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Abnormal Suppression of LLPS of Protein in Mild Salt Concentrations

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Abstract: Liquid-liquid phase separation (LLPS) is a significant biological phenomenon that plays a crucial role in biomaterials science and protein crystallization. In this study, we explored the phase behavior of proteins in various metal cationic solutions containing polyethylene glycol. As expected, we observed that low salt concentrations promoted LLPS, consistent with previous studies. However, LLPS was suppressed as salt concentrations increased further. Specifically, the critical protein concentration required for LLPS rose with higher salt concentrations. We found that the promotion and suppression of LLPS are influenced by the valence of the ions. Notably, divalent cationic salts exhibited a more pronounced promotional effect on protein phase separation at low salt concentrations than monovalent cationic salts. In contrast, at higher salt concentrations, monovalent cationic salts had a stronger inhibitory effect on LLPS compared to divalent cationic salts. This can be attributed to the ionic shielding effect at low salt concentrations and the formation of a hydration layer at higher salt concentrations, which increases short-range repulsive interactions between protein molecules. Interestingly, the suppressive effect was absent in trivalent cationic solutions.

Keywords: liquid-liquid phase separation; ovalbumin; ion valence state; salt concentration

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1. Introduction

Liquid-liquid phase separation (LLPS) is recognized as a key mechanism for various structural and functional processes in cell biology. LLPS underlies the formation of membraneless organelles and facilitates the enrichment of specific biomacromolecules, such as proteins and nucleic acids. Notable examples include the formation of organelles like nucleoli and P granules in the nucleus and cytoplasm, enabling organisms to perform specific biochemical reactions and metabolic activities[1-3]. Additionally, LLPS plays an increasingly important role in various biological functions and is associated with numerous diseases[4].

In typical cells, biomolecular condensates play a crucial role in the crowded cytoplasmic environment. Polyethylene glycol (PEG), a hydrophilic nonionic compound, is commonly used in vitro to replicate this crowded environment. Due to its unique chemical and physical properties, PEG is widely soluble in water and most organic solvents. It affects protein behavior through excluded-volume effects and direct interactions with proteins, such as hydrophobic interactions or hydrogen bonding[5, 6]. Factors influencing protein phase separation phenomena include solution concentration, pH, temperature, and the presence of excipients[7]. Additionally, the effects of different types of salts on protein behavior are significant, as salts can influence protein charge states, solubility by changing ionic strength, and phase separation processes[8-10]. Earlier studies have demonstrated the specific role of salts in protein behavior, particularly those related to the Hofmeister salt series[11-13]. However, there is a lack of extensive understanding of the

synergistic regulation of protein co-solutes such as PEG and salts, to provide new perspectives on protein phase transition mechanisms.

Egg white albumin (ovalbumin, OVA) constitutes 65% of the total protein in egg whites and is the main component of egg protein. Due to its good solubility, stability, and biocompatibility, ovalbumin is widely used in food, medicine, and biomaterials, making it an important model protein for studying phase behavior in biochemical studies[14-16].

In this study, we investigated the effect of PEG and various valence state cationic salts on the phase behavior of OVA by observing droplet morphology using microscopy and measuring solution turbidity. By exploring the effects of different valence state cationic salts on protein LLPS at low and mild salt concentrations, we assessed the influence of salt on phase separation in the presence of PEG. We found an unexpected suppression of LLPS of protein in mild salt concentrations, which is not typically observed in the low salt concentration range.

