilization effect by NaI for this peptide was found to be much weaker compared to the negatively charged $(AE)_6$ due to the (electrostatically induced) depletion of Na^+ at the peptide backbone. This demonstrated that I^- alone was not responsible for denaturation, but rather assisted and amplified cationic action. This exemplified the synergetic mechanisms behind specific ion-induced (de)stabilization of protein secondary structures and its sensitive dependence on local value and sign of the charge on the peptide.

Already the early simulation studies of proteins in aqueous solutions of simple salts showed

that the remaining part of the protein surface, i.e., polar and hydrophobic amino acid groups, do not strongly attract ions.[35, 112, 113] Therefore, these regions do not contribute significantly to the preferential binding of salts to the protein surface when compared to the charged side chains and the backbone. Nevertheless, hydrophobic surface groups can contribute to salt exclusion and, in general, these interactions may be characterized in terms of a direct or reversed Hofmeister series depending on the combination of surface polarity and hydrophilicity/hydrophobicity employed. [11, 15, 87]

3. **Beyond Hofmeister**

Beyond separate cationic and anionic series

Global salt and cosolvent effects have been widely investigated in considerable detail. These studies have addressed solubilities, cloud-point temperatures and equilibrium constants for (i.e., transition free energies, ΔG) of biomolecules.[9, 28, 29, 70, 73, 77, 80-82, 89, 101] Despite the diversity of these studies and the various specific salt effects that were found, only a limited number of physical scenarios seem to be relevant on a macroscopic level. In the vast majority of experiments, linear changes in the thermodynamic properties with salt concentration were found, often up to surprisingly high concentrations. This linear behavior provides justification for the m-value description and the transfer models introduced in the 1960s.[77, 80-82, 114, 115]

Less frequently, nonlinearities in biomolecular thermodynamic quantities as functions of concentration of salts (e.g., in $T_0(c)$, $\Delta G(c)$, $K(c)$, etc.) due to the addition of salts or cosolvents were reported at higher concentrations (c > 4 M). Such nonlinearities were believed to originate from solution non-ideality. [77, 80, 116] Qualitatively different nonlinearities (again in $T_m(c)$, $\Delta G(c)$, or $K(c)$) due to the addition of salts may appear in the sub-molar salt concentration range. [70, 117, 73, 118]

In this case, the thermodynamic properties are non-monotonic in *c*, which are signified, e.g., by a reentrant collapse transition (at fixed temperature) of the polymer, undergoing a collapsed → swollen → collapsed transition with increasing cosolvent concentration. These cases were reported only recently for the action of weakly hydrated anions, such as ClO_4^-, SCN^-, or I^-, on thermoresponsive polymers.[70, 73, 117] Note that such a cononsolvency effect[119] can also be observed in polymer solubility in mixed solvents (e.g., water/methanol) under some conditions,[120] where the polymer is completely soluble in the pure solvents, but not in their mixtures.[56, 119-122]

A unified model of salt effects on protein stability, covering these three main regimes during the reentrant transitions, was established recently by coarse-grained simulations, Flory-theory, and simple statistical mechanics considerations.[123] Figure 8A-E summarizes and illustrates the different levels of description of the three distinct regimes of the concentration dependent cosolvent (e.g., salt) action, found in ternary solutions (i.e., containing biomolecules in water and salt). These are: *(i) Collapse due to depletion (exclusion).* Under conditions where the cosolvent (e.g., Na_2SO_4) is depleted, the state of the polymer with a smaller exposed surface area is preferred. *(ii) Swelling due to weak attraction (weak binding).* In contrast, in the weakly attractive regime (e.g., for GndCl or urea), the state of the polymer with the larger exposed surface area is preferred. In these cases the cosolvent effect on $\Delta G(c)$ is linear. [70, 73, 75] This situation can be well described by *m*-value-type models and the transition thermodynamics is similar to that in neat water. This is related to the fact that these cases are essentially weak perturbations from neat water conditions. *(iii) Collapse and reentrant swelling due to strong attraction (strong binding).* For certain co-solutes (such as ClO_4^-, I^-, SCN^-) the binding at the polymer surface is relatively strong, but decreases with the concentration of the co-solute. [70, 73, 75] This effectively leads to the opening up of larger polymer surface areas at low salt concentrations and to collapsed states at higher salt

concentration. This behavior was captured in calorimetry experiments [55-57] as well as in MD studies. In this strongly binding ("bridging" or "weak crosslinking") regime, [123] the addition of cosolvent leads to tightly collapsed polymer states, maximizing contacts of monomers and cosolvent due to enthalpic reasons, at low cosolvent concentrations. The same enthalpic gain leads to polymer swelling at high cosolvent concentrations.[123] This happens due to the fact that the extended polymer conformation provides more exposed surface area for interactions, which is also entropically favorable in terms of exchange of nearby cosolvent molecules.

The thermodynamic expansion model[75] can be further used to interpret these results. The description by the thermodynamic model, as shown in Figure 8A-C provides the effects of salt depletion, weak adsorption, and strong bridging on the polymer thermodynamics parameters, such as transition enthalpy, measured in calorimetry studies.[56] It also provides preferential binding coefficients, measurable in fields such as dialysis and accessible via computer simulations (Figure 8C). The coarse-grained simulations, [121, 123] converging the average knowledge gained from all-atom simulations, [40, 41, 43, 44, 112] allow one not only to assess the stability of polymer chains in different co-solvent regimes but also to extract the excess adsorption of the cosolvent molecules as well as thermodynamic features. Consequently, the trends in experimental data can be quantitatively analyzed and the corresponding microscopic details revealed. [117, 123]

Specific binding sites

So far, we have mainly discussed ion interaction with biomacromolecules and small molecules containing amide bonds. Clearly, the role of ions in biology is far richer and more complex with large concentration gradients often existing across the lipid membrane. For example, the concentration of K^+ in the cytoplasm is about two orders of magnitude higher than its

concentration in the extracellular fluid, while the reverse is true for Na^+. Such a strong gradient is not due to a Hofmeister effect, as the difference in the ion pairing interactions for these ions with charged carboxylic acid groups, phosphate groups or neutral protein backbones is only very modest, but rather due to the action of specialized ion pumps and channels. However, the coordination number differences of these cations within ion channels or other specialized binding sites are often significant. It has been demonstrated that the coordination number for cations plays a crucial role in ion channel selectivity and can account for the significant differences in the interactions of K^+ or Na^+ with these biological entitites.[124-126] In fact, the very existence of these specialized sites can override Hofmeister effects. This is responsible for many of the ion selective behaviors observed in cells. In other words, while Hofmeister series effects are always present as a background, they can be overridden by steric arrangements of specific chemical sites.

