Bulk Phase Behavior vs Interface Adsorption: Specific Multivalent Cation and Anion Effects on BSA Interactions

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ABSTRACT: Proteins are ubiquitous and play a critical role in many areas from living organisms to protein microchips. In humans, serum albumin has a prominent role in the foreign body response since it is the first protein which will interact with, e.g., an implant or stent. In this study, we focused on the influence of salts (i.e., different cations (Y3+, La3+) and anions (Cl−, I−) on bovine serum albumin (BSA) in terms of its bulk behavior as well as the role of charges for protein adsorption at the solid–liquid interface in order to understand and control the underlying molecular mechanisms and interactions. This is part of our group’s effort to gain a deeper understanding of protein–protein and protein–surface interactions in the presence of multivalent ions. In the bulk, we established two new phase diagrams and found not only multivalent cation-triggered phase transitions, but also a dependence of the protein behavior on the type of anion. The attractive interactions between proteins were observed to increase from Cl− < NO3− < I−, resulting in iodide preventing re-entrant condensation and promoting liquid–liquid phase separation in bulk. Using ellipsometry and a quartz-crystal microbalance with dissipation (QCM-D), we obtained insight into the growth of the protein adsorption layer. Importantly, we found that phase transitions at the substrate can be triggered by certain interface properties, whether they exist in the bulk solution or not. Through the use of a hydrophilic, negatively charged surface (native silica), the direct binding of anions to the interface was prevented. Interestingly, this led to re-entrant adsorption even in the absence of re-entrant condensation in bulk. However, the overall amount of adsorbed protein was enhanced through stronger attractive protein–protein interactions in the presence of iodide salts. These findings illustrate how carefully chosen surface properties and salts can directly steer the binding of anions and cations, which guide protein behavior, thus paving the way for specific/triggered protein–protein, protein–salt, and protein–surface interactions.

INTRODUCTION

Salts are essential for life as we know it. Humans need to ingest adequate amounts of salts via their diet1 in order to maintain biological and physiological functions in the body.2 The absence or excess of salts can lead to diseases such as renal edema, Addison’s disease, congestive heart failure,2 Parkinson’s disease,3 Alzheimer’s,4 or hypertension.1 For certain bacteria, called halobacteria, a high salt concentration is required for survival,5 while in plants it can induce cell death due to abiotic stress.6

Salts consist of cations and anions, which have different properties and promote ion-specific interactions, thus facilitating different functions in biomolecules, such as proteins, e.g., via ion–protein interactions.7 To decode their role and more specifically their binding mechanisms to proteins, numerous studies were conducted in the past decades.8–10 Nevertheless, there are still contradictory opinions and open questions concerning the interplay of electrostatic, hydrophobic, van der Waals, and entropic interactions between proteins and salts in bulk and at solid interfaces.

The chloride anion (Cl−) plays a central role in the human body. It is the body’s principal anion and the second main contributor to blood plasma tonicity11 and has a key role in the regulation of body fluids, preservation of electrical neutrality, acid–base balance,12 muscular activity, and osmotic pressure.11 In the human body, a chloride imbalance can induce diseases such as dystrophia myotonica, cystic fibrosis, chronic pancreatitis, epilepsy, cataracts, or Barter’s disease.13,14 In addition, it is used to diagnose other diseases and deficiencies.14 In biopharmaceutical drugs, chloride is, e.g.,
added to liquid antibody solutions for long-term stability\textsuperscript{15} or used to study alcohol degradation in the liver.\textsuperscript{16}

Another essential anion, which is primarily ingested through fish and dairy products, is iodide (I\textsuperscript{−}).\textsuperscript{17} I\textsuperscript{−} is, among others such as I\textsubscript{2}, I\textsubscript{3}−, IO\textsuperscript{−}, HIO\textsuperscript{−}, and H\textsubscript{2}IO\textsubscript{5}−, one form of iodine (potentially) present in the human body.\textsuperscript{18} In this study, we focus on I\textsuperscript{−}. It is most prominently known due to its role in thyroid hormone production,\textsuperscript{19} but it is also found in the saliva, stomach, intestines, kidneys, ovaries, and bloodstream.\textsuperscript{18}

An iodide deficiency can lead to goiter and hypothyroidism.\textsuperscript{18} Especially during pregnancy, an adequate level of iodide is crucial to prevent mental retardation and cretinism in newborns.\textsuperscript{18} In medicine, the antioxidant nature of iodide is used to combat free radicals and peroxides. While it is known for its disinfection/antimicrobial properties,\textsuperscript{20} another important aspect is its positive effect in the treatment of cardiovascular diseases,\textsuperscript{21} respiratory disorders,\textsuperscript{22} inflammatory skin diseases, and especially degenerative eye diseases.\textsuperscript{17}

All of these aspects illustrate the importance of studying protein–ion interactions. Some important properties of these interactions are summarized in the so-called Hofmeister series.\textsuperscript{23–25} Anions are hereby ordered according to their propensity to salt-in (stabilizing) or salt-out (destabilizing) the protein:

\[
\begin{align*}
\text{SO}_4^{2−} & > \text{PO}_4^{3−} > F^{-} > \text{CH}_3\text{COO}^{-} > \text{Cl}^{-} \\
& > \text{Br}^{-} \approx \text{NO}_3^{-} > \Gamma > \text{ClO}_4^{-} > \text{SCN}^{-}
\end{align*}
\]

