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Phase stability of aqueous mixtures of bovine serum albumin with low molecular mass salts in presence of polyethylene glycol

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Abstract

The stability of bovine serum albumin (BSA) solutions against phase separation caused by cooling the system is studied under the combined influence of added poly(ethylene glycol) (PEG) and alkali halide salts in water as solvent. The phase stability of the system depends on the concentration of the added PEG and its molecular mass, the concentration of the low molecular mass electrolyte and its nature, as also on the *p*H of the solution. More specifically, the addition of NaCl to the BSA-PEG mixture promotes phase separation at *p*H = 4.0, where BSA carries the net positive charge in aqueous solution, and it increases the stability of the solution at *p*H=4.6, i.e., near the isoionic point of the protein. Moreover, at *p*H = 4.6, the cloud-point temperature decreases in the order from NaF to NaI and from LiCl to CsCl. The order of the salts at *p*H = 4.0 is exactly reversed: LiCl and NaF show the weakest effect on the cloud-point temperature and the strongest decrease in stability is caused by RbCl and $NaNO_3$. An attempt is made to correlate these observations with the free energies of hydration of the added salt ions and with the effect of adsorption of salt ions on the protein surface on the protein–protein interactions. Kosmotropic salt ions decrease the phase stability of BSA-PEG-salt solutions at *p*H < *p*I, while exactly the opposite is true at *p*H = *p*I.

Keywords

Bovine serum albumin solution; Polyethylene glycol; Cloud–point temperature; Solubility; Salt specific effects

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

5. CRediT authorship contribution statement

Hurija Džudžević Čančar, Matic Belak Vivod, Miha Lukšič: Methodology, Validation, Writing - Original Draft, **Hurija Džudžević Čančar, Matic Belak Vivod**: Investigation, **Miha Lukšič**: Software, Visualization, **Vojko Vlachy, Miha Lukč**: Conceptualization, Resources, Supervision, Project administration, Funding acquisition, Writing - Review & Editing.

1. Introduction

Proteins have ionizable amino acid residues on their surface that can be charged in aqueous solution. For this reason, proteins in water exhibit certain properties which are common to aqueous polyelectrolyte (polyampholyte) solutions. For example, in addition to the *p*H of the solution, they are also sensitive to the amount of added low molecular mass electrolytes (simple salts) as well as to their chemical nature. Many phenomena involving protein solutions that are important in industrial and technological processes are therefore salt-specific [1–15]. For example, protein transport across membranes, salting-in and salting-out, crystallization, to name a few, are strongly influenced by the type of salts added.

One of these phenomena is protein aggregation, which poses a serious challenge to the pharmaceutical industry [16,17]. Indeed, biopharmaceutical formulations must be free of aggregates to ensure high potency and low viscosity. The importance of these phenomena has led to a long-standing interest in the study of salt-specific effects, which is reflected in the so-called Hofmeister series. The latter is defined as a list of ions ranked according to their efficiency in affecting the solubility of proteins [1,6]. The specific effects of buffers at the same nominal *p*H of protein solutions have recently been critically studied by Salis and Monduzzi [13]. Indeed, buffer ions compete with ions of the added strong electrolyte for adsorption on the protein surface. Clearly, studies of aqueous electrolyte-protein solutions leading to a better understanding of the protein–protein interaction mediated by water and salts are of great importance for both life sciences and industry.

Formulations of proteins often contain also uncharged polymers. Poly(ethylene glycol), PEG, is one such example and plays a versatile role, for example, in pharmaceutical applications [18]. Knowledge of polymer-protein interactions and their dependence on the molecular mass of the polymer as well as on its concentration are key factors when it comes to polymer-protein formulations in bio-related applications [19]. Nowadays, PEG is used as one of the most effective crystallization agents. Studies on the effect of PEG on solubility or liquid–liquid phase separation in protein solutions have mostly focused on salt-free protein PEG systems (e.g. [20–23]), while there are significantly fewer studies involving protein-PEG mixtures with added salts (e.g. [24–26]).

To learn more about protein aggregation and the influence of salts and polymers on this process, we performed an experimental study of BSA-PEG-salt mixtures in acetate buffer at *p*H = 4.6, i.e., near the isoionic point of BSA (*p*I ≈ 4.6) and separately at *p*H = 4.0, where the net charge of the protein is positive. Note that the literature values for *p*I vary somewhat depending on the experimental method and conditions used [27]. In the experimental procedure used here, the solution is cooled until the first cloudiness appears. Following many others, we call this temperature the cloud-point temperature, T_c. For the reader's orientation, lower values of the cloud-point temperature indicate higher stability of the solution against aggregation/phase separation (colloidal stability).

In the first part of the study, we varied the amount and molecular mass of PEG, which was added to the BSA-buffer mixture. The concentration of the protein was fixed at 90

mg/mL, while the concentration of the acetate buffer was 0.1 mol/L. Next, we examined the effect of adding NaCl on the T_c of the PEG-BSA-buffer mixtures at *p*H values of 4.6 and 4.0. Interestingly, the effect (an increase or decrease in T_c when salt was added) depended qualitatively on *p*H of the solution. These interesting results prompted us to investigate in more detail the cloud-point temperatures of BSA mixed with PEG and various low molecular mass salts. The salts we studied included NaF, NaBr, $NaNO_3$, NaI, and the series of alkali chlo-rides from lithium to cesium chloride. We varied the amount of PEG added and in some case also its molecular mass. The idea was to examine the salt-specific effect in BSA-PEG-salt mixtures under a wide range of experimental conditions.

A related earlier study was published in Ref. [24], in which the lysozyme-electrolyte mixture was titrated with PEG to investigate protein solubility under such conditions. The results showed that the solubility of lysozyme at 25 °C decreased with the addition of PEG; the solubility was lower for a PEG with a higher degree of polymerization. Also, increasing the NaCl concentration decreased the solubility of lysozyme in the presence and absence of PEG. The solubility experiments were performed at *p*H values of 4.0 and 6.0, in both cases the lysozyme was net positively charged. The *p*I of lysozyme is 11.2, where this protein is unstable against conformational changes, so virtually no measurements were made in this *p*H range. Important qualitative differences between the results for lysozyme and BSA mixtures with PEG and low molecular mass salts will be discussed.