The behavior of divalent alkali earth cations and, in particular, transition metal ions is very different from that of monovalent alkali metal cations. Magnesium, calcium, zinc, and divalent ions formed from first row transition metal elements are exploited in numerous metalloproteins and serve as the basis for a myriad of catalysts and structural elements. Such transition metal cations are distinct from Hofmeister ions in that they can form coordination complexes in which substantial charge is transfer between the metal center and the binding ligand. These interactions often lead to much tighter interactions than those of Hofmeister-type ion pairing and different rules apply. The generic ion specificity for first row transition metal ions to amines and thiols in coordination complexes follows a distinct rank ordering of behavior, which is called the Irving-Williams series and is listed as follows:[127]

$$Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$$

The main driving force in the Irving-Williams series is the charge transfer between the transition metal center and its ligands, which reaches a maximum for Ni^{2+} and Cu^{2+}, but is weaker for the ions to their left. Zn^{2+} also shows much less charge transfer because it possesses filled *d* orbitals and is, therefore, technically not a transition metal. It should be noted that the first row transition metals can also follow a Hofmeister series when charge transfer processes with ligands, such as amines or thiols, are not involved.

Conclusions

Molecular simulations together with spectroscopic and thermodynamic experiments have allowed us to understand the basic principles that govern the interactions of ions with proteins in aqueous solutions leading to salting out and salting in effects. The gist of Hofmeister effects, i.e., the ordering of ions according to their ability to salt out proteins, lies in local interactions at their surfaces. Straightforward thermodynamic reasoning leads to the notion that the more strongly attracted an ion is to a protein in solution, the less efficient it is in its salting out and *vice versa*. In particular, the crucial regions of protein surfaces interacting with ions are the backbone and charged side chains, with polar and hydrophobic side chains playing a much smaller role. Both simulations and experiments confirm that cations follow standard Hofmeister ordering with strongly hydrated cations interacting more strongly and thus being less efficient in salting out than weakly hydrated ones. This is true both at the protein backbone and at negatively charged side chains, with the former interactions being significantly weaker than the latter. The picture for anions is more complex with backbone and side chain interactions being oppositely ordered. At the backbone, they follow the normal anionic Hofmeister series with weakly hydrated anions interacting more strongly and thus being less efficient in salting out than strongly hydrated ones. However, at the positively charged side chains, the anionic ordering is *reversed*. As a result of the

above considerations, cations follow the Hofmeister series for protein salting out behavior, while for anions this is true only for proteins where the backbone effect is stronger than that of the positively charged side chains. For strongly positively charged proteins such as lysozyme at low to neutral pH values, the anions can actually follow a reversed Hofmeister series.

No matter how powerful they appear to be, the Hofmeister rules governing protein salting out/salting in and their molecular rationalizations are only of an approximate nature. The first and foremost approximation lies in separating the effects of ions of opposite polarities into distinct cationic and anionic series. This is not the full story since ions interact with their counter-ions both in the bulk solution and at the surfaces of proteins. Beyond the need to satisfy the electroneutrality condition in both environments, there may be particularly strong cation-anion interactions for specific salts that render the separate treatment of cations and anions in the Hofmeister series questionable. For example, guanidinium is known to interact strongly with proteins in its most common chloride salt. Such cation-protein interactions can be, however, significantly diminished for guanidinium when paired with sulfate. In other words, it is not only the interaction of a given ion with the protein surface that must be considered, but also its interactions with counter-ions.

The importance of the Hofmeister series goes beyond salting out of proteins, which is just one macroscopic manifestation of ion specific effects. The complementary process of salting in is intimately connected with protein (de)stabilization and denaturation, which remains a major scientific and technological challenge. Next, there is a plethora of biological functions that are controlled by ions, ranging from homeostasis and calcium signaling, to key roles of specific cations in metalloproteins. Most of these processes go beyond generic Hofmeister interactions and involve specific steric arrangements, e.g., in active sites of enzymes. The question that poses itself,

motivating further research, is as follows: How much does Nature exploit Hofmeister effects in biological function and to what extent does it overrule them via forming specific binding sites?

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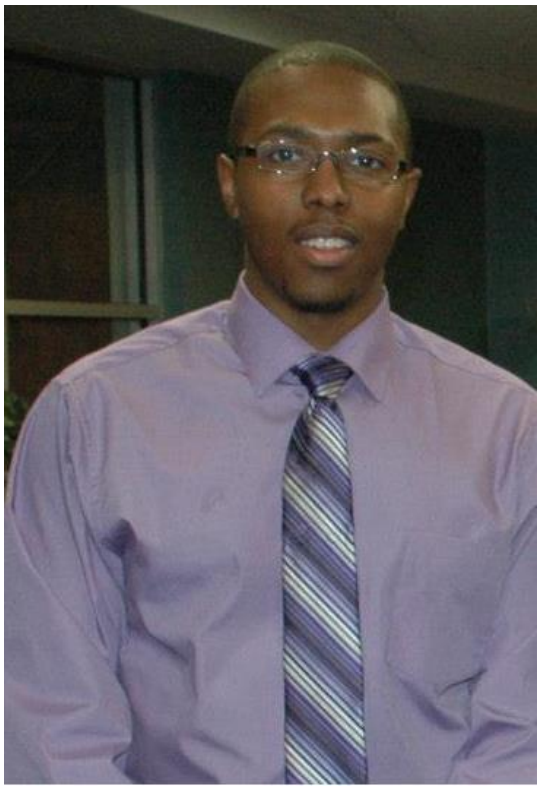
Jana Hladílková earned her MS. degree in Chemical Technology at the University of Chemistry and Technology in Prague in 2010. She obtained her PhD. in Chemistry at the Charles University in Prague in 2014 working with Pavel Jungwirth on modeling Hofmeister ion effects on peptides and proteins. Since 2014 she has been a postdoc in the group of Mikael Lund at the Lund University in Sweden. Her research is focused on ion and pH effects on assembling of biomolecules at a variety of surfaces using, among other simulation approaches, a pH sensitive coarse-grained model.