(1)

For cations, the corresponding series reads:\textsuperscript{25}

\[
\begin{align*}
\text{Na}^{+} & > \text{K}^{+} > \text{Rb}^{+} > \text{Ca}^{2+} > \text{Ni}^{2+} > \text{Mg}^{2+} > \text{Fe}^{2+} \\
& > \text{Zn}^{2+} > \text{Al}^{3+} > \text{Fe}^{3+}, \text{Cr}^{3+} > \text{NH}_4^{+}
\end{align*}
\]

(2)

Typical physiologically relevant cations are mostly metal ions and have valencies from monovalent (e.g., Na\textsuperscript{+}) to trivalent (e.g., Al\textsuperscript{3+}) ions.\textsuperscript{26} The trivalent lanthanum cation (La\textsuperscript{3+}), which is less known in the physiological context, can be used on the one hand as a model cation due to its similar size to calcium, e.g., muscle contraction.\textsuperscript{27} On the other hand, it can block unwanted binding of physiological cations with similar size. For example, La\textsuperscript{3+} was found to inhibit the growth of cancerous cells in colon cancer,\textsuperscript{28} leukemia,\textsuperscript{29} and skin cancer\textsuperscript{30} and is thus a component in anticancer drugs already tested in vivo and in vitro.\textsuperscript{28} In the same sense, it can act either as a neurotoxin\textsuperscript{31} or trigger the release of neurotransmitters.\textsuperscript{32} In plants, La\textsuperscript{3+} acts as a chemical fertilizer\textsuperscript{33} and relieves the plant of salinity-induced oxidative stress.\textsuperscript{34}

Yttrium (Y) belongs to the group of transition metals, but its chemistry is similar to that of lanthanides.\textsuperscript{35} Y\textsuperscript{3+} is used in chemotherapy as a treatment for liver cancer\textsuperscript{36} and in radioimmunotherapy as part of yttrium-labeled antibodies.\textsuperscript{37} In dentistry, elementary yttrium is incorporated into dental implants for better osteoblast adhesion.\textsuperscript{38} For a further discussion of selected properties and applications of multivalent ions, see ref 7.

Due to the attractive interaction between opposite charges, cations can bind to negatively charged molecules. Under certain conditions, cations can thereby induce attractive forces, e.g., between macro-anions\textsuperscript{39} and bridge particles of equal charge, such as polyelectrolytes,\textsuperscript{40} surfactants,\textsuperscript{41} or actin filaments.\textsuperscript{42} Cations can induce charge inversion in biological membranes,\textsuperscript{43} anionic liposomes\textsuperscript{44} and globular proteins.\textsuperscript{45}

Our group observed a complex phase behavior for globular proteins (human and bovine serum albumins, β-lactoglobulin, ovalbumin) in the presence of multivalent cations such as Cd\textsuperscript{2+}, Zn\textsuperscript{2+}, La\textsuperscript{3+}, Al\textsuperscript{3+}, Y\textsuperscript{3+}, Ho\textsuperscript{3+} and Fe\textsuperscript{3+}.\textsuperscript{35,46–50} The phase diagram features phenomena such as liquid–liquid phase separation (LLPS), protein crystallization, and re-entrant condensation (RC) due to cation-induced charge inversion.\textsuperscript{35,46–50}

In this study, we focus on the effect of anions (Cl\textsuperscript{−}, I\textsuperscript{−}) and cations (La\textsuperscript{3+} vs Y\textsuperscript{3+}) on the bulk behavior of bovine serum albumin (BSA) as well as on its adsorption behavior on a negatively charged, hydrophilic surface (native silica). Serum albumin is often the first protein to adsorb to a solid interface\textsuperscript{51} in contact with blood serum. It is the most abundant blood protein and has a well-studied structure,\textsuperscript{52} making it an ideal and important protein to study. In terms of substrate properties, hydrophilic surfaces are highly hemocompatible\textsuperscript{53,54} Negatively charged surfaces are important for initiating blood clotting\textsuperscript{55} by inducing protein adsorption and platelet adhesion,\textsuperscript{56} besides activating further proteolytic systems in the blood plasma.\textsuperscript{56,57} Thus, native silica is a good model surface to study protein adsorption in addition to its well-defined properties and smooth surface.

In the following, we shed light on the dominant interactions guiding protein cluster formation and protein adsorption and the influence of multivalent salts on these behaviors. We aim to obtain a comprehensive picture of the underlying mechanisms and interactions driving these phase behaviors via ultraviolet–visible (UV–vis) spectroscopy, optical microscopy, Fourier transform infrared spectroscopy (FTIR), and pH measurements. Depending on the ion type, composition, and valency, different protein behaviors are observed due to varying type and strength of interactions. Second, we studied protein adsorption by attenuated total reflectance (ATR)–FTIR, ellipsometry, and quartz-crystal microbalance with dissipation (QCM-D) measurements.