2. Materials and Methods

Bovine serum albumin (BSA; purity ⩾ 96%, $M_w \approx 66.5$ kDa, fatty acid free; lot number: SLBM9552V), poly(ethylene) glycol (PEG; $M_w \approx 6000$, 10000, and 20000 g/mol), and NaI were purchased from Sigma Aldrich. Other chemicals were supplied by Merck (LiCl, NaCl, KCl, CsCl, NaF, NaBr, $NaNO_3$, CH_3COONa, 100% CH_3COOH and 1 mol/L NaOH solution), and by Riedel-De Haän (RbCl).

All solutions were prepared with Mili Q water at room temperature. A 0.1 mol/L acetate buffer was prepared by adding 1 mol/L NaOH solution to an appropriate amount of acetic acid in water to obtain the desired *p*H value of 4.6 and 4.0. *p*H was measured by Iskra pH meter model MA5740 (Ljubljana, Slovenia), using a combined glass microelectrode InLab 423 from Mettler Toledo (Schwerzenbach, Switzerland).

BSA-buffer solutions were prepared by weighing the dry protein and dissolving it in a 0.1 mol/L acetate buffer (*p*H = 4.6 and 4.0). All solutions were filtered through 0.45 μm Minisart Sartorious filters. Solutions were dialyzed extensively (three changes of fresh buffer solutions every 8 h) against the same buffer using a dialysis cassette (Slide-A-Lyser Dialysis Cassette G2 Thermo Scientific, M_w cut-off 3.5 kDa). The concentration of BSA was determined by UV–vis spectrophotometer (Varian Cary 100 Bio) at 280 nm using extinction coefficient 0.667 $mLcm^{-1}mg^{-1}$ [22]. The BSA stock solutions used had a protein concentration of approximately 160 mg/mL.

PEG-buffer solutions were prepared by weighing dry PEG (6000, 10000, and 20000 g/mol) and dissolving it in a 0.1 mol/L acetate buffer (*p*H = 4.6 and 4.0). Stock solutions were prepared with PEG concentration of 250 mg/mL.

All low molecular mass salts were first dried at 105 °C for 1.5 h and then left in the desiccator to cool. 0.1 mol/L salt solutions were prepared by weighing an appropriate amount of the dry salt and dissolving it in the 0.1 mol/L acetate buffer solution with *p*H = 4.6 and 4.0.

BSA-PEG and BSA-PEG-salt solutions were prepared by mixing the appropriate volumes of the BSA, PEG and salt stock solutions just prior to the measurements. ThermoFisher Scientific pipettes were used, and volume additivity was assumed. The *p*H of all solutions was measured and no deviations beyond the experimental uncertainty (±0.04) were found upon the salt addition. The indicated concentrations of BSA, PEG and low molecular mass salts denote the corresponding concentrations of these species in the prepared aqueous-buffer formulation (in all formulations the concentration of the buffer species is 0.1 mol/L).

Measurements of the cloud-point temperature, T_c (defined as the temperature at which the first opacity of the solution occurs after cooling), were determined by monitoring the absorbance of the solution at λ = 340 nm as a function of temperature. An Agilent Technologies Cary 100 UV–vis spectrophotometer coupled to a Cary temperature controller (Agilent) was used. The solutions were kept in a micro quartz cuvette (Black Wall 0.7 mL, 1 cm optical path length). The determined values of cloud-point temperatures may depend on the cooling rate (see, e.g., Ref. [28]). First, cooling rate optimization was performed, with T_c recorded at 0.1, 0.2, 0.25, 0.5, and 1 °C/min. For all T_c measurements reported here, we used the cooling rate of 0.25 °C/min, which was found to give the most reliable results and was also consistent with our previous measurements. UV–vis spectroscopy does not allow us to determine the nature of the phase transition. However, in most cases, the formulation cooled below the cloud-point temperature separated into two liquid phases (protein-rich and protein-poor), and in some cases an amorphous protein precipitate was observed.

3. Results and Discussion

In the following sections, the results of measurements of the cloud-point temperature, T_c, of aqueous BSA-PEG and BSA-PEG-salt solutions in 0.1 mol/L acetate buffer at *p*H = 4.6 and 4.0 are presented. The dependence of T_c on the concentration of PEG, the concentration of the salt as well as on the average molecular mass of PEG and on the identity of the salt was investigated. Ion-specific trends were correlated with the free energy of hydration of the salt ions and with the affinity of the ions for the protein surface, which affects the net charge of the protein and the associated protein–protein interactions. The BSA concentration was 90 mg/mL in all cases. PEG was added to the protein solutions to bring the T_c into the experimentally measurable temperature range (above approximately −8 °C).

The protein isoionic point, *p*I, depends to some extent on the properties of the solution (amount of protein and salt, identity and concentration of buffer) and on the experimental method used to determine *p*I. Literature data on the isoionic point of BSA, determined by

osmometry and electrophoresis, range from *p*I = 4.4 to 4.7 [29,30]. Values obtained from ζ-potential measurements range from 4.1 to 5.1 [27,31–33]. For a 1 mg/mL BSA in aqueous solution, Salis et al. determined the isoionic point of BSA in the presence of 100 mM NaCl to be at *p*H = 4.6. The second minimum of the virial coefficient was also found at *p*H ≈ 4.6 [34]. Therefore, in this work *p*H = 4.6 was taken as the isoionic point. If the *p*H of the solution is lowered below the isoionic point of the protein, it will result in a positively charged protein surface. At *p*H = 4.0, which was used in the present work, the net charge of the BSA molecule was estimated to be about $+7e_0$ [31,35].

It should be noted that the distribution of ionizable amino acid residues on the surface of the protein is not uniform, resulting in an asymmetry in the charge distribution. As a result, the protein has a dipole moment (as well as higher moments, such as the quadrupole moment [36]) that depends on *p*H. At the isoionic point, where the net charge of the protein is zero, the dipole moment is nonvanishing. The effect of added salts on the electrostatic screening of like-charge repulsion and dipolar attraction can therefore be *p*H-dependent, meaning that salts can decrease or increase the T_c value of the protein solution depending on the *p*H value of the mixture.