2. Results

2.1. The phase behavior of ovalbumin and PEG-8000

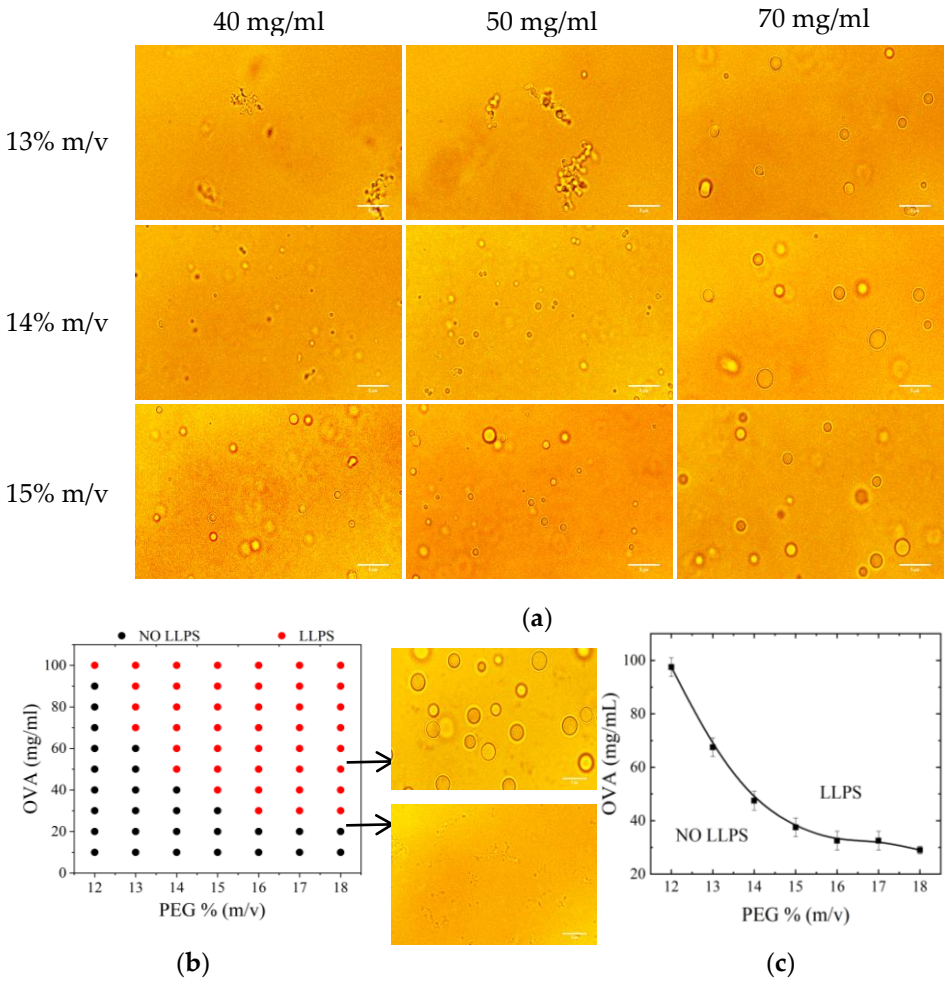

Figure 1. (**a**) Representative microscopic images of OVA under different experimental conditions; (**b**) Diagram of phase separation states based on droplet formation and representative micrographs (red data point: LLPS, Typical micrograph of LLPS occurring (18% m/v PEG-8000, 50 mg/ml OVA); black data point: NO LLPS, Typical micrograph of LLPS not occurring (18% m/v PEG-8000, 20 mg/ml OVA); (**c**) Critical concentration plot for the occurrence of LLPS based on droplet formation. pH = 7, room temperature.

The phase separation behavior of OVA (10-100 mg/mL) with different concentration gradients from PEG-8000 (12%-18% m/v) was systematically investigated at room temperature (pH = 7 Tris buffer system). Representative microscopic images are shown in Figure 1(**a**). Under fixed OVA concentration conditions, as the PEG-8000 concentration increased from 13% m/v to 15% m/v, the system underwent a transition from free protein aggregates to a typical droplet-like condensate and this morphological change clearly characterizes the occurrence of liquid-liquid phase separation. It was also shown that the critical concentration of PEG-8000 required for induced phase separation was negatively correlated with the protein concentration, and increasing the concentration of PEG-8000 could significantly reduce the critical concentration of protein required for induced phase separation. This phenomenon can be attributed to the exclusion volume effect and is consistent with the findings of existing studies that PEG promotes phase separation of biomolecules through an entropy-driven mechanism[17, 18].

In order to visually characterize the phase separation behavior, the phase separation states of the system were mapped in this study based on microscopic observations (Figure 1(**b**)). It shows two typical states: the LLPS state with droplet-like condensate in the microscopic image and the NO LLPS state with free protein aggregates in the microscopic image. Based on the state diagram analysis, the critical concentration boundary for the OVA-PEG-8000 system to undergo phase separation was further identified in this study (Figure 1(**c**)), which provides important basic data for the subsequent study of the effect of salt concentration on the behavior of the protein phase.

2.2. Driving Forces of Liquid-Liquid Phase Separation in OVA/PEG-8000

Multivalent interactions (including hydrophobic interactions and electrostatic interactions) are the key drivers in regulating liquid LLPS, and these interactions determine the phase separation behavior by affecting the interactions between macromolecules. In-depth elucidation of the molecular mechanisms of liquid-liquid phase separation is important for the precise regulation of the phase separation process as well as for providing support for research in related fields.

2.2.1. Effect of hydrophobic interactions on liquid-liquid phase separation of OVA/PEG-8000

In our case, 1,6-hexanediol (1,6-HD) was used as a hydrophobic interfering agent to investigate the phase separation mechanism of the OVA-PEG-8000 system. 1,6-HD, as a classical hydrophobic interfering agent, acts mainly by disrupting the weak interactions between the protein molecules, in particular by weakening the hydrophobic interactions, and thus inhibits the liquid-liquid phase separation[19]. Under the experimental conditions of fixed OVA concentration (60 mg/mL) and PEG-8000 concentration (15% m/v), we systematically investigated the effects of different concentrations of 1,6-HD (5%, 10%, and 20%) on the phase separation behavior, as shown in Figure 2(**a**). Meanwhile, the solution absorbance values were measured as shown in Figure 2(**b**).