### EXPERIMENTAL SECTION

**Materials and Bulk Phase Behavior.** We obtained the materials used in this study from Sigma-Aldrich (now Merck), namely, BSA with a purity of ≥98% (product no. A7906), yttrium chloride (YCl\textsubscript{3}) with a purity of 99.99% (product no. 451363), lanthanum chloride (LaCl\textsubscript{3}) with a purity of 99.99% (product no. 449830), yttrium iodide (YI\textsubscript{3}) with a purity of 99.9% (product no. 413011), lanthanum iodide (LaI\textsubscript{3}) with a purity of 99.9% (product no. 413674), and deuterium oxide (D\textsubscript{2}O) with a purity of 99.9% (product no. 151882). Salt stock solutions with a concentration of 100 mM in deagassed Milli-Q water were prepared for the bulk and adsorption measurements. The Milli-Q water has a total organic carbon (TOC) value of 1.7 ppb or μg/L and a resistivity of 18.2 MΩ cm at 25 °C. BSA has a net negative charge of −10 e at neutral pH.\textsuperscript{45} To determine the protein stock solution concentration via the Beer–Lambert law, a UV–vis spectrometer (Cary 50 UV–vis spectrometer, Varian Technologies, USA) was used to perform absorbance measurements. Most proteins containing aromatic amino acids show an absorbance maximum at 280 nm; thus, a range from 200 to 400 nm was scanned for each concentration determination.

For the phase diagram determination, the protein solutions were prepared with degassed Milli-Q water at protein concentrations (c\textsubscript{p}) of 1, 2, 5, 20, 50, 80, and 100 mg/mL. The salt concentration (c\textsubscript{s}) was varied from 0 to 60 mM. The phase diagrams shown in Figure 1 were determined by eye, UV–vis transmittance measurements, and optical microscopy.\textsuperscript{35} The first phase transition from regime I to II at the specific salt concentration c\textsubscript{s} is defined by the onset of turbidity (Figures S1 and S2, and Table S1) and LLPS by the formation of a
and angle calibration of the ellipsometer) are substantially smaller.

RESULTS AND DISCUSSION

Interface Studies/Adsorption. All adsorption measurements were performed in situ, and the adsorption time was set to 1 h, which was sufficiently close to equilibrium conditions. All samples for ellipsometry and QCM-D were centrifuged and only the supernatant was used (clear solution necessary to perform ellipsometry).

To ensure the reproducibility of our findings and to estimate real standard deviations as statistical error bars, all measurements were repeated at least three times. The systematic errors (e.g., wavelength and angle calibration of the ellipsometer) are substantially smaller than the statistical error bars. In all figures, the mean value of those measurements with real standard deviation is plotted.

Ellipsometry. The substrates used were p-doped (boron) Si wafers with (100) orientation, which were purchased from Micro-Chemicals GmbH (Germany) (product no. WSM606752XB134SN1). The substrates had a thickness of 675 ± 25 μm² and a resistivity of 1–10 Ω·cm. The substrate size was 16 to 8 mm with a native silica layer of roughly 1.7 nm on top, which was measured for each sample individually. The substrates were cleaned with acetone, isopropanol, and water, respectively, for 10 min in each solution in the ultrasonic bath. The contact angle of the substrate with water was around 30°, and the substrate roughness was below 1 nm. We utilized a VASE M-2000 ellipsometer by J.A. Woollam (USA) to perform adsorption measurements with a homemade, solid–liquid cell at 68°, the Brewster angle of native silica in water.56–62 For data collection and analysis, we used the CompleteEASE software of J. A. Woollam by creating a model including the optical properties of the individual layers. Specifically for BSA, a Cauchy layer with A = 1.43, B = 0.01, and C = 0 was chosen (in the literature, the value of A varies between 1.42 and 1.45 for BSA).53–60

QCM-D Measurements. QCM-D measurements were conducted with the Q-Sense Analyzer of Biolin Scientific (Sweden).58–60 The measurements were performed with the QSoft software and analyzed with DnDnd and QTools of Biolin Scientific. The quartz sensors used were native silica-coated (product no. QS-QSS303). The flow cell allowed in situ cleaning with 2% Hellmanex, ethanol, and water. The setup had the option of inverting the cell with the substrate on top of the solution, which was utilized, thus avoiding sedimentation effects. Since the dissipation (D) was greater than 0, a viscoelastic (Voigt) model was used for the data analysis of BSA. If the viscoelastic properties of the adsorbed material are too pronounced, the oscillating sensor gets damped too strongly (D ≠ 0) and the direct correlation between frequency drop and mass uptake described by the Sauerbrey model is not valid anymore.58 For more information on the Voigt model, see refs 68, 71, and 72. In combination with the ellipsometer, we were able to determine the associated water (d_{water}) within our adsorption layer.57,74 Since ellipsometry does not account for the water content in the adsorbed protein layer thickness determined (d) and the QCM-D cannot differentiate between water and proteins adsorbed to the interface, the extracted adsorbed layer thickness d_{QCM-D} contains both. More details on data analysis and fitting parameters can be found in ref 75.