3.1. Higher molecular mass PEGs are more effective in reducing the phase stability of aqueous-buffer BSA solutions

Fig. 1 shows the results for the cloud-point temperatures, T_c, of BSA-PEG solutions in acetate buffer as a function of PEG concentration. Data are given for PEG-10k (i.e. the average molecular mass of PEG equals 10, 000 g/mol) and PEG-6k (6, 000 g/mol). The concentration of protein was fixed at 90 mg/mL, and no salt was added to these solutions.

As expected based on previous experimental and theoretical studies, the presence of PEG decreases the phase stability of the protein solution and the cloud-point temperature increases with the increase of the amount of PEG in the mixture. The effect of polymer addition is significant: changing the PEG-10k concentration from 26 to 32 mg/mL at *p*H = 4.6 (Fig. 1a), increases the cloud-point temperature from about −3 to +20 °C. Similarly, for PEG-6k–protein mixtures, an increase from +7 to +24 °C is observed when the concentration of PEG changes from 34 to 39 mg/mL. It is also clear that PEGs with higher molecular mass are more effective in reducing the phase stability of the protein formulation; both facts have been established previously (*cf.* Refs. [37–40,24], and references therein). Unfortunately, the measurements could only be made in an interval of T_c values from about +40 to −8 °C. In most cases, this fact prevents reasonable extrapolations to a zero PEG concentration, i.e., to PEG free BSA-buffer mixtures.

In Fig. 1b we plot the T_c against the PEG-10k concentration at *p*H = 4.0. From this data, it is quite clear that the protein solution is more stable at this *p*H because the protein molecule is net positively charged. One needs to add more PEG (almost twice as much as in the case of *p*H = 4.6) to make the protein solution unstable (to observe the cloudiness) under these conditions. Note that the concentrations of the protein and buffer are the same in both panels of Fig. 1.

The effect of the molecular mass of PEG on the cloud-point temperature at *p*H = 4.6 is summarized in Fig. 2. T_c of solutions containing 90 mg/mL BSA and 30 mg/mL PEG are shown for PEG-6k, PEG-10k and PEG-20k. Lai et al. have systematically investigated the influence of the molecular mass of PEG on the fluorescence properties of bovine serum albumin under physiological *p*H conditions [41]. They suggest that a certain amount of PEG can bind to both the hydrophobic patches on the protein surface and the positively charged regions, but the binding is weak. The hydrodynamic diameter of PEG at infinite dilution is estimated to be 45, 60.4, and 90 Å for PEG-6k, 10k, and 20k, respectively [42], and the hydrodynamic diameter of BSA is estimated to be approximately 74 Å [43]. For PEG-6k and PEG-10k, the coil size of the polymer is smaller than the size of the BSA molecule, while in the case of PEG-20k, the polymer molecule is larger than the protein. Increasing the molecular mass of PEG can lead to conditions where more than one BSA molecule can bind to one PEG molecule. Such conditions promote aggregation of BSA. From the point of view of depletion interactions [44], the tendency to destabilize the solution when the molecular mass of PEG is increased can be explained as follows: When two protein molecules approach each other, their depletion layers overlap and exclude the polymer molecules from this region. This increases the osmotic pressure, resulting in an attractive interaction between the proteins. For PEGs with higher molecular mass, the depletion layer becomes larger and the attractive interaction becomes effective at larger protein–protein distances. Under otherwise equal conditions, PEGs with larger molecular mass decrease the phase stability of the protein solution to a greater extent. The effect of molecular mass of PEG on T_c of BSA-PEG solutions is strong; increasing the M_w of PEG from 6, 000 to 20, 000 g/mol increases T_c from approximately −8 to 13 °C (*cf.* Fig. 2). Similar trends were observed for lysozyme-PEG mixtures [45].

3.2. *p*H of the BSA-PEG-salt solution determines whether addition of sodium chloride will stabilize or destabilize the system

One of the most interesting results of this study is shown in Fig. 3. Here we present the effect of increasing concentration of added salt (NaCl) on the cloud-point temperature of the BSA–PEG mixture at *p*H = 4.6 and 4.0. As before, the concentration of BSA and the acetate buffer are 90 mg/mL and 0.1 mol/L, respectively. Fig. 3a shows the results for solutions at *p*H = 4.6 and for PEG with molecular mass of 6,000 and 10,000 g/mol. In both cases, the concentration of PEG was equal to 30 mg/mL. Fig. 3b shows the effect of increasing NaCl concentration on T_c for solutions containing BSA and PEG-10k (c_{PEG} = 33 mg/mL) at *p*H = 4.0.

It is easy to note a qualitative difference between the two salt concentration dependencies: While at *p*H = 4.6 (close to the isoionic point) an addition of NaCl increases the stability of the system (Fig. 3a), an opposite effect is observed at *p*H = 4.0, where BSA carries a net positive charge (Fig. 3b). By adding a low molecular mass electrolyte to the protein mixture, we increased the ionic strength of the original solution. According to a somewhat simplified application of the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, an addition of salts (by shielding the protein–protein net charge interaction) causes a decrease in stability of the system, i.e., an increase in the cloud-point temperature. As we show here, the effect depends

on the *p*H of the solution. The 10% difference in the concentration of PEG cannot account for these qualitative differences.

A non-monotonic salt dependence of the potential of the mean force between proteins (associated with the *p*H of the solution), was attributed to the asymmetric charge distribution of the proteins. Phillies [46] as well as Tavares, Bratko, Prausnitz and others [47], have addressed the situation in which the forces between proteins were strongly influenced by dipolar interactions. This is a region of parameters where dissolved electrolytes screen the charge–charge repulsion more strongly than the dipole attraction. In our case, this would be for *p*H = 4.6 (*cf.* Fig. 3a). The protein model with distributed charge was also studied by Kalyuzhnyi and Vlachy [14], who showed that the second virial coefficient, B_2, can have a nonuniform dependence on the concentration of the added salt (see Fig. 3 in Ref. [14]). There are not very many experimental studies showing this effect; notable exceptions are Refs. [48,49]. The most recent contributions in this direction are those of Curtis and his group [50,51]. These authors measured an interaction parameter k_D by dynamic light scattering and obtained the osmotic second virial coefficient by static light scattering in solutions of monoclonal antibodies.