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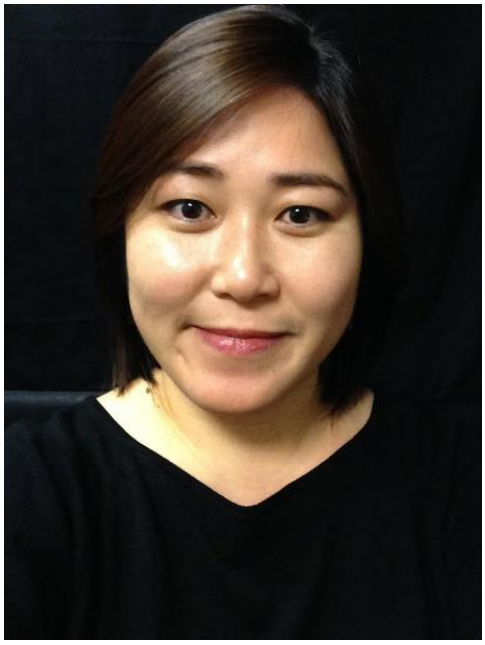

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Jan Heyda is an Assistant Professor of Physical Chemistry at the University of Chemistry and Technology in Prague. He obtained his M.Sc. in chemistry and mathematics in 2008 and earned his Ph.D. in theoretical physical chemistry at the Charles University in 2011, working with Pavel Jungwirth. He was an Alexander von Humboldt Research Fellow at the Helmholtz-Zentrum Berlin in 2012-2014. His research interests encompass understanding of the thermodynamic background of salt-specific effects in solubilities of small organic molecules, stability of biomolecules, and phase behavior of thermoresponsive materials via application of atomistic computer simulations and Kirkwood-Buff theory. Related interests include the application of theoretical tools in physical chemistry techniques, such as osmometry, densimetry, or liquid-liquid equilibria, and in electrophoresis.

Joachim Dzubiella received his doctorate in 2002 under the supervision of Prof. C. N. Likos and Prof. H. Löwen in Theoretical Soft Matter Physics at the Heinrich-Heine Universität in Düsseldorf, Germany. After postdoctoral stays with Prof. J.-P. Hansen in Cambridge, UK, and Prof. A. J. McCammon in San Diego, USA, he returned to Germany in 2006 to head an Emmy-Noether research group at the Technical University Munich. Since 2010 he is a group leader at the Helmholtz-Zentrum Berlin and a Professor for Theoretical Physics at the Humboldt-Universität zu Berlin. Research in his group is devoted to the modeling and simulation of soft matter and complex fluids.

Paul S. Cremer received his B.A. degree from the University of Wisconsin-Madison in 1990 and Ph.D. in Chemistry from the University of California-Berkeley in 1996. Following postdoctoral work at Stanford University from 1996-98, he was a Professor of Chemistry at Texas A&M University from 1998 to 2012. Since 2013, he has been the J. Lloyd Huck Chair in Natural Sciences at the Pennsylvania State University. His research interests involve understanding the interactions of ions with proteins, peptides, small molecules, and phospholipid membranes. His group also explores interfacial

water structure, designing assays with supported lipid membranes, and the employment of microfluidic platform architectures.

Pavel Jungwirth received his PhD. at the Charles University in Prague in 1993. After a postdoctoral stay at the Hebrew University in Jerusalem and at the University of California Irvine (1994-5) he was a group leader at the Heyrovsky Institute in Prague. At present, he is a Distinguished Chair at the Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences and a Professor (external faculty) in physics at the Charles University in Prague. With his group he employs state-of-the-art computational techniques spanning from classical molecular dynamics to ab initio quantum chemistry in direct contact with experimental biochemistry and molecular spectroscopy to unravel the action of ions in biological contexts involving proteins and/or cellular membranes in their native aqueous environment. His related interests encompass modeling of direct and indirect radiation damage to DNA and the structure and dynamics of solvated electrons. In the past decade, he has been obsessed with Hofmeister ion effects on proteins and the Feature Article may be viewed as a sort of a therapy replacing the obsession with molecular understanding.

Figure Captions

Figure 1: A commemorative plaque at the building of the Charles University in Prague, where Hofmeister carried out his groundbreaking experiments, unraveled during a Hofmeister symposium in 2010. The bilingual inscription (in Czech and German), which also includes the original anionic series, reads: "Professor Franz Hofmeister (1850–1922), who carried out research in this building, predicted that amino acids in proteins are connected by a peptide bond and, in 1888, derived the lyotropic (Hofmeister) series of ions." (Photo courtesy of P. Jungwirth.)

Figure 2: Modern version of the cationic and anionic Hofmeister series and the accompanying physical properties including the salting out ability (adapted with permission from Ref. 8 and http://tinyurl.com/ed5gj).

Figure 3: A) The $\Delta\,\delta$ chemical shift for each proton after subtraction of the linear term along with the residual LCST after the linear part is deducted as a function of salt concentration for $(VPGVP)_{120}$ in aqueous NaSCN salt solutions. Figure 3A is adapted with permission from ref. 44. B) The VSFS spectra of air/PNIPAM/water interface at 1M sodium salts of Hofmeister anions as indicated in the legend (except NaF and Na_2SO_4 were 0.8 M salts). C) FT-IR spectra of the amide I region for butyramide molecule in aqueous solution i) pure D_2O, ii) 5M NaCl, and iii) 5M $CaCl_2$, D) the SFG spectra of air/butyramide/water interface at different chloride salts of Hofmeister cations, as indicated in the legend at the subphase.

Figure 4: Distributions of NaSCN and Na_2SO_4 near a PNIPAM surface are present in the left column, in the form of spatial density at contour levels (presented in backets), which is a multiple

of the ion or water density in the bulk, of ions (Na^+ green (1.5x), SO_4^{2-} silver (1.5x), SCN^- yellow (3x)) and water (red (1.5x)). In the middle column, this information is condensed in proximal distribution function from the PNIPAM surface (Na^+ green, SO_4^{2-} gray, SCN^- yellow) and water (red)). In the right column, we present the thermodynamic preferential binding coefficient Γ (integrated information). The preferential hydration of PNIPAM in Na_2SO_4 contrasts strongly with preferential binding of NaSCN (blue lines for over effect of salt), having primarily the origin in the different affinity of anions (compare the gray and yellow lines for effect of SO_4^{2-} vs. SCN^- anions). Note, that the information about the distribution is (partly) accessible via spectroscopic measurements, while the preferential binding is thermodynamically relevant.