ATR-FTIR Measurements. ATR-FTIR measurements allow for structure determination of adsorbed proteins to a solid interface and were conducted with the Thermo Nicolet iS50 with Specac Gateway ATR insert.66,67 The measurement software was Omnic, and the following settings were chosen for the absorbance measurements on the Si block: gain, 1; aperture, 10; scan no., 294; resolution, 4 cm⁻¹. The evanescent wave penetrates through the substrate a few micrometers into the bulk solution. Thus, the measured signal contains not only the adsorbed proteins but also the signal of the surrounding bulk proteins. To check for the influence of the bulk proteins on the absorbance data, we flushed the cell and checked if the signal of the reversibly adsorbed proteins in water changed compared to the adsorption layer in bulk, which was not the case (Figure S6). The measurements were conducted in H₂O, but checks were made in D₂O (data not shown), to ensure the background subtraction was sufficient even though the H₂O and amide-I signals are overlapping. The data were corrected for the background, but not for the baseline, which explains the small offset at 0 absorbance of the individual curves (Figure S5). The slight differences in the amide-II band and amide-I peak position compared to the FTIR measurements shown in Figure 2 are due to the use of H₂O as the solvent instead of D₂O.

We studied the effect of different anions and multivalent cations on the protein bulk phase behavior and the adsorption
La3+ can be rationalized by weaker protein interactions. While other mechanisms may also play a role, it is reasonable to assume that the bigger the cation (lower charge density), the weaker are the attractive interactions it can induce. A detailed study on the role of cations on the BSA phase behavior was performed by Martsaarskaia et al.53 Our findings are consistent with the previous work on chloride salts illustrated in Figure 1c and d.

Interestingly, no re-entrant condensation occurs with either iodide salt. This implies that no transition into regime III is observed and thus at high \( c_p \) the protein-salt solution remains in regime II. Additionally, in the case of the iodide salts, LLPS begins at lower \( c_p \) compared to BSA in the presence of chloride salts (Figure 1c and d). This indicates much stronger BSA–BSA attractive forces in the presence of iodide salts, for which protein–protein interactions are attractive even at very high \( c_p \). This behavior is in good agreement with the behavior found for BSA with nitrate salts. Note that the respective phase diagrams of BSA with nitrate salts are shown in Figure S3 as a reference for the reader. Braun et al.78 already observed attractive interactions of BSA at very high \( c_p \) in their case with nitrate salts (LaNO\(_3\) and YNO\(_3\)), indicated by the phase diagram and measured \( B_s/B_H^\infty \) values. A systematic change in phase behavior of BSA with Cl\(^-\), NO\(_3\)^-, and I\(^-\) can be observed: \( c^* \) shifts to higher \( c_p \). LLPS occurs at lower \( c_p \) and RC vanishes. Thus, ranking the anions from inducing strong attractive interactions from weakest to strongest: Cl\(^-\) < NO\(_3\)^- < I\(^-\). Multiple factors contribute to this behavior, which are explained in the following sections and supported by relevant anion properties in Table 1.

**Hydration and Protein Stability.** According to the Hofmeister series, iodide is more prone to cause destabilization of the protein (denaturation) than chloride,92 which could prevent re-entrant condensation (\( c^* \)). Yet, at low and moderate ionic strengths (<0.1 M), weakly hydrated anions such as iodide neutralize the electrostatic repulsive forces and thermostabilize BSA more efficiently than strongly hydrated anions, leading to the reverse Hofmeister series: SCN\(^-\) > I\(^-\) > NO\(_3\)^- > Br\(^-\) > Cl\(^-\) > SO\(_4\)\(^{2-}\).

\[
\text{SCN}^- > \text{I}^- > \text{NO}_3^- > \text{Br}^- > \text{Cl}^- > \text{SO}_4^{2-}
\]  

(3)

This order is based on the hydration of an ion, which is linked to its ion radius, heat capacity \( C_{p,\text{ion}} \), ionic B coefficient \( B_s \), and structural entropy \( S_{\text{str}} \) listed in Table 1. These properties also determine whether the ion is making or breaking the water structure around itself according to Marcus83,86,88 and can be...
expressed with the water structure parameter $\Delta G_{\text{HB}}$.

Thus, in this $c_\text{b}$ range (<0.1 M), iodide stabilizes the BSA structure better than nitrate and nitrate does so better than chloride. In fact, $\text{Cl}^-$ is known to have little effect on the water structure or protein stability,

and thus has a passive role in this context.

In order to assess the influence of iodide on the secondary structure of BSA, FTIR measurements were performed (see Figure 2). The secondary structure of BSA is stable over a LaI$_3$ concentration range from 0 to 20 mM with a prominent peak around 1650 nm

D$_2$O (amide-I). This peak is associated with $\alpha$-helices, which make up roughly 66% of BSA in its native shape

and indicates an intact globular structure. Performing multiple measurements at different $c_\text{b}$ values of 0, 0.4, 3, and 20 mM did not show any significant structural changes in the amide-I band due to salt type or concentration. Thus, we can exclude that denaturation or strong structural changes in the protein structure are the cause for the suppression of re-entrant condensation, i.e., the absence of regime III.