3.3. Phase stability of aqueous BSA-PEG-salt solution is salt-specific: the Hofmeister series

In this section, we examine the salt-specific effect in BSA-PEG-salt mixtures. This phenomenon plays an important role in biology and engineering and is discussed in many papers and books, to name a few [1–15]. Our experimental results are shown in Figs. 4–6.

First, we show in Fig. 4a the cloud-point temperature results for a series of sodium salts with different anions: NaF, NaCl, NaBr, $NaNO_3$, and NaI at *p*H = 4.6. Two PEG samples differing in molecular mass (6,000 and 10,000 g/mol) and concentration (30 and 36 mg/mL) were studied. The cloud-point temperature decreases in the order NaF > NaCl > NaBr > $NaNO_3$ > NaI. This means that addition of NaI stabilizes the system more than addition of NaF. In other words: We need to cool the protein-PEG solution mixed with iodide salt to a lower temperature to induce the phase separation. This arrangement applies to both PEG species (PEG-6k and PEG-10k) present in such a mixture.

The effect of cation variation (chloride salts) on T_c is shown in Fig. 4b. The solution conditions (protein, PEG, and salt concentrations) and, most importantly, the *p*H are the same as in Fig. 4a. It seems that the PEG-6k does not affect the salt specificity very much here. The ability of cations (in pair with chloride ions) to salt-out BSA follows the direction from LiCl to CsCl (i.e., the cloud-point temperature decreases in the order LiCl > NaCl > KCl > RbCl > CsCl). We also see that cations are less effective than anions.

These results are confronted with those presented in Fig. 5, where the cloud-point temperatures are shown for solutions with *p*H equal to 4.0. Note that under these conditions the protein has a net positive charge and therefore anions are the counterions. Again, panel a of Fig. 5 shows the effect of salt anions and panel b that of cations. Fig. 5a shows the results for the addition of NaF, NaCl, NaBr and $NaNO_3$. As we can see, the stability of the solution toward phase separation strongly decreases in the direction from sodium fluoride to sodium

nitrate (the cloud-point temperature decreases in the order $NaNO_3$ > NaBr > NaCl > NaF). The difference in T_c values is about 20 °C: Nitrate ions stabilize the solution much less than fluoride ions (when their partner in salt formation is sodium ion). This result is qualitatively different from that shown in Fig. 4a (*p*H = 4.6), where the ion ordering is just the opposite.

In Fig. 5b we show the effects of varying the cation in a set of chloride salts on T_c. Similar to Fig. 4b, data are shown for LiCl, NaCl, KCl, and RbCl. Here, RbCl is most effective and LiCl least effective in triggering the phase separation: T_c decreases in the order RbCl > KCl > NaCl > LiCl. This result is in qualitative contradiction with the results displayed in Fig. 4b, which are for solutions with *p*H = 4.6. In conclusion, qualitative differences in solution stability with respect to the nature of the added low molecular mass salt are observed for the solutions with *p*H values of 4.0 and 4.6.

The relative effectiveness of the anions on the stability of the protein solution shown in Figs. 4a and 5a follows a different sequence at and below the isoionic point. The phenomenon has been studied in several papers (e.g., [52–55,14,56]); the most comprehensive analysis of these and several other experimental results related to Hofmeister effects was presented by Schwierz et al. [56]. For anions all theoretical studies agree (though with different arguments) that the salts follow the indirect Hofmeister series below the *p*I and the direct series above it. In the case of cations the situation is not so clear. First, the effects are here much smaller. Second, to quote Ref. [56], cations are less “regular than anions”, so we can have a direct or reverse (even partially reverse) order, depending on the protein [56–58]. Note that the sequence of the salts in the Hofmeister series also depends on the concentration of the added salt [7,14,56,17] and the hydrophobicity [55,59,56] of the protein. In our case (Figs. 4b and 5b), both the anionic and cationic series reverse sequence when the *p*H is changed from 4.0 to *p*H = *p*I = 4.6.

Of the ionizable groups on the protein surface, the carboxylate group ($-COO^-$) can be classified as kosmotropic, whereas the protonated amine group ($-NH_3^+$) is chaotropic. The ion-specific affinities of salt ions to charged amino acid residues follow Collins’ law of matching water affinities [2,5,11,60]: an anion and a cation form a strong contact pair if they have similar (matching) water affinities (i.e., both ions are kosmotropes or both ions are chaotropes). Therefore, the affinity of cations to $-COO^-$ groups follows the series: Li^+ > Na^+ >Rb^+ > Cs^+ (from kosmotropic to chaotropic cation), while for anions interacting with $-NH_3^+$, the affinity order is: I^- > NO_3^- > Br^- > Cl^- > F^- (from chaotropic to kosmotropic anion) [56]. For example, when NaI is added to the protein solution, the I^- ions most effectively “neutralize” the contribution of the positively charged groups to the net charge of the protein. In the case of added NaF, the effect of F^- is weaker than in the case of added NaI. When LiCl is added to the protein solution, the Li^+ ions interact most effectively with the $-COO^-$ groups on the protein and “neutralize” the contribution of the negatively charged groups to the net charge of the protein. In the case of added CsCl, the effect of Cs^+ is weaker than in the case of added LiCl.

To explain our experimental results (Figs. 4 and 5) in terms of the binding affinities between ions and proteins, we need to consider the effects of anions and cations on the net charge

of the protein at a *p*H < *p*I and at a *p*H = *p*I. When the *p*H of the solution is below the isoionic point of the protein (*p*H < *p*I), there is an excess of $-NH_3^+$ groups on the protein surface compared to $-COO^-$ groups. The addition of I^- or NO_3^- ions results in a stronger "neutralization" of the charge of the $-NH_3^+$ groups than F^- ions. However, this reduces the net positive charge of the protein. The result is a diminished electrostatic repulsion between the protein molecules what makes the solution less stable. The perturbation will be stronger in the case of added NaI (or $NaNO_3$) than NaF, i.e. the cloud-point temperature will be lower for a protein–NaF mixture than for a protein–NaI (or protein-$NaNO_3$) mixtures. This is what we observe in our experiments (see Fig. 5a). At a *p*H < *p*I, the protein molecule becomes more positively charged due to the interaction of the cations with the $-COO^-$ groups. This makes the protein molecules more repealable and the effect of the added cation is stabilizing. Kosmotropic cations (e.g. Li^+) have a greater effect than chaotropic (e.g. Rb^+ or Cs^+). This means that the solution with added LiCl has a lower value of cloud-point temperature than the protein solution with added RbCl or CsCl. This is exactly the result shown in our Fig. 5b.