Figure 5: A) The LCST response of ELP DV_2F-64 as a function of monovalent chloride salts concentration. B) The interpretation of cation-specific effects for monovalent cations on negatively charged elastin in the framework of the extension of the thermodynamic model in eqn (4), which accounts for the electrostatic interactions. [75, 79] The nonspecific electrostatic interactions are introduced via Donnan potential, which is universal and dominates the effect of cation (inset). The remaining effect of salt on LCST can be well modelled with the salt-specific parameters of the reference neutral ELP. C) LCST response of ELP DV_2F-64 as a function of concentration and identity of divalent metal chlorides. Experiments in A) and C) were performed with 10 mg/mL ELP in 10 mM Tris buffer at pH 9.76. D) The LCST curves for 6.4 mg/ml ELP KV_6-112 at pH 7 as a function of salt concentration for a series of sodium Hofmeister anion salts. The inset shows the plot the correlation between the partial molar volumes of anions vs. B_{max} constant. Figures 5A and 5C are adapted with permission from ref. 74

Figure 6: Spatial distribution of anions (sodium salts top to bottom: Na_2SO_4, NaCl, NaBr, NaI, NaSCN) near zwitterionic triglycine oligopeptide is present on the left column. In the middle column, the proximal distribution functions of anions are evaluated with respect to three distinct methylene groups with α-protons 1 (red, NH_3^+ terminus), 2 (green, NH-CH_2-CO), and 3 (blue, COO^- terminus). In the right column, we present the thermodynamic preferential binding coefficient Γ evaluated in regions adjacent to the three methylene groups (see inset and legend). Note that only the overall proximal distribution function $g^{prox}(r)=\Sigma_{i=1,2,3}\ g^{prox}_i(r)$ is normalized to 1 and that the thermodynamically relevant preferential binding coefficient is the sum of the partial contributions of distinct parts of the surface $\Gamma=\Sigma_{i=1,2,3}\ \Gamma_i$.

Figure 7: A) OTS-covered quartz/water interfaces at pH 10.0 in contact with 0.10 mM sodium salt solutions display a direct Hofmeister effect. Legend indicates the salt identity. B) Schematic shows Na^+ partitions more effectively than Cl^- to the OTS covered negatively charged quartz/water interface (top), but with larger anions like SCN^- that are less excluded from the negatively charged quartz/water interface than Cl^- (bottom). Figure 7A and 7B are adapted with permission from ref. 111.

Figure 8: A summary of salt-specific regimes on the collapse-swelling equilibrium of neutral thermoresponsive polymer ELP $(V_5)_{120}$ based on experimental LCST data, thermodynamic modelling, and generic Langevin coarse-grained simulations and all-atom simulations. (A) The experimental LCST data on neutral ELP $(V_5)_{120}$ in three guanidinium salts (GndCl brick brown, GndSCN dark yellow, Gnd_2SO_4 gray, see the legend) is presented as symbols and the thermodynamic fit of experimental LCST data is presented as lines. (B) The calculated transition

free energy of swelling, ΔG, at T_0=302K, is presented in (B), and (C) the difference in preferential binding of salt to collapsed and swollen state, $\Delta\Gamma$, (evaluated at T_0=302K). (D) The three regimes of salt action are described in terms of generic Langevin dynamics simulations providing the microscopic view. From left to right - collapse due to depletion (exclusion), swelling due to weak attraction (weak binding), and collapse and reentrant swelling due to strong attraction (strong binding).[118] (E) Spatial distribution of guanidinium salts, which were found in experiment to represent the three different regimes of salt action (Gnd_2SO_4, GndCl, and GndSCN; Gnd^+ purple, SO_4^{2-} gray, Cl^- gold, SCN^- yellow) near the ELP pentapeptide as obtained in all atom MD simulations with explicit water solvent.

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Figure 1:


Franz Hofmeister
V TÉTO BUDOVĚ PŮSOBIL CHEMIK PROF. FRANZ HOFMEISTER (1850-1922),
KTERÝ PŘEDPOVĚDĚL, ŽE AMINOKYSELINY SE V BÍLKOVINÁCH POJÍ PEPTIDICKOU
VAZBOU, A KTERÝ V ROCE 1888 NAVRHL LYOTROPNÍ (HOFMEISTEROVU) ŘADU IONTŮ:
$C_4H_4O_6^{2-} > SO_4^{2-} > HPO_4^{2-} > C_3H_5O(CO_2)_3^{3-} >$
$CH_3CO_2^- > HCO_3^- > CrO_4^{2-} > Cl^- > NO_3^- > ClO_3^-$
IN DIESEM GEBÄUDE FORSCHTE DER BERÜHMTE CHEMIKER
PROF. FRANZ HOFMEISTER (1850-1922), WELCHER DIE PEPTIDBINDUNGEN
DER AMINOSÄUREN IN EIWEISSEN VORHERSAGTE UND 1888 DIE LYOTROPISCHE
(HOFMEISTER) REIHE DER IONEN VORSCHLUG.
M. KNOBLOCH

Figure 2:

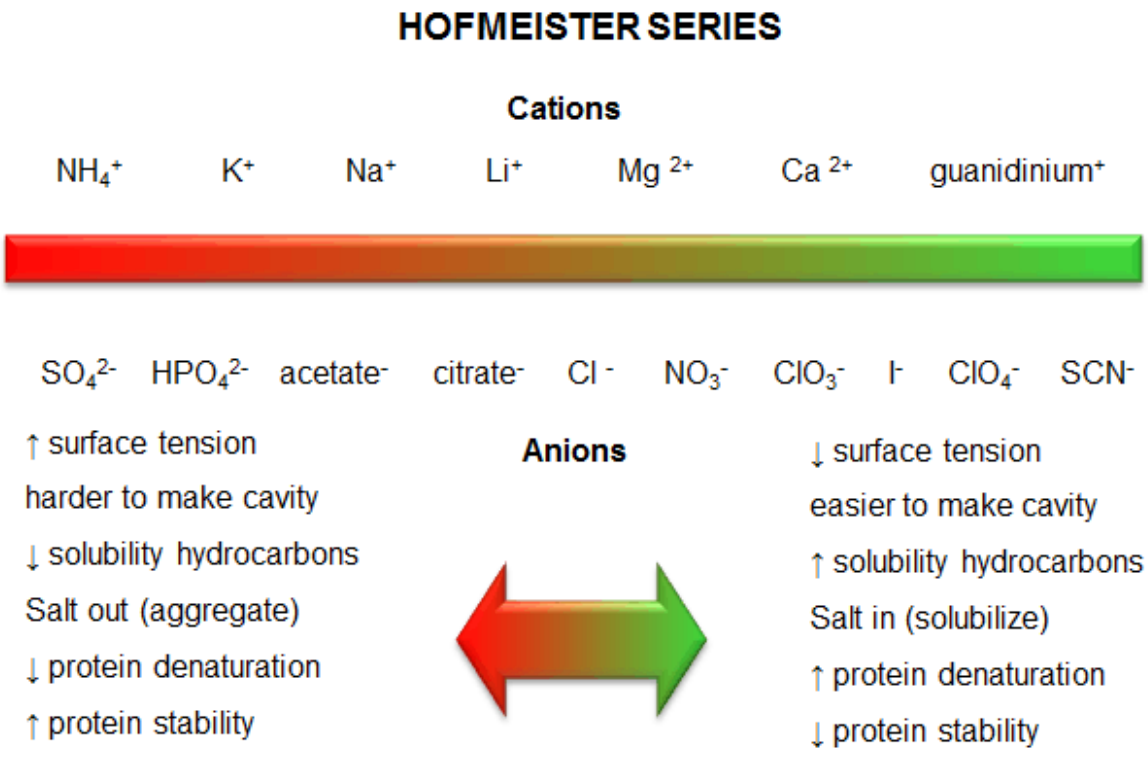

HOFMEISTER SERIES
Cations
NH_4^+ K^+ Na^+ Li^+ Mg^{2+} Ca^{2+} guanidinium$^+$
SO_4^{2-} HPO_4^{2-} acetate$^-$ citrate$^-$ Cl^- NO_3^- ClO_3^- I^- ClO_4^- SCN^-
Anions
↑ surface tension
harder to make cavity
↓ solubility hydrocarbons
Salt out (aggregate)
↓ protein denaturation
↑ protein stability
↓ surface tension
easier to make cavity
↑ solubility hydrocarbons
Salt in (solubilize)
↑ protein denaturation
↓ protein stability

Figure 3:

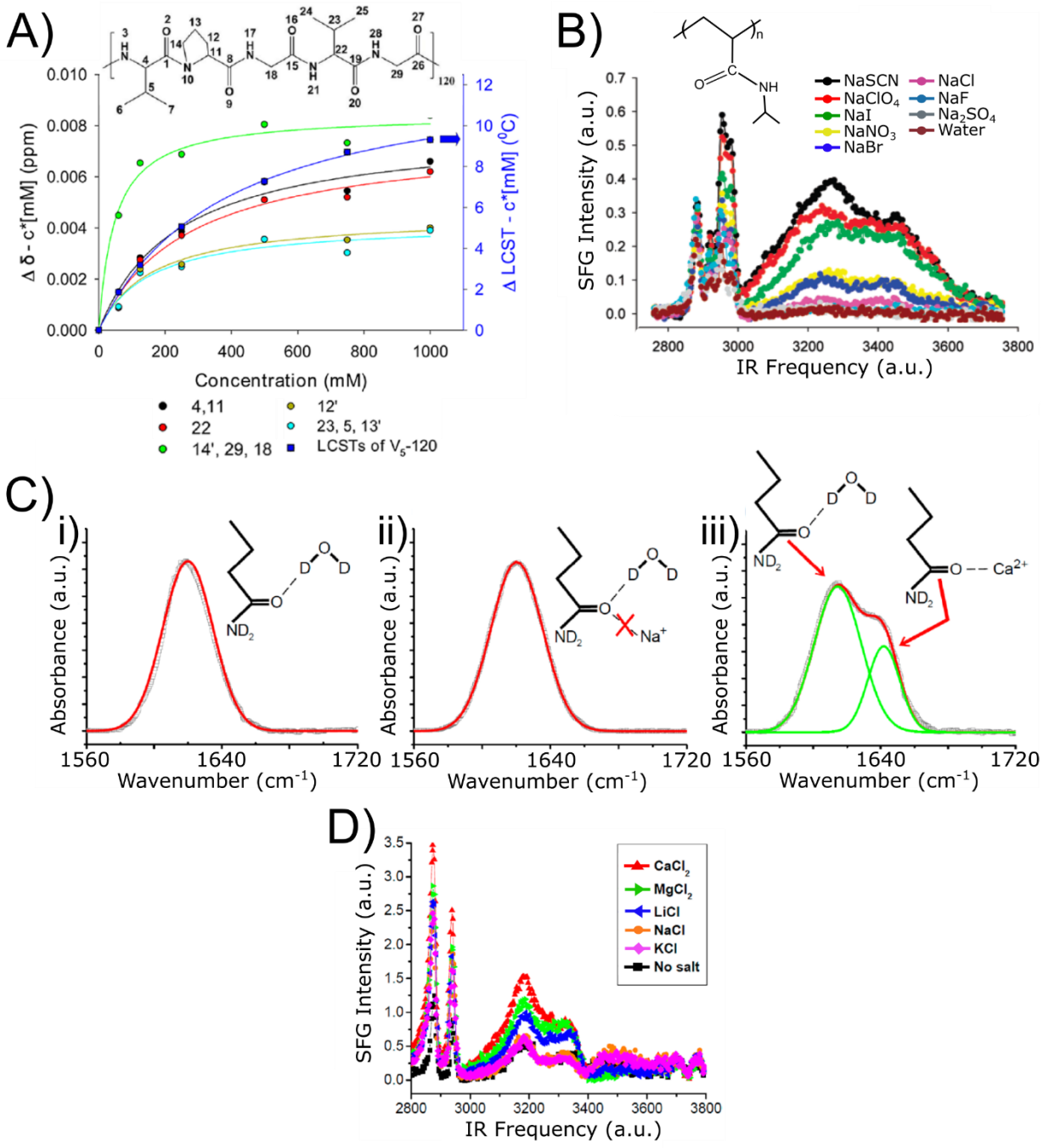

A)
Δ δ - c*[mM] (ppm)
Δ LCST - c*[mM] (°C)
Concentration (mM)
4,11
22
14', 29, 18
12'
23, 5, 13'
LCSTs of V5-120
B)
SFG Intensity (a.u.)
IR Frequency (a.u.)
NaSCN
NaClO4
NaI
NaNO3
NaBr
NaCl
NaF
Na2SO4
Water
C)
i)
ii)
iii)
Absorbance (a.u.)
Wavenumber (cm-1)
ND2
Na+
Ca2+
D)
SFG Intensity (a.u.)
IR Frequency (a.u.)
CaCl2
MgCl2
LiCl
NaCl
KCl
No salt

Figure 4:

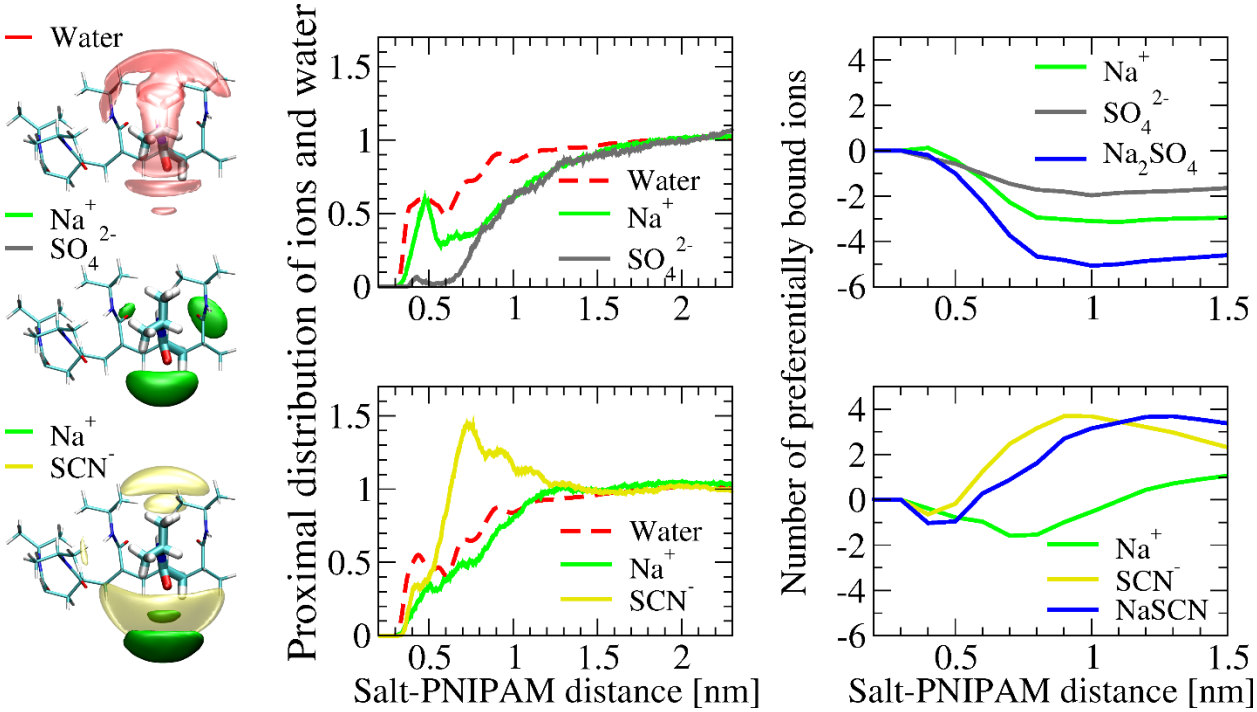

Water
Na+
SO4 2-
Na+
SCN-
Proximal distribution of ions and water
Water
Na+
SO4 2-
Water
Na+
SCN-
Salt-PNIPAM distance [nm]
Number of preferentially bound ions
Na+
SO4 2-
Na2SO4
Na+
SCN-
NaSCN
Salt-PNIPAM distance [nm]

Figure 5

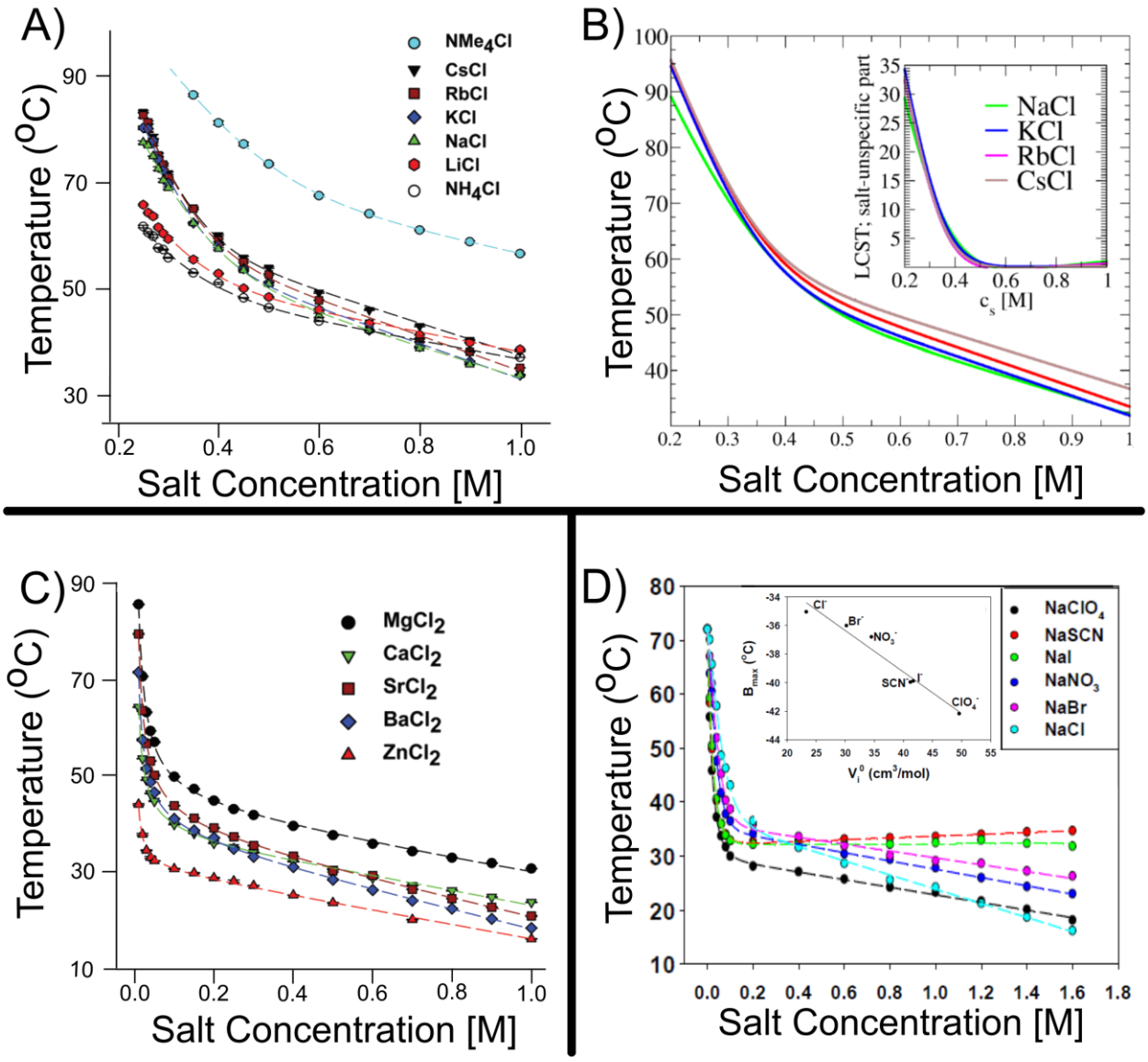

A)
Temperature (°C)
Salt Concentration [M]
NMe4Cl
CsCl
RbCl
KCl
NaCl
LiCl
NH4Cl
B)
Temperature (°C)
Salt Concentration [M]
LCST; salt-unspecific part
cs [M]
NaCl
KCl
RbCl
CsCl
C)
Temperature (°C)
Salt Concentration [M]
MgCl2
CaCl2
SrCl2
BaCl2
ZnCl2
D)
Temperature (°C)
Salt Concentration [M]
Bmax (°C)
Vi0 (cm3/mol)
Cl-
Br-
NO3-
SCN-
I-
ClO4-
NaClO4
NaSCN
NaI
NaNO3
NaBr
NaCl