**pH Changes.** The addition of salt and subsequent salt-induced water hydrolysis and ionization of hydrophilic protein residues can change pH and thus, in principle, protein behavior. Importantly, though, previous studies have shown that the trivalent cations (Y$^{3+}$ and La$^{3+}$) used here do not induce significant pH changes.

To determine the effect of anions on the pH, the pH of BSA-LaI$_3$ samples was measured after preparing the protein/salt mixtures (Figure 3). The pH decreases slightly with increasing salt concentration, yet the drop in pH does not correlate with the phase transitions seen in the bulk. The pH does not change significantly with time or $c_\text{p}$ either. This pH trend of LaI$_3$ is similar to that found for YCl$_3$ in our previous publication as well as similar to the pH variations of pH-neutral salts (i.e., YCl$_3$ and LaCl$_3$).

We thus conclude that, while pH effects may contribute to the protein phase behavior in some form, they do not qualitatively change the bulk behavior and are not primarily responsible for the absence of regime III observed with iodide salts as opposed to the chloride salts.

**Amount of Anions Bound to BSA.** The literature consistently reports that more iodide anions are bound to BSA per molecule than nitrate ions and even less chloride anions (see Table 1). If the number of anions bound depends on the anion type, it can explain why a certain anion type has a stronger influence on the protein phase diagram than another, which results, e.g., in the disappearance of re-entrant condensation. The number of anions bound increases as Cl$^- < NO_3^- < I^-$, which reflects the order of ion-induced attractive interactions in the phase diagram. Thus, it is reasonable to assume that iodide has a stronger influence on protein–protein interactions, which again emphasizes the general weak effect of Cl$^-$ on the protein stability or water structure.

To obtain a comprehensive understanding on the different binding affinities of anions to BSA, different mechanisms and properties leading to the binding affinity are described and discussed in the following sections.

**Strength of Protein–Ion Interaction.** Weakly hydrated anions bind directly to proteins, causing the protein to maximize its solvent accessible surface area and the bulk solution to become a better solvent. In turn, strongly hydrated anions interact indirectly through bound water molecules with the protein, thus reducing the proteins surface area by making it more compact. The bulk solution becomes a worse solvent.

Anions with a lower charge density bind more tightly to the protein. This implies that iodide binds more tightly to BSA than nitrate and nitrate stronger than chloride (see Table 1 for anion radius and surface charge). In other (positively charged) proteins, it was found that iodide can bridge proteins, thus promoting anion-induced cluster formation.

One indicator for iodide-mediated protein–protein bridging, as well as cation bridging of BSA molecules in solution, is that, at high $c_\text{b}$ for both LaI$_3$ and YI$_3$, the protein cluster sizes increase until they start to sediment at $c_\text{b} > c_\text{s}$ (LLPS) and the volume of "dense" (sedimented) protein phase further increases with increasing $c_\text{b}$ (Figure S1a and b). This is also inversely reflected in the $c_\text{b}$ values of the "dilute" (upper) phase, which decreases with increasing $c_\text{b}$ (Figure S5). The combination of increased $c_\text{b}$ and volume of the sedimented phase indicates consistently attractive interactions between proteins even at high $c_\text{b}$ preventing re-entrant condensation. This is consistent with results by Braun et al. for BSA/La(NO$_3$)$_3$$_3$ which found stable $B_2/B_\text{HS}$ values at $\approx 2.25$ for high $c_\text{b}$ and $c_\text{p}$ at which re-entrant condensation vanished. In systems in which re-entrant condensation is always present (i.e., LaCl$_3$ and YCl$_3$), the $B_2/B_\text{HS}$ values start to increase at high $c_\text{b}$, illustrating the decreasing attractive force due to cation-induced overcharging effects of the proteins. The decreased $c_\text{p}$ of the dilute phase of YI$_3$ compared to LaI$_3$ further supports the finding of stronger BSA–BSA interactions in the presence of Y$^{3+}$ and is consistent with the trend found for LaCl$_3$, compared to YCl$_3$ by Braun et al.

**Binding Sites.** The literature distinguishes between specific and nonspecific, high and low affinity, and polar and nonpolar ion-binding sites on proteins. It appears to be established that chloride binds to cationic/basic binding sites of BSA, HSA, and different (nonspecific) binding mechanism for iodide to positively charged protein groups of BSA, while others find a different (nonspecific) binding mechanism for iodide to nonpolar groups of HSA, human carbonic anhydrase II, lysozyme, human carbonic anhydrase II, and peptide. The same applies to the binding of other anions such as anionic dyes, anionic amphiphiles, or anionic ligands to BSA, all of which bind preferentially to hydrophobic groups. In some cases, an interplay of electrostatic and hydrophobic interactions is needed, in which the proximity of positive and nonpolar groups has a favorable effect on anion binding. BSA has numerous binding sites with different binding affinities due to specific and nonspecific binding mechanisms. In any case, the binding mechanism of iodide is...
much more complex than the binding of chloride.\textsuperscript{105} Chloride binds only to positively charged sites, whereas, depending on the bulk properties (i.e., charge and polarity of protein), iodide will bind specifically to positively charge sites and/or nonspecific to hydrophobic sites with different affinities.\textsuperscript{104,110,116} In comparison, multivalent cations bind to acidic areas (surface-exposed carboxylic groups)\textsuperscript{79,117,118} on the protein via an endothermic, electrostatically and entropy-driven process.\textsuperscript{119}