The experimental results showed that the overall phase separation behavior of the system did not change significantly with increasing 1,6-HD concentration. It is worth noting that when the concentration of 1,6-HD reached 20%, microscopic observation showed a slight change in droplet morphology, which was characterized by incomplete droplet edges. However, this change in morphology did not significantly affect the overall phase separation behavior of the system. This observation was further confirmed by absorbance value measurements, which showed that even at the highest concentration of 1,6-HD (20%), the absorbance value of the system decreased only slightly, but remained relatively stable overall, with no apparent discontinuities. This result is consistent with the microscopic observation of incomplete droplet edges, suggesting that the addition of 1,6-HD did not result in a significant change in the phase separation behavior of the system.

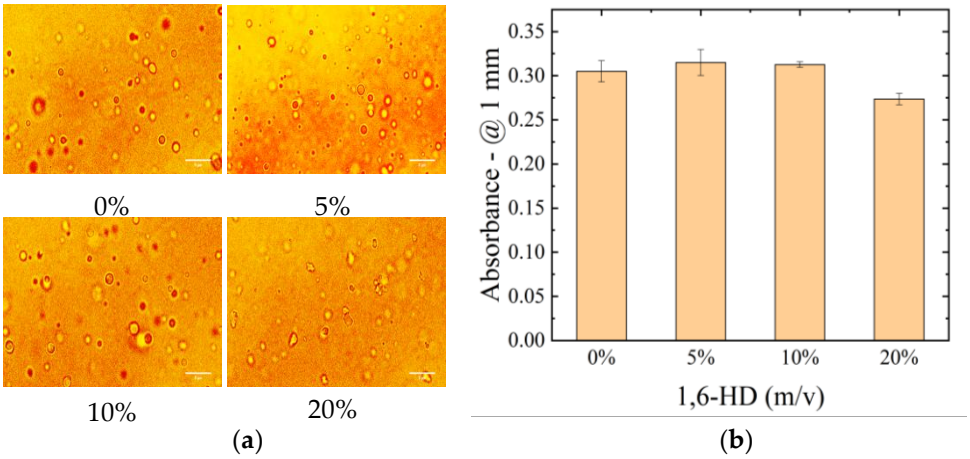

Figure 2. (**a**) Microscopic images under different concentrations of 1,6-HD treatment; (**b**) Absorbance values under the corresponding conditions. System: 15% m/v PEG-8000, 60 mg/ml OVA, 1,6-HD concentrations of 0% m/v, 5% m/v, 10% m/v , 20% m/v. pH = 7 at room temperature.

The above experimental results suggest that the contribution of hydrophobic interactions is relatively limited in the liquid-liquid phase separation of the OVA-PEG-8000 system. Other dominant interaction mechanisms may exist in this system.

2.2.2. Effect of electrostatic interactions on liquid-liquid phase separation of OVA/PEG-8000

Based on the above experiments confirming the limited contribution of hydrophobic interactions to the phase separation of the OVA-PEG-8000 system, we further explored the potential role of electrostatic interactions in this process. As a typical modulator of electrostatic interactions, 6-A significantly inhibits the LLPS process by attenuating the electrostatic interactions between protein molecules[19]. Based on this, this study analyzed the effect of 6-A on the phase separation behavior of OVA-PEG-8000 system by systematically varying its concentration (0-10% m/v).

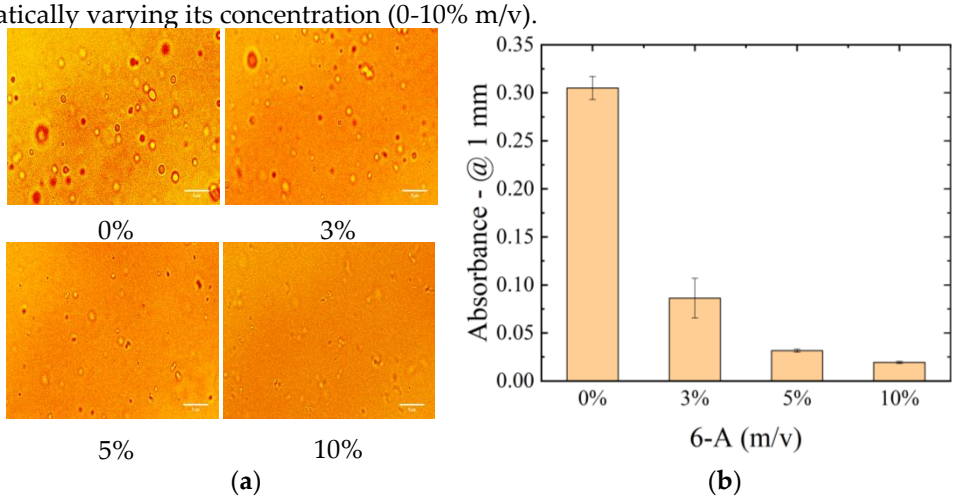

Figure 3. (**a**) Microscopic images under different concentrations of 6-A treatment; (**b**) Absorbance values under the corresponding conditions. System: 15% m/v PEG-8000, 60 mg/ml OVA, 6-A concentrations of 0% m/v, 3% m/v, 5% m/v, and 10% m/v, respectively, pH = 7 at room temperature.