When *p*H of the solution is equal to the isoionic point of the protein (*p*H = *p*I), there is an equal number of $-COO^-$ and $-NH_3^+$ groups on the protein surface. By adding NaI to the solution, more $-NH_3^+$ groups are "neutralized" than in the case of adding NaF. This makes the surface of the protein more net negative in the case of the added NaI. Due to the greater electrostatic repulsion of the net negatively charged protein molecules, the solution with NaI added will be more stable than the protein–NaF mixture, i.e., the cloud-point temperature will be lower for the protein–NaI solution than for the protein–NaF mixture. This is the result we observed in experiments (see Fig. 4a). As a direct consequence of the water asymmetry, the anions have a stronger hydration than the cations: (i) since the oxygen atom of water is quite electronegative, it is easier for water to accept a negative charge from the anions than to accept a positive charge from the cations, and (ii) anions interacting with the hydrogen atoms of water can facilitate inter-shell hydrogen bonding between water molecules [2,60]. A number of experimental studies have confirmed that the influence of anions on protein solutions is much greater than the influence of cations [56,55] (even at *p*H $\gg$ *p*I [57]). When some of the salt anions bind to the protein surface of a net neutral protein molecule (*p*H = *p*I), the effect is the same as when the protein is in a solution with *p*H slightly above *p*I (*p*H > *p*I). When cations bind with $-COO^-$ groups, they shift the slightly negative net charge of the protein back to a more electroneutral molecule. Such a situation leads to a destabilizing effect of the cations, and the chaotropic cations have a greater effect than the kosmotropic ones. This means that the solution with added LiCl has a higher value of the cloud-point temperature than the protein solution with added CsCl. Again, the result is consistent with experimental observations shown on Fig. 4b.

In several of our previous works, we have shown that thermodynamic properties, such as enthalpy changes upon dilution or mixing in polyelectrolyte solutions [59,62,63,57], or alternatively, cloud-point temperature data for protein solutions [28] correlate well with hydration properties of low molecular mass salt ions. The correlation of the T_c with the Gibbs free energy of hydration, $\Delta_{hyd}G$, of the low molecular mass salt ions used in this work

is examined in Fig. 6. The $\Delta_{hyd}G$ values are taken from Ref. [61]. Panel a shows the T_c values as a function of $\Delta_{hyd}G$ of the anions for two different *p*H values of the BSA-PEG-salt mixtures. The concentration of PEG-10k is 30 mg/mL at *p*H = 4.6 (filled circles) and 33 mg/mL (filled squares) at *p*H = 4.0. As we can see, the cloud-point temperatures for solutions with *p*H = 4.6 (filled circles) decrease with increasing $\Delta_{hyd}G$ of anions. On the other hand (filled squares), the dependence at *p*H = 4.0 has an opposite trend: the T_c values increase strongly due to an increase (less negative values) of $\Delta_{hyd}G$. In agreement with the trends in Figs. 4a and 5a, we may state that introducing a more kosmotropic anion into the protein solution at *p*H < *p*I causes the solution to become less phase stable, while the opposite is true at *p*H = *p*I.

It is of interest to also examine the behaviour of cations under such conditions (Fig. 6b). A similar pattern to that for anions emerges: for *p*H = 4.6 (around the isoionic point, filled squares) T_c increases with an increase in the Gibbs free energy of cation hydration. The opposite is true for *p*H = 4.0, where BSA has a net positive charge. The dependence here is nearly linear (the dashed lines are, however, provided merely to guide the eyes). The effect of PEG seems to be somewhat stronger here. An introduction of a more kosmotropic cation into the protein solution at *p*H < *p*I causes the system to become less stable, whereas the opposite is true at *p*H = *p*I (see Figs. 4b and 5b).

To the best of our knowledge, no studies have systematically investigated the combined effect of PEG and salts on the phase stability of protein formulations as a function of *p*H, i.e., for *p*H < *p*I, *p*H ≈ *p*I, and *p*H > *p*I. The solubility of lysozyme in protein-PEG-salt mixtures at 25 °C was determined for *p*H ≪ *p*I in Ref. [24]. Here, the effect of salts could be explained by screening the charged amino residues of the protein, and the effect of PEG was explained by depletion forces. For crystalline-salt solutions, Finet et al. [53] performed SAXS measurements at *p*H values below and above the *p*I. They observed the Hofmeister series reversal when the conditions changed from *p*H < *p*I to *p*H > *p*I, which is also consistent with the observations of this study. Recently, we performed NMR relaxometry studies on lysozyme-salt and BSA-salt solutions [57,58]. These studies have shown that Hofmeister effects and the underlying specificity of ion-water-protein interactions are correlated with the geometrical features of the respective protein surface, namely the surface roughness of the protein. When polymers are present in the protein-salt solution, the protein-polymer surface interactions certainly play a role beyond depletion interactions and may have an effect on the Hofmeister series. Further studies on the combined effect of salts and polymers on protein stability below, at, and above the protein isoionic point are certainly welcomed.

4. Conclusions

The results of the research described in this article may be briefly summarized as follows: (i) Addition of PEG to the solution of BSA with or without low molecular mass salt promotes protein aggregation. The effect is stronger with higher molecular masses of PEG. These results are expected and have been observed previously with aqueous solutions of other proteins. (ii) Addition of NaCl to the BSA-PEG solution can lead to qualitatively different effects depending on *p*H of the solution studied. At *p*H = 4.6, an addition of salt increases

the stability of the system towards phase separation, while the same process at *p*H = 4.0 decreases the formulation's stability. (iii) *p*H of the solution determines the order of salts in their role of stabilizing (or de-stabilizing) the solution. For *p*H = 4.6, i.e., near the isoionic point of BSA, the stability of the solution increases against phase separation from NaF (least stable) to NaI (most stable) and from LiCl to CsCl. An opposite ordering is observed in the case of the solution at *p*H = 4.0, where BSA has a net positive charge. (iv) The correlation between the cloud-point temperature and the Gibbs free energy of hydration of the salt ions confirms the crucial role of water in determining the salt-specific effects.