**Competing Interactions.** In addition to the properties of the anion, the trivalent cations have to be considered as well. Anion–cation chlоро-complex formation can be excluded since those start to form at 0.2 M for yttrium salts and 0.4 M for lanthanum salts, respectively.\textsuperscript{120,123} Nitrate-complexes start to form at 0.12 M for lanthanum salts and 0.18 M for yttrium salts,\textsuperscript{122,123} which are \( c_s \) values outside of the phase diagram shown in Figure 1. Iodo-complexes start to form at lower \( c_s \) than chlоро-complexes, yet this should not influence our measurements.\textsuperscript{124} Thus, anion–cation complexes do almost certainly not contribute to the effects studied. However, anions could assist and amplify the effect of cations on protein–protein interactions.\textsuperscript{111,115} Depending on the cation type and charge of the protein in system, the anion can support the destabilizing or stabilizing role of the cation on the protein structure.\textsuperscript{111,125}

Overall, electrostatic (and hydrophobic) ion–protein and protein–protein interactions and the special role of multivalent ions are key for the understanding of the observed (bulk) behavior. The increasing role and effect of anions from Cl\textsuperscript{−} < NO\textsubscript{3}\textsuperscript{−} < I\textsuperscript{−} on the phase diagram can be explained with the combination of an increasing number of ions bound to BSA, stronger binding, increasing protein-stabilizing role of ions, potentially anion bridge formation and increasing role of nonspecific protein–protein interactions, as well as cation bridges. These properties appear to be responsible for preventing the system from undergoing re-entrant condensation triggered by trivalent cations, which means that it remains in regime II with dominant attractive forces between protein molecules, for the iodide salts and partially for nitrate salts.

**Protein Adsorption.** In this section, we discuss how the presence of different salts (LaCl\textsubscript{3}, YCl\textsubscript{3}, LaI\textsubscript{3}, YI\textsubscript{3}) in BSA solutions (\( c_p = 20 \text{ mg/mL} \)) influences the adsorption behavior of BSA to a net negatively charged, hydrophilic substrate and to which extent this is related to the bulk protein phase behavior.

**Salt-Dependent Protein Adsorption (Ellipsometry).** The thickness of the adsorbed amount of BSA on native silica, \( d \), is plotted as a function of ratio of salt/protein concentration (\( c_s/c_p \)) in Figure 4. The effective measured \( d \) with ellipsometry assumes a volume fraction of 1, which is laterally averaged over the measured surface.

Different aspects need to be considered when comparing the different adsorption trends. These include the position of the adsorption maximum, the amount of protein adsorbed, and the overall shape of the curve. Without salt, adsorption of \( d \approx 1 \text{ nm} \) can be observed. This can be attributed to positively charged side chains of the protein interacting with the negatively charged substrate, as well as contributing hydrophobic and hydrogen bond interactions,\textsuperscript{112} leading to sparse adsorption at the interface.

The initial increase of adsorption can be ordered according to LaI\textsubscript{3} < LaCl\textsubscript{3} < YI\textsubscript{3} < YCl\textsubscript{3} shown in the inset of Figure 4, meaning that the smallest amount of YCl\textsubscript{3} is needed to achieve the thickest adsorption layer at low \( c_s \). This behavior reflects the bulk phase transition \( c^* \) from regime I to regime II at \( 2 > 1.5 \approx 1.5 > 1.3 \text{ mM salt} \), respectively, which occurs at lower \( c_s \) for the yttrium salts than for the lanthanum salts due to the stronger attractive intermolecular forces induced by Y\textsuperscript{3+} (see Figure 1). For iodide salts, more salt has to be added to the protein solution to observe the transition from regime I to II at \( c^* \) than for chloride salts. This is due to the fact that iodide has a dominant role in the protein behavior hindering “simple” charge screening through multivalent cations in bulk and at the interface.

The shape of the adsorption curve maximum is similar to that of the bulk interactions. In bulk, regime II of the BSA–LaCl\textsubscript{3} system is very narrow compared to that of YCl\textsubscript{3} (Figure 1), which is reflected in the width of the maximum. For the iodide salts, the LLPS regime starts at a lower \( c_s \) (3 mM) for YI\textsubscript{3}, than for LaI\textsubscript{3} (5 mM) (Figure 1a and b). This bulk instability induces stronger adsorption, explaining the position of the curve maximum (Figure 4).