The experimental results showed (Figure 3(**a**)) that the degree of phase separation of the OVA-PEG-8000 system gradually weakened with the increase of 6-A concentration. When the concentration of 6-A reached 10%, the droplet-like condensate almost completely disappeared, indicating that 6-A could effectively inhibit the phase separation pro-

cess of the system. To quantitatively characterize this phenomenon, we measured the absorbance value of the solution at 600 nm by ultra-micro UV-Vis spectrophotometer (Figure 3(**b**)). The results showed that the absorbance values decreased significantly with increasing 6-A concentration, which further confirmed the inhibition of phase separation by 6-A.

This result suggests that electrostatic interactions play a key regulatory role in the phase separation process of the OVA-PEG-8000 system.

2.3. The effect of cations with different valence states on the liquid-liquid phase separation of OVA/PEG-8000

Salt ions are able to modulate the ionic strength of the solution and thus affect the electrostatic interactions of proteins, and this effect depends on the type and concentration of the salt[20, 21]. On the basis of clarifying the dominance of electrostatic interactions, we further explored the role of cations with different valence states (Na^+, K^+, Mg^{2+}, Ca^{2+}, Y^{3+}) on the OVA/PEG-8000 system phase behavior modulation.

The modulation of NaCl concentration on the liquid-liquid phase separation behavior of OVA/PEG-8000 system can be described as follows. Firstly, the phase separation behavior of the system was observed in real time using an optical inverted microscope by adding different concentration gradients of NaCl (5-100 mM) at a fixed concentration of PEG-8000 (15% m/v) (Figure 4(**a**)). The results showed that NaCl concentration significantly modulated the phase separation behavior of the OVA/PEG-8000 system. In the low salt concentration range of 5-10 mM, the critical protein concentration required for phase separation decreased with increasing salt concentration, indicating that the low salt condition promotes phase separation. However, when the NaCl concentration was in the range of 50-100 mM, the system required a higher protein concentration for phase separation to occur, showing an inhibitory effect of medium salt conditions. Overall, the regulation of LLPS by salt concentration showed a non-monotonic trend of promotion followed by inhibition. Overall, the regulation of LLPS by salt concentration showed a non-monotonic trend of promoting and then inhibiting effects.

To further validate the microscopic observations, we assessed the turbidity by measuring the absorbance values of the solutions under different conditions using a UV-Vis spectrophotometer (Figure 4(**b**)). It was found that at low salt concentrations of 5 mM and 10 mM, the absorbance values of the salt-containing samples were higher than those of the unsalted control, which is consistent with the previously mentioned idea that low salt promotes LLPS. On the contrary, at mild salt concentrations of 50 mM and 100 mM, the absorbance values of the corresponding samples showed a decreasing trend, which supports the conclusion that mild salt environments are unfavorable to the occurrence of LLPS of ovalbumin in the crowded state.

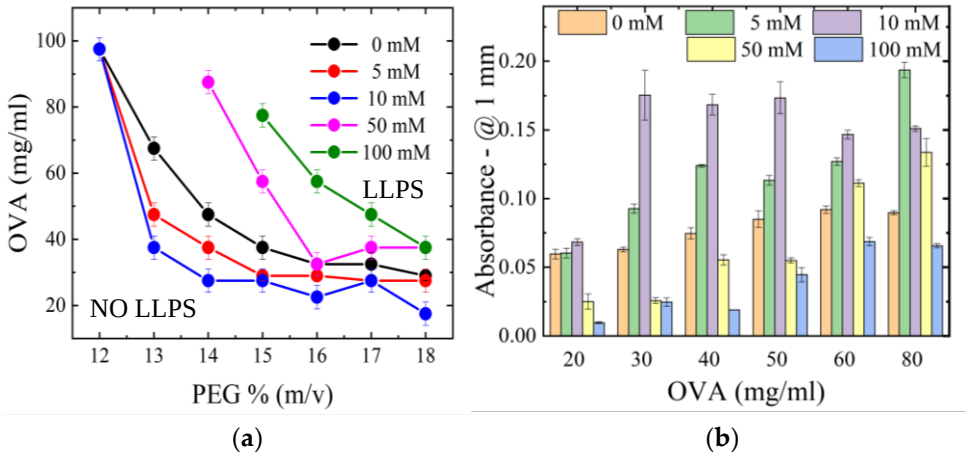

Figure 4. (**a**) Changes of phase separation boundaries of OVA/PEG-8000 system at different NaCl concentrations. Referring to Figure 1(**c**), black solid lines indicate the phase boundaries when no

NaCl was added, and colored solid lines indicate the corresponding phase boundaries at different NaCl concentrations: red (5 mM), blue (10 mM), pink (50 mM), and green (100 mM); (**b**) Changes in absorbance values at corresponding concentrations. Salt concentration: orange (0 mM), green (5 mM),purple (10 mM),yellow (50 mM),blue (100 mM). PEG-8000 was 15% in the system.