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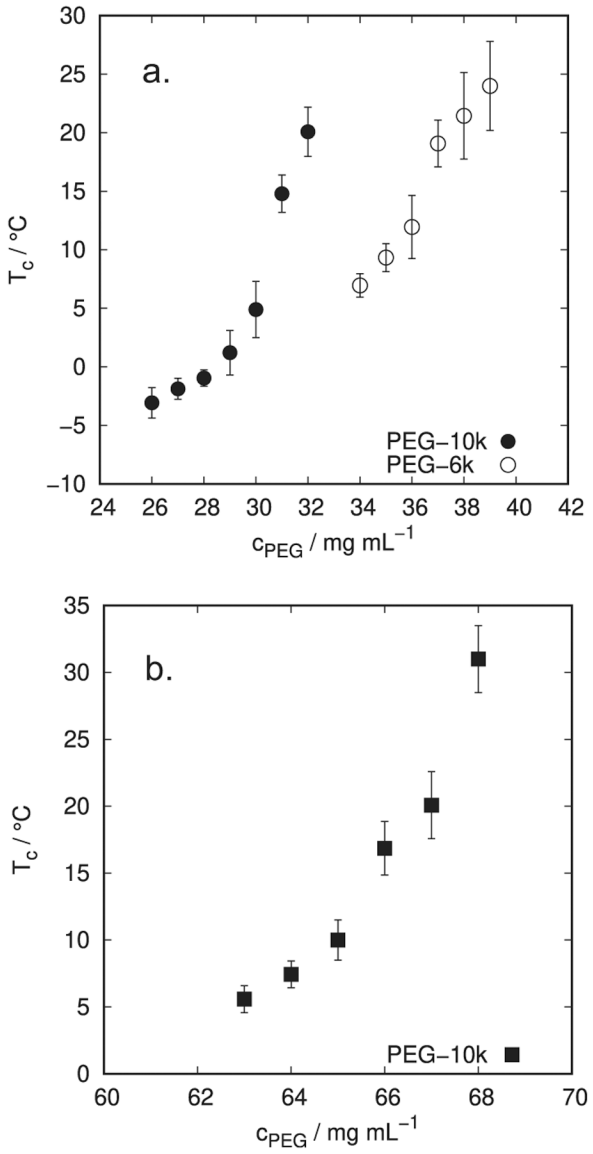

Fig. 1.

Cloud-point temperature, T_c, of BSA-PEG mixtures as a function of PEG concentration, c_{PEG}, at (*a*) *p*H = 4.6 and (*b*) 4.0. Filled symbols are for PEG-10k (10, 000 g/mol), and empty symbols are for PEG-6k (6, 000 g/mol). The concentrations of acetate buffer and BSA were set to 0.1 mol/L and 90 mg/mL, respectively.

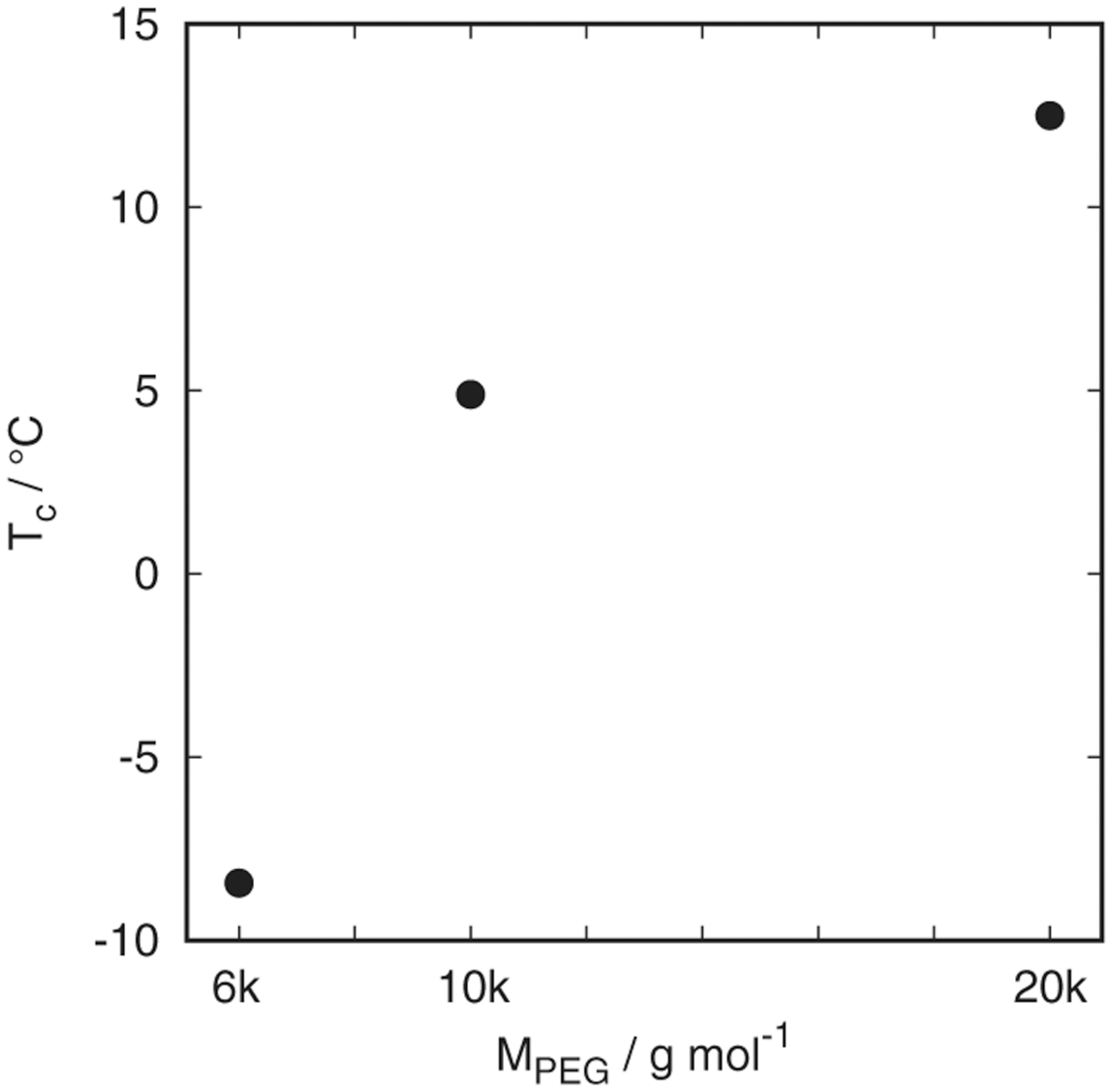

Fig. 2.