Another interesting observable is the maximum adsorbed amount, which follows the order LaCl\textsubscript{3} < YCl\textsubscript{3} < YI\textsubscript{3} < LaI\textsubscript{3}. The weaker adsorption for LaCl\textsubscript{3} in comparison with YCl\textsubscript{3} is due to weaker attractive protein–protein and subsequently protein–substrate interactions. The general adsorption trend of LaCl\textsubscript{3} and YCl\textsubscript{3} can be explained using the ion-activated attractive adsorption model.\textsuperscript{126} It assumes negatively charged patches on the protein and the substrate to which cations can bind and also form ion bridges between protein molecules and between protein and substrate. In this context, the model neglects the anions (here: chloride), since they have no strong impact on the overall bulk\textsuperscript{99} or adsorption behavior. Adsorption is guided by the number of multivalent cations bound to the proteins and the substrate and an attractive wall potential experienced by the proteins.\textsuperscript{126} This obviously applies to both LaCl\textsubscript{3} and YCl\textsubscript{3}.

The iodide salts show higher maximum adsorption in comparison to the chloride salts. It is important to bear in mind that the iodide salts induce a strong LLPS in regime II in the bulk solution (Figure 1). In a previous publication, we studied the correlation of metastable LLPS formation (i.e., bulk instability) in bulk with enhanced protein adsorption and found a wetting transition induced by LLPS at the solid

![Figure 4](https://dx.doi.org/10.1021/acs.langmuir.0c02618)

**Figure 4.** Ion effect on protein adsorption. Ellipsometric measurements of the adsorbed protein layer thickness \( d \) as a function of \( c_s/c_p \) at 20 mg/mL BSA and room temperature for LaCl\textsubscript{3} (light green), YCl\textsubscript{3} (orange), YI\textsubscript{3} (blue), and LaI\textsubscript{3} (dark green). The data for YCl\textsubscript{3} is taken from ref 126. The absolute value of \( d \) increases as LaCl\textsubscript{3} < YCl\textsubscript{3} < YI\textsubscript{3} < LaI\textsubscript{3}. For better visibility, the inset shows a magnification of the initial adsorption increase from 0 to 5 \( c_s/c_p \) together with a guide to the eye.

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These results are in good agreement with our findings and further support the wetting layer transition at bulk instability also for iodide salts. Here, LaI$_3$ leads to more pronounced adsorption compared to YI$_3$, which appears counterintuitive at first. Even though yttrium induces stronger interprotein attraction, it appears that this trend is reversed if the counterion is iodide. This shows that the ion-activated adsorption model cannot be applied to the iodide salts since the iodide anions compared to chloride ions have a strong impact on the phase behavior, as well as on adsorption, and the model does not account for the anions in solution. Note that in regime II only the dilute phase can be used for ellipsometry measurements. The dilute phase shows a $c_s$-dependent decrease in $c_p$ (see Figure S5). This effect may contribute in some form to the smaller amount of adsorbed protein in the presence of YI$_3$ compared to LaI$_3$, but it does not alter the dominant interaction and interaction strength of the bulk solution. It seems that a “stronger” cation such as Y$^+$ induces interactions which interfere with and diminish the effect of iodide. However, with a “weaker” cation such as La$^+$, iodide has a more prominent effect on the amount of protein adsorbed, indicating a pronounced formation of anion bridges and nonspecific protein–protein binding.

At high $c_p$ we observe re-entrant adsorption for all salts. For the chloride salts, this was expected since these salts undergo re-entrant condensation at $c_{**}$ from regime II to regime III in...
the bulk, which is defined by charge inversion of the proteins due to trivalent cation binding and thus a decrease in attractive forces leading to smaller clusters and to a decrease in adsorption. These results are consistent with previous findings in polyelectrolytes and proteins and can be explained with the ion-activated adsorption model. For the iodide salts, re-entrant condensation in the bulk was not observed. Re-entrant adsorption on a solid interface, however, was observed. For a hypothesis rationalizing this behavior, it helps to consider the possible protein-protein vs protein-surface interactions. Due to the surface being negatively charged and hydrophilic, anions are not likely to bind to the substrate with high surface excess since iodide prefers to adsorb to nonpolar and/or positively charged surfaces. This means that, in the vicinity of the substrate with restricted properties, charge inversion mediated by trivalent cations can occur. In the bulk, in contrast, this is not the case due to the complex protein surface of BSA containing nonpolar, polar, negatively and positively charged areas. Consequently, the variety of possible protein-protein and protein-salt interactions hinder re-entrant condensation.

**Global Protein Structure on Substrate (ATR-FTIR).** To exclude denaturation at the interface as the primary source for re-entrant adsorption at high $c_w$ we performed ATR-FTIR measurements on BSA in the presence of LaCl$_3$ and LaI$_3$ on a Si block (Figure 5), which showed essentially intact secondary protein structures. Both salts showed maximum absorbance at 1657 cm$^{-1}$, which correlates with its dominant $\alpha$-helix structure. The absorbance signal is much more intense for LaI$_3$, which can be explained by an increased adsorption compared to LaCl$_3$ and is consistent with the ellipsometry findings. The amide-I band does not significantly change its shape with increasing concentration of the same salt, but it differs slightly between the two salts.