Based on the regulation law of NaCl on the phase separation behavior of the OVA/PEG-8000 system, we further explored the effect of the divalent salt ($MgCl_2$) on this system, as shown in Figure 5(**a**).The regulation of the phase separation behavior of the OVA/PEG-8000 system by $MgCl_2$ showed a nonmonotonic character similar to that of NaCl.

The experimental results showed that the critical protein concentration required for LLPS to occur decreased and the overall boundary shifted downward when the salt concentration was in the range of 5-10 mM. $MgCl_2$ decreased to a greater extent compared with NaCl, indicating that the divalent facilitation was stronger than the monovalent one, which was attributed to the fact that the divalent salt had a higher charge density, and it could shield the electrostatic repulsion between protein molecules more effectively and promote the aggregation of protein molecules. The critical line for the occurrence of liquid-liquid phase separation was significantly shifted upward at a salt concentration of 100 mM, suggesting that LLPS was inhibited to a certain extent at higher salt concentrations. It is noteworthy that the boundary of $MgCl_2$ was shifted upward to a lesser extent at a salt concentration of 100 mM compared to the monovalent salt NaCl, suggesting that the inhibitory effect of divalent salts was weaker than that of monovalent salts. This may be related to the change in the structure of protein solvation layer and the efficiency of electrostatic shielding.

Meanwhile, the absorbance values in Figure 5(**b**) further quantitatively verified the trend of the effect of salt concentration. When the salt concentration was lower than 10 mM, the absorbance values when phase separation occurred were higher than those without salt addition. At a salt concentration of 5 mM, the absorbance values of divalent salts were higher than those corresponding to monovalent salts, which is consistent with the above results at low salt concentrations. With the increase of salt concentration, the absorbance value gradually decreased, and when the salt concentration was 100 mM, the absorbance was significantly lower than that of the unadded salt, indicating that there was indeed an inhibitory effect at higher salt concentrations.

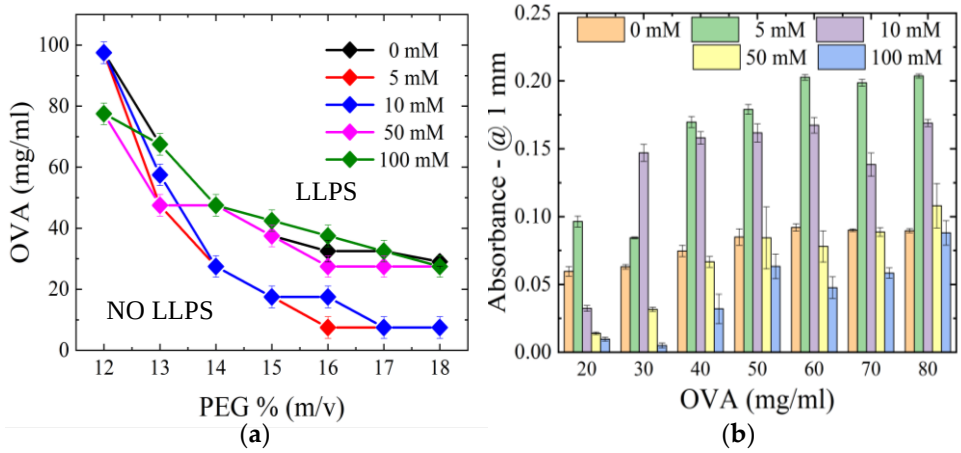

Figure 5. (**a**) Variation of phase separation boundaries of OVA/PEG-8000 system at different $MgCl_2$ concentrations. Referring to Figure 1(**c**), the black solid line indicates the phase boundary when no $MgCl_2$ was added, and the colored solid lines indicate the corresponding phase boundaries at different $MgCl_2$ concentrations: red (5 mM), blue (10 mM), pink (50 mM), and green (100 mM); (**b**) Change in absorbance values at corresponding concentrations. Salt concentration: orange (0 mM), green (5 mM), purple (10 mM), yellow (50 mM) and blue (100 mM). System: 15% m/v PEG-8000. pH = 7, room temperature.

Based on the non-monotonic regulation laws of monovalent (NaCl) and divalent ($MgCl_2$) salts on the phase separation behavior of the OVA/PEG-8000 system, the present study further systematically investigated the role of ionic species in regulating the phase separation process. Based on the controlled experimental conditions of 15% m/v PEG-8000 and 60 mg/mL OVA, four salts with different concentration gradients (5 mM, 10 mM, 50 mM, 100 mM, and 500 mM) were added, namely, NaCl and KCl, $MgCl_2$, and $CaCl_2$. The absorbance values of the solutions under each experimental condition were quantitatively determined by an ultra-micro UV-Vis spectrophotometer , and the results are shown in Figure 6.