Cloud-point temperature, T_c, of BSA-PEG mixtures as a function of polymer molar mass, M_{PEG}. Results are for solutions of BSA (90 mg/mL) and PEG (30 mg/mL) in acetate buffer (0.1 mol/L) with *p*H = 4.6. The designations 6k, 10k, and 20k denote the molecular mass of PEG, i.e., 6,000, 10,000, and 20,000 g/mol, respectively. The solution with PEG-6k is stable at temperatures above −8.5 °C, while the solution with PEG-20k is stable above 12.5 °C.

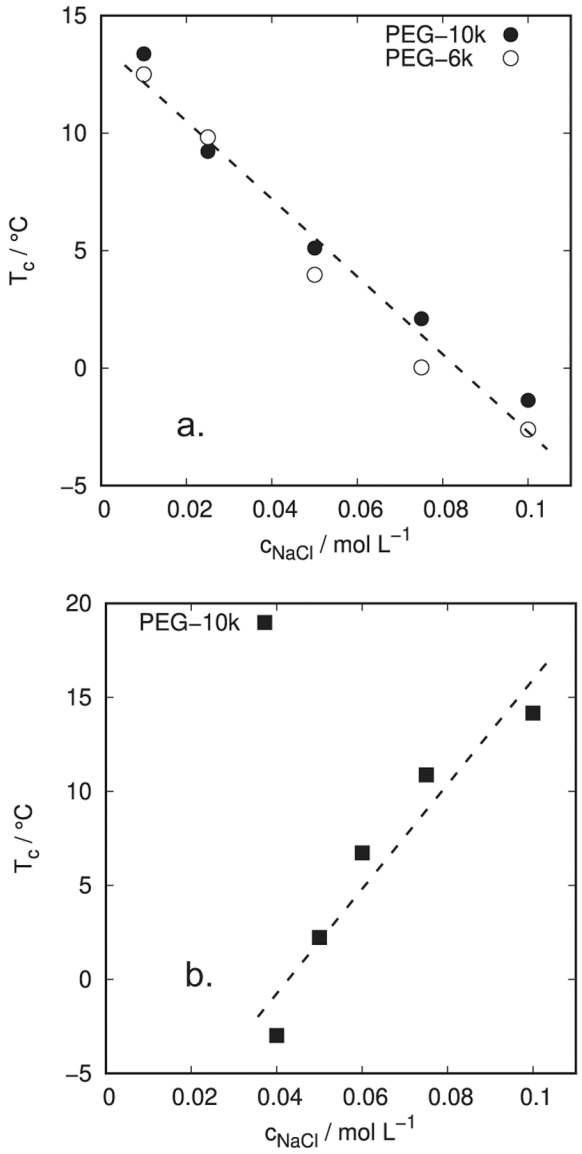

Fig. 3.

Cloud-point temperature, T_c, of BSA–PEG–NaCl mixtures as a function of the salt concentration, c_{NaCl}, at (*a*) *p*H = 4.6 and (*b*) 4.0. Filled symbols are for PEG-10k (10000 g/mol), and empty symbols are for PEG-6k (6000 g/mol). The concentrations of acetate buffer and BSA were set at 0.1 mol/L and 90 mg/mL, respectively. The concentration of PEG-10k was 30 mg/mL at *p*H = 4.6 (filled circles) and 33 mg/mL at *p*H = 4.0 (filled squares), while the concentration of PEG-6k was 36 mg/mL at *p*H = 4.6 (empty circles). The dashed lines are guides for the eyes.

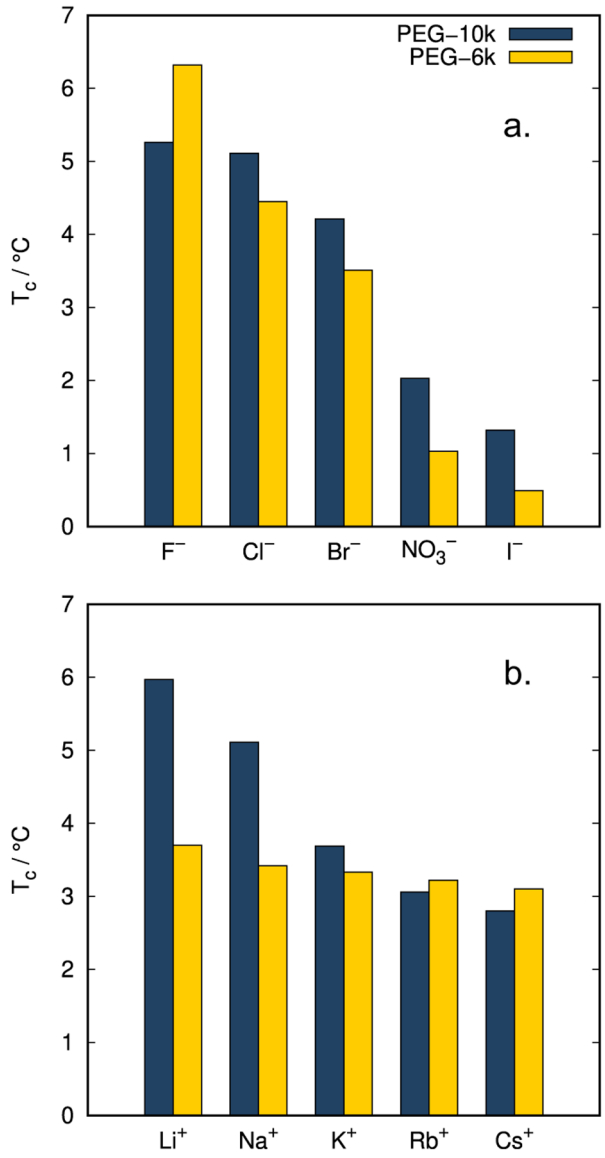

Fig. 4.