Figure 6:

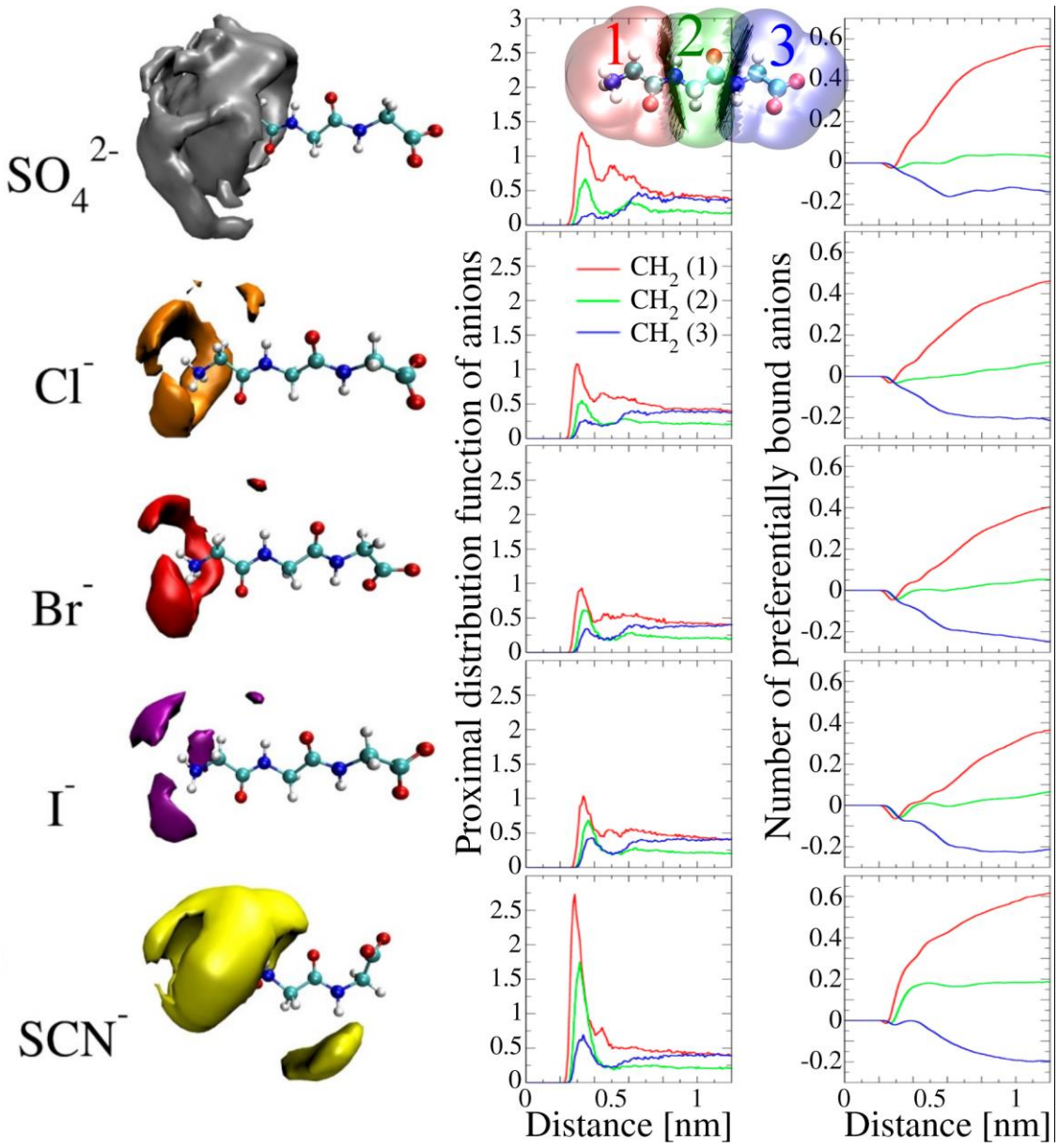

SO4 2-
Cl-
Br-
I-
SCN-
1
2
3
CH2 (1)
CH2 (2)
CH2 (3)
Proximal distribution function of anions
Number of preferentially bound anions
Distance [nm]
Distance [nm]

Figure 7:

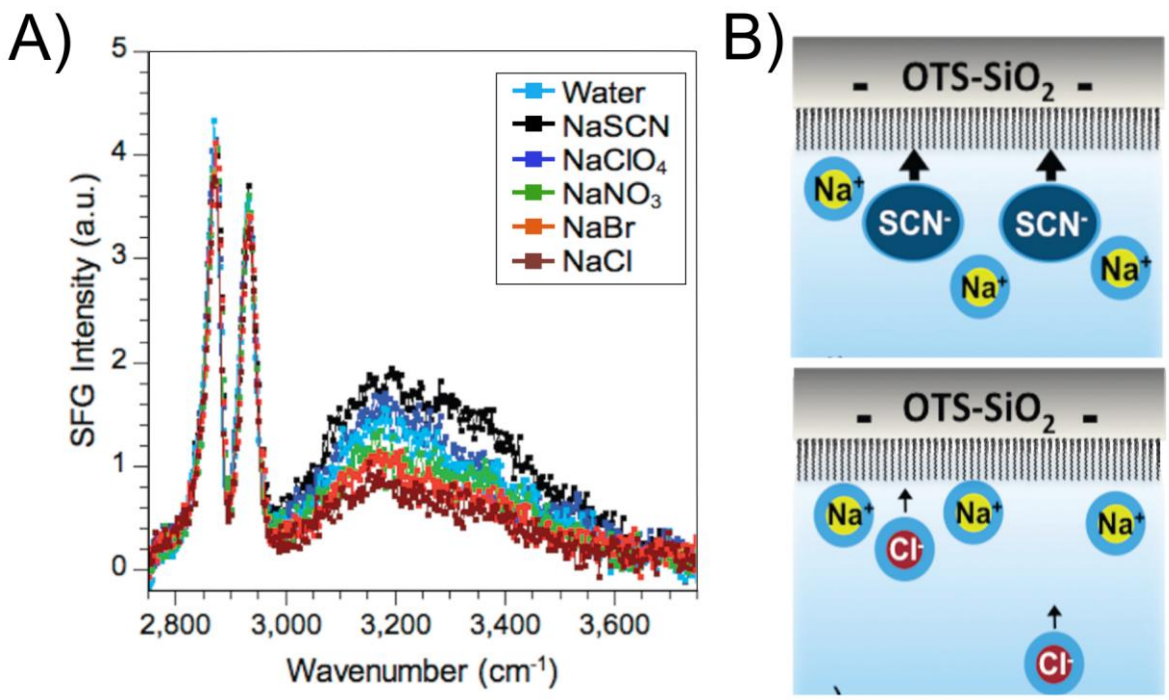
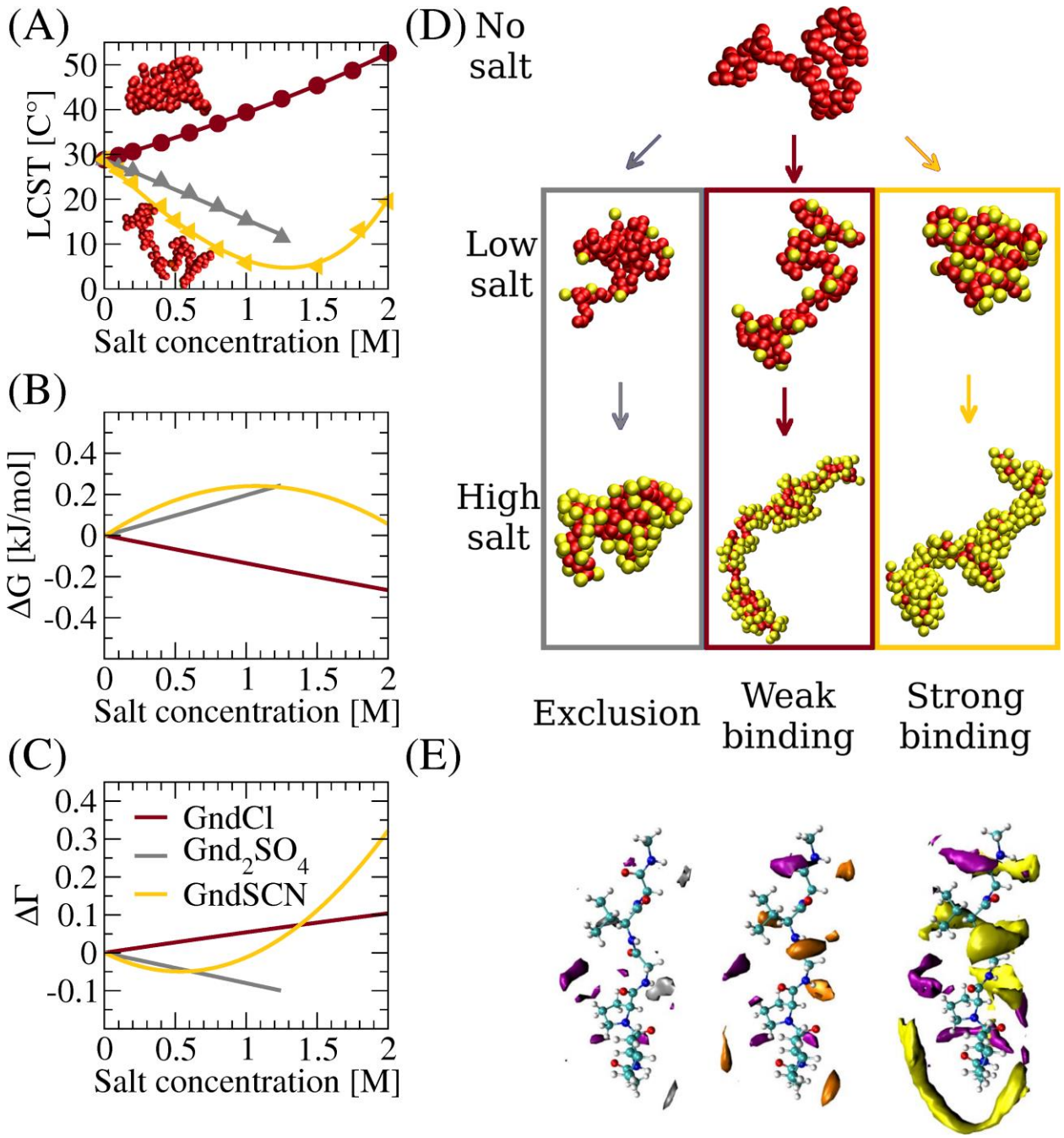
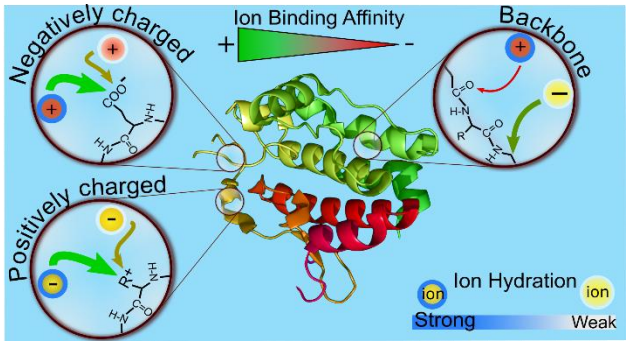

A)
SFG Intensity (a.u.)
Wavenumber (cm-1)
Water
NaSCN
NaClO4
NaNO3
NaBr
NaCl
B)
OTS-SiO2
Na+
SCN-
Cl-

Figure 8:


(A)
LCST [C°]
Salt concentration [M]
(B)
ΔG [kJ/mol]
Salt concentration [M]
(C)
GndCl
Gnd2SO4
GndSCN
ΔΓ
Salt concentration [M]
(D)
No salt
Low salt
High salt
Exclusion
Weak binding
Strong binding
(E)

TOC GRAPHIC


Negatively charged
Ion Binding Affinity
+
-
Backbone
Positively charged
Ion Hydration
ion
ion
Strong
Weak