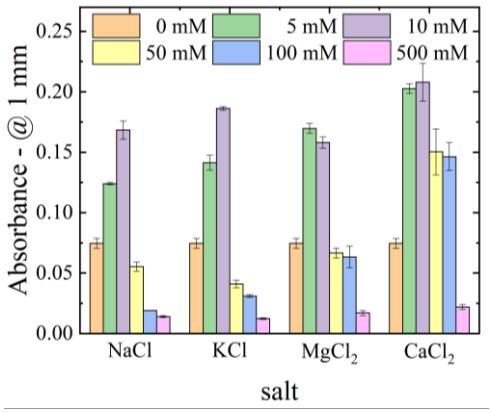

Figure 6. Change in absorbance values of the effect of adding different concentrations of NaCl, KCl, $MgCl_2$ and $CaCl_2$ on OVA/PEG-8000. System: 40 mg/ml OVA,15% PEG-8000, pH = 7, room temperature. Salt concentration: orange (0 mM), green (5 mM), purple (10 mM), yellow (50 mM), blue (100 mM), pink (500 mM).

The results showed that at low salt concentrations (5 mM, 10 mM), both monovalent and divalent salts, the absorbance values of the solutions in which liquid-liquid phase separation of OVA/PEG-8000 occurred were higher than those of the solutions when no salt was added. This phenomenon indicates that the low salt concentration promotes the liquid-liquid phase separation, and with the increase of salt concentration, when the monovalent salt concentration increased to 50 mM, 100 mM, the absorbance values decreased significantly, and the decrease of the divalent salt with the increase of salt concentration was weaker than that of the monovalent salt, especially $CaCl_2$. However, the absorbance values were significantly decreased when the salt concentration continued to increase to 500 mM. It indicated that higher salt concentration inhibited the liquid-liquid phase separation. Meanwhile, optical inverted microscope was used to observe the microscopic state of some solutions, and the results are shown in Figure 7. The droplet size showed a non-monotonic change with increasing salt concentration. Monovalent and divalent salts generally increased at low salt concentrations (5-10 mM), and the droplet size was larger for divalent than monovalent. This is consistent with the fact that divalent has a higher charge state significantly promoting liquid-liquid phase separation of proteins. In contrast, the droplet size decreased at higher salt concentrations, with monovalent salts showing droplet contraction at 50 mM, while divalent salts contracted significantly only at higher salt concentrations. This suggests that monovalent salts exhibit more significant phase separation inhibition effects at higher salt concentrations, reflecting the specificity of different valence ions.

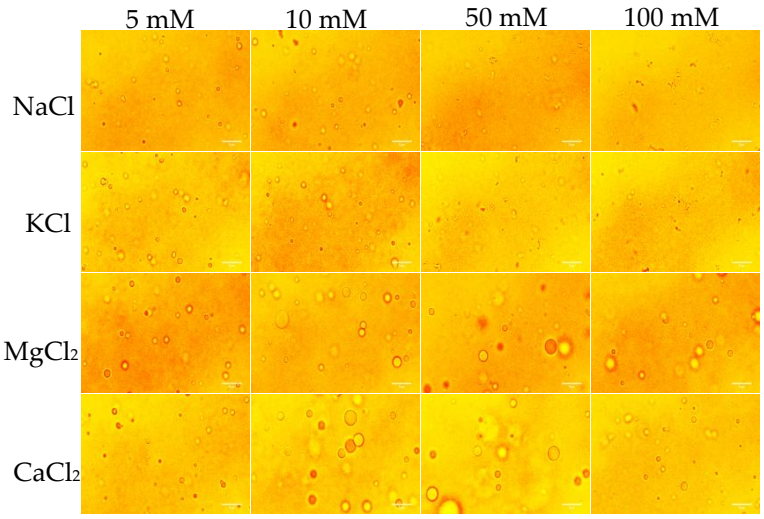

Figure 7. Microscopic images of the effect of NaCl, KCl, $MgCl_2$ and $CaCl_2$ on OVA/PEG-8000. System: 40 mg/ml OVA,15% PEG-8000, pH = 7, room temperature. Salt concentration: 5 mM, 10 mM, 50 mM, 100 mM.

The effect of trivalent cations (Y^{3+}) on the phase separation process of this system was further investigated in this study with the aim of elucidating the effect of different ionic valence states on the phase behavior of phase separation.