Cloud-point temperature, T_c, of BSA–PEG–salt mixtures as a function of the anion of sodium salts (*a*) or cation of chloride salts (*b*) at *p*H = 4.6. Concentrations of the acetate buffer and BSA were fixed at 0.1 mol/L and 90 mg/mL, respectively, and the concentration of the salt was 0.05 mol/L. The concentration of PEG-10k (blue bars) was 30 mg/mL and of PEG-6k (yellow bars) it was 36 mg/mL.

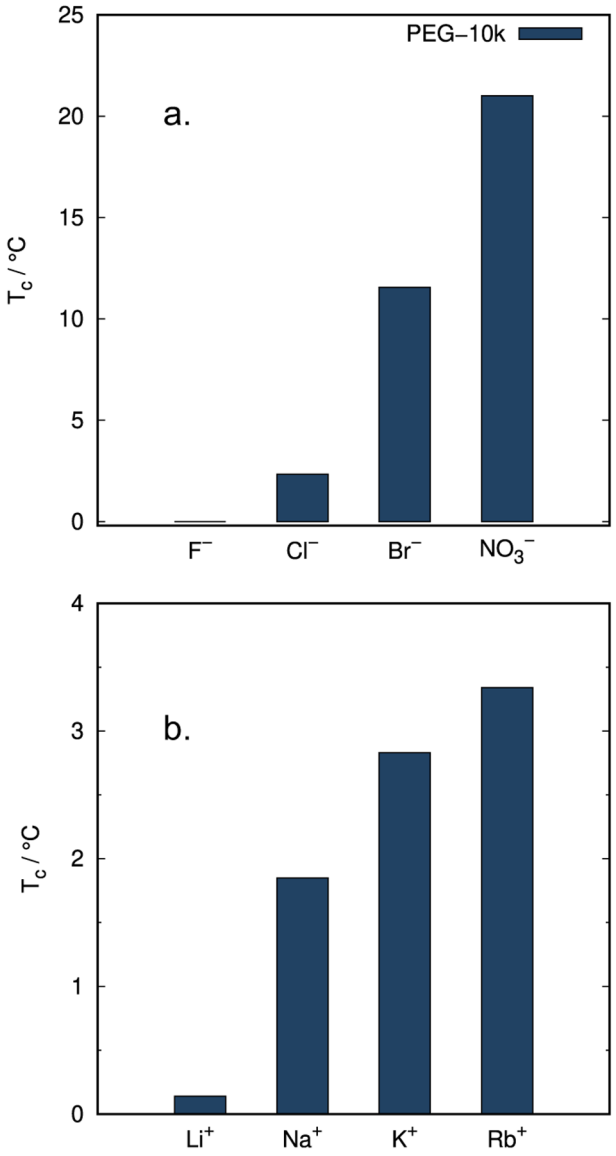

Fig. 5.

Cloud-point temperature, T_c, of BSA–PEG–salt mixtures as a function of the anion of sodium salts (*a*) or cation of chloride salts (*b*) at *p*H = 4.0. Concentrations of the acetate buffer and BSA were fixed at 0.1 mol/L and 90 mg/mL, respectively, and the concentration of the salt was 0.05 mol/L. The concentration of PEG-10k was 33 mg/mL.

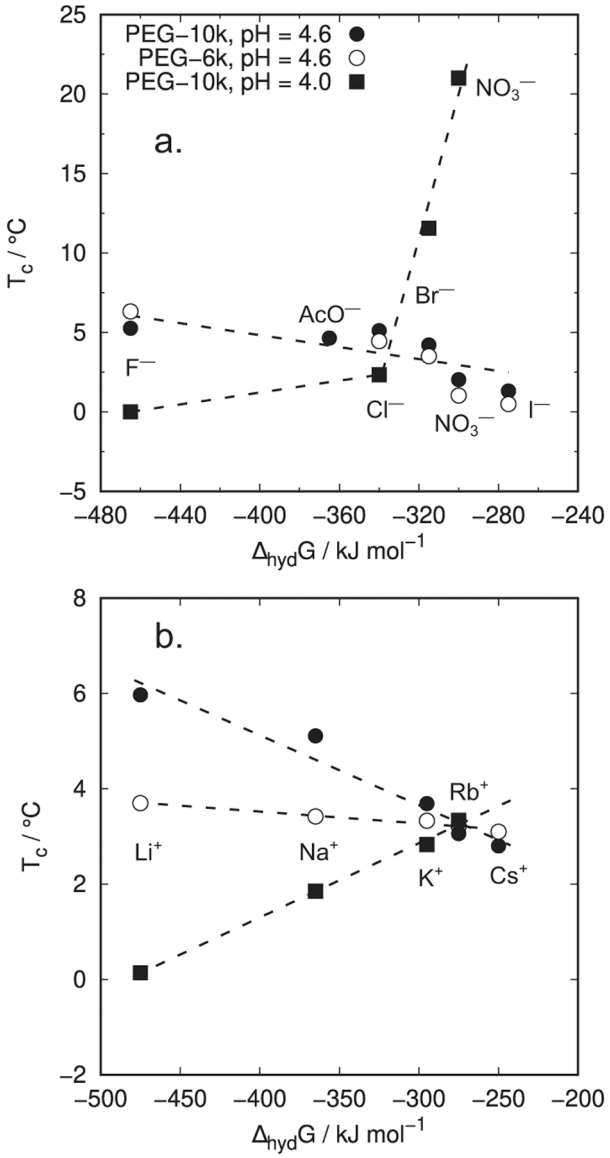

Fig. 6.

Cloud-point temperature, T_c, of BSA-PEG-salt mixtures as a function of the hydration Gibbs free energy, $\Delta_{hyd}G$ [61], of the (*a*) anion or (*b*) cation of the added sodium or chloride salt, respectively. The concentrations of the acetate buffer, BSA, and salt were fixed at 0.1 mol/L, 90 mg/mL, and 0.05 mol/L, respectively. The concentration of PEG-10k was 30 mg/mL at *p*H = 4.6 (filled circles) and 33 mg/mL (filled squares) at *p*H = 4.0, while the concentration of PEG-6k (empty circles) was 36 mg/mL. The dashed lines are linear fits and serve as a guide to the eye. Note the difference in scale between panels (*a*) and (*b*).