**Protein Layer Structure and Kinetics (QCM-D).** Complementary measurements were conducted with QCM-D, which on the one hand confirmed the results of our ellipsometry data and on the other hand provided additional insights into the structure and properties of the adsorption layer.

For data analysis, we used the Voigt viscoelastic model to calculate the thickness of the adsorbed layer from the measured frequency and dissipation changes (for examples of raw data, see Figure 6). Already from the raw data, the difference between LaCl$_3$ and LaI$_3$ is obvious. For all samples in the presence of the respective salt, the frequency drop is lower in the presence of LaI$_3$. This means that more proteins are adsorbed to the substrate, leading to a stronger damping of the oscillating substrate compared to the same $c_w$ of LaCl$_3$. In addition, the dissipation is higher in the LLPS regime, indicating a more diffuse layer formation. Similar to the ellipsometry data (Figure 4), we observe re-entrant adsorption for both data sets. The calculated adsorbed protein layer thickness $d_{\text{QCM-D}}$ is plotted in Figure 7a. The overall adsorbed $d_{\text{QCM-D}}$ of BSA/LaI$_3$ is enhanced compared to LaCl$_3$, which is in good agreement with the results shown in Figure 4. By rinsing the QCM-D cell with water, the amount of irreversibly adsorbed proteins was determined (Figure 7, black symbols). These make up only a small portion of the full adsorption layer of roughly 10 nm and can be assumed to be the first monolayer of proteins directly in contact with the substrate.

A big advantage of QCM-D in combination with ellipsometry is that we are able to determine the associated water $d_{\text{assoc}}$ within our adsorption model (Figure 7b). The associated water consists of a hydration layer around the proteins, hydro-dynamically bound water to the substrate and water trapped within the adsorption layer. With the information on the water content in the adsorption layer, the layer morphology can be better understood. In a previous publication, we established experimentally and theoretically the correlation between the formation of a wetting layer at the bulk instability induced by LLPS and the subsequent formation of a wetting layer. Note that the offset between the two maxima is due to the different positions of regime II in the bulk.

**CONCLUSIONS**

In this study, we focused on the effect of anions and cations on the protein bulk behavior and adsorption behavior. Interestingly, we reveal that the co-ion (anion) changes the balance and weakens the effect of trivalent cations in the bulk, making re-entrant condensation disappear and liquid-liquid phase separation appear. Chloride does not appear to significantly affect the phase diagram of BSA, whereas iodide promotes stronger attractive protein-protein interactions. This illustrates a dominant role of anions on the phase behavior of globular proteins in the presence of multivalent cations.

By choosing specific substrate properties (here: imitating the bulk properties), the influence of ions on protein adsorption can be controlled. In the BSA-YCl$_3$ system studied in ref 126,
the bulk and adsorption behavior were highly similar due to the negative charge of the proteins, as well as the substrate, and the passive role of chloride. By extending this study to salts with different cations and anions, we have shown that the adsorption behavior is not solely guided by the dominant electrostatic bulk interactions but can also be triggered by surface parameters. This explains the observations presented here: re-entrant adsorption in the absence of re-entrant condensation in bulk. The higher adsorbed amount supports the dominant role of iodide and the hypothesis of anion bridging and nonspecific binding in bulk.

Through the use of suitable anions and cations, the dominant interactions in bulk can be tuned, while in the adsorption process substrate properties can effectively reduce or even uncouple those interactions. The regulation of interactions on a molecular level opens up new avenues for drug design and biomaterials. In addition to its medical relevance, the use of iodide is potentially very useful for studies of our system using X-rays due to its high scattering power and contrast with light elements (i.e., water and proteins).

## Associated Content

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.langmuir.0c02618.

- Phase transition determined by UV–vis transmittance measurements; dilution series and bulk microscopy images; UV–vis transmittance measurements; BSA phase diagram in the presence of nitrate salts; complete FTIR absorbance spectrum; protein concentration of dilute phase; ATR-FTIR absorbance measurements after rinsing (PDF)

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### Abbreviations

Y³⁺, yttrium cation; La³⁺, lanthanum cation; Cl⁻, chloride anion; I⁻, iodide anion; NO₃⁻, nitrate anion; BSA, bovine serum albumin; QCM-D, quartz-crystal microbalance with dissipation monitoring; SiO₂, silicon dioxide; LLPS, liquid–liquid phase separation; RC, re-entrant condensation; FTIR, Fourier transform infrared spectroscopy; ATR, attenuated total reflectance; HSA, human serum albumin; B₁/₂eff, reduced second virial coefficient; c₀, salt concentration; c*, first phase transition; c**, salt concentration of re-entrant condensation; d₃₂ protein concentration; rₑff, effective ion radius; Cₛᵦᵣ, heat capacity; dₚ/Δcₚ, ionic aqueous surface tension; B, Jones–Dole ionic B coefficient; Sₑ, structural entropy; ΔGₙₑw, water structure parameter; dᵣ, adsorbed protein layer thickness; dₓ, adsorbed protein layer thickness via QCM-D; dₛₑw, associated water in the adsorbed layer

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