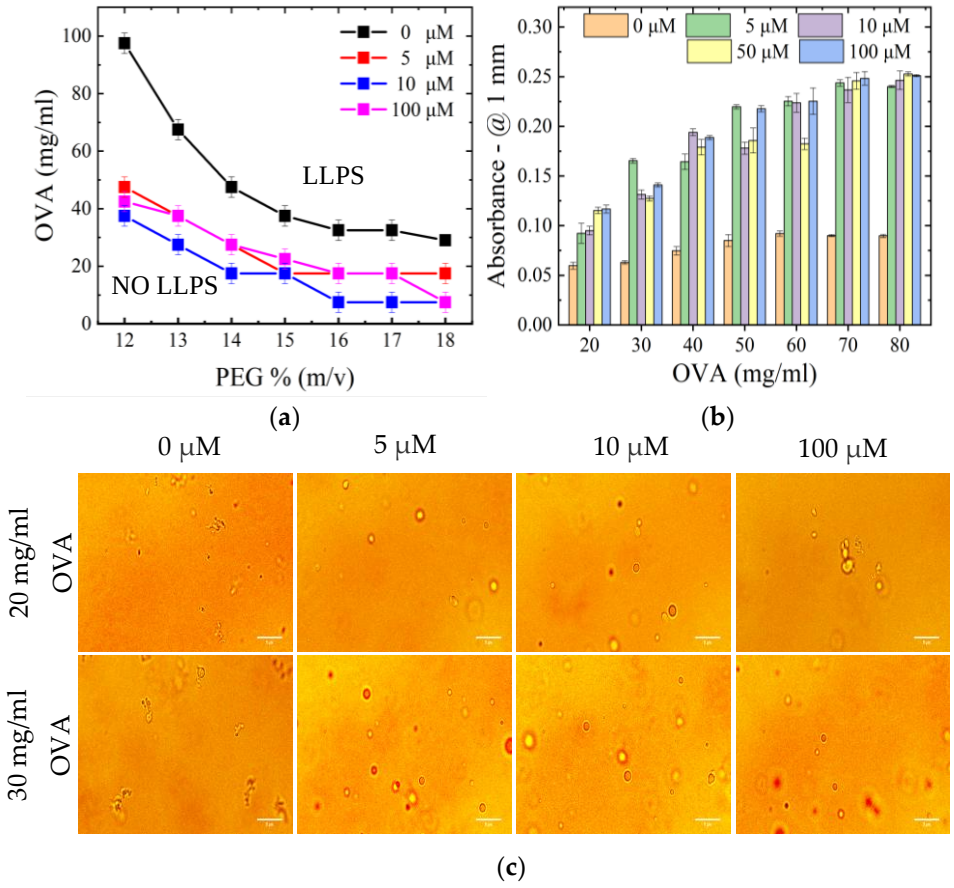

Figure 8. (**a**) Variation of phase separation boundaries of the OVA/PEG-8000 system at different YCl3 concentrations. Referring to Figure 1(**c**), the black solid line indicates the phase boundary without YCl_3 addition, and the colored solid lines indicate the corresponding phase boundaries at different YCl_3 concentrations: red (5 μM), blue (10 μM), pink (50 μM) and green (100 μM); (**b**) Change in absorbance values at corresponding concentrations. Salt concentrations: orange (0 μM), green (5

μM), purple (10 μM), yellow (50 μM) and blue (100 μM); (**c**) Microscopic images of the effect of YCl_3 on OVA/PEG-8000. System: 20/30 mg/ml OVA, 15% PEG-8000, salt concentrations: 5 μM, 10 μM, 50 μM, 100 μM, pH = 7, room temperature.

The results in Figure 8(**a**) show that yttrium chloride is able to promote liquid-liquid phase separation of proteins at the order of micromolar. The phase boundaries of the OVA/PEG-8000 system with the addition of 5-100 μM salt concentration were significantly lower than the phase separation boundaries of the system without salt. Meanwhile, the absorbance values measured by ultraviolet spectrophotometer (Figure 8(**b**)) showed that the absorbance values of the solutions with salt addition were all higher than those without salt addition, which to some extent reflected that the degree of liquid-liquid phase separation occurred became stronger. When the concentration of trivalent cations increased to the millimolar scale, the visibility of the solution was very low, which is presumed to be due to the overly strong interactions between the trivalent cations and the proteins. All these phenomena indicate that the trivalent salt has no inhibitory effect on the phase separation of OVA/PEG in the range of salt concentration of this study. The microscopic images of some of the solutions are shown in Figure 8(**c**), With the increase of salt concentration (0-100 μM), the proteins formed droplet-like condensates or formed aggregates from free small aggregates. This suggests that the presence of trivalent cations contributed to stronger interactions between proteins and promoted coalescence.

2.4. Influence of other factors on ion-modulated phase separation

To assess the effect of PEG molecular weight, we tested PEG-4000 and PEG-8000 under identical conditions (OVA = 60 mg/mL, 18% m/v PEG, NaCl/$MgCl_2$ concentration gradient).

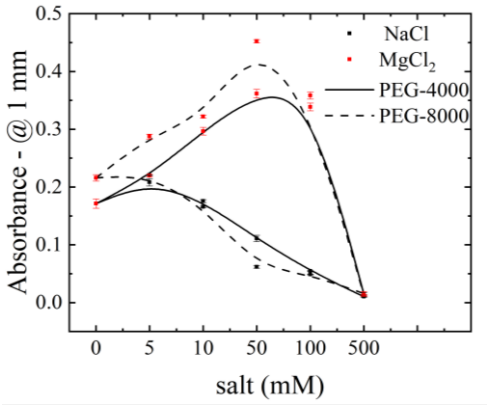

Figure 9. Comparative absorbances of OVA with PEG-4000/PEG-8000 at different salt (NaCl, $MgCl_2$) concentration measured at 60 mg/ml OVA, 18% m/v PEG and pH = 7 in room temperature.

Figure 9 shows that both molecular weights exhibit a non-monotonic trend of promotion by low salt and inhibition by high salt, suggesting that molecular weight size does not influence the observed effects.

Buffer components may affect protein phase separation behavior through ion-specific binding. To rule out Tris-specific artifacts, we explored the trend of absorbance values with increasing salt concentration under identical conditions (15% PEG-8000, 40 mg/mL OVA) in phosphate buffer (PB, pH = 7) .

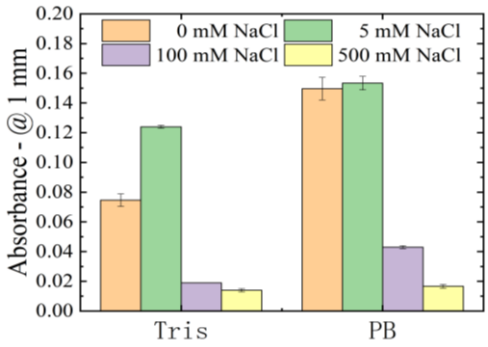

Figure 10. Comparison of absorbances of OVA/PEG in different buffer systems containing NaCl (Tris, PB). System: 40 mg/ml OVA, 15% m/v PEG-8000. NaCl concentration: 0 mM, 5 mM, 100 mM, 500 mM. pH = 7, room temperature.

As shown in Figure 10, the initial absorbance in salt-free PB was higher than that in Tris buffer, which can be considered to be related to the solution environment of PB itself. Overall, the regulation trend of PB under low-salt slightly promoting and mild-salt inhibiting conditions was highly similar to that in Tris buffer. This phenomenon confirms that this pattern is not significantly interfered by the chemical specificity of the buffer and confirms the universality of ionic valence regulation.

3. Discussion

In this study, we determined the occurrence of liquid-liquid phase separation by qualitatively observing the solution microstates through optical inverted microscopy and quantitatively measuring the solution turbidity in response to the solution absorbance values by UV-Vis spectrophotometry, and analyzed the effect of different valence cation eyes on the phase behavior of the OVA/PEG-8000 system. Previously it was determined that the system undergoes phase separation and that electrostatic interactions have a more significant effect than hydrophobic interactions.

Possible regulatory mechanisms for the effect of different valence salts on the phase separation of the system were considered in terms of electrostatic interactions. we have attempted to propose a mechanism to explain the experimental phenomena in this study. In the present system, the volume exclusion effect of PEG all leads to an increase in the effective concentration of solutes in solution, which further affects the aggregation behavior of biomolecules, similar to the regulation of the basal phase behavior of bovine γ-globulin by PEG, which alters the effective concentration of proteins through the volume exclusion effect, thus affecting the phase separation threshold[22]. On this basis, the ionic valence states at low salt concentrations play a decisive role in the contribution to the electrostatic shielding through the modulation of the Debye length (a characteristic length describing the spatial extent of the classical shielding effect in electrolyte solutions)[23]. Divalent cations have a high charge density, which can significantly shorten the Debye length, enhance the shielding of the protein surface charge, and weaken the intermolecular repulsion, so that phase separation can be triggered at lower protein concentrations. While low valence ions have low charge density, the electrostatic shielding effect (ions neutralize or shield the protein surface charge through electrostatic interaction, altering intermolecular repulsion) generated at the same low salt concentration is weaker, and a higher protein concentration is required to overcome the electrostatic repulsion between proteins to experience phase separation. Therefore, divalent ions have a stronger facilitating effect than monovalent ions at low salt concentrations. Related studies have shown that the strength of competitive interactions between ions and biomolecules or solvents is determined by two key factors: the electrostatic shielding effect and hydration entropy (the ability of ions to disrupt or enhance the network of water molecules, affecting the stability of the hydration layer on the surface of the protein)[24]. The addition of polyethylene glycol to the electrolyte solution causes a change in the dielectric constant of the

solution (a physical quantity characterizing the solvent's ability to shield charges), which in turn affects the activity coefficient of the salt, and the extent of this change is dependent on the salt concentration[25].

As the salt concentration increases, the dielectric constant in solution weakens, ions strongly affect the protein and water molecule arrangement in the solvent, where the effect of hydration entropy competes with and is stronger than the effect of electrostatic shielding effect. In conjunction with the idea that cation hydration is preferred in studies of salt solubilisation and salt precipitation mechanisms of proteins with cations, the stability of protein hydration corresponds to salt ions in the order $Ca^{2+} < Mg^{2+} < K^{+} < Na^{+}$ based on the charge density and ionic radius[26]. Divalent ions interfere with the stability of the hydration layer on the surface of proteins more than monovalent ions, making the interaction between proteins and PEG more intense. Therefore, at mild salt concentrations, monovalent ions require a higher critical protein concentration to experience phase separation compared to divalent ions. Within the scope of the system we studied, trivalent cations are more focused on neutralizing the surface charge of proteins, and their contact with proteins greatly weakens the electrostatic repulsion between proteins and promotes protein aggregation. For example, the BSA globular protein LLPS was investigated based on the fact that the protein and YCl_3 can produce strong interactions leading to phase separation[27]. In addition, this may also be related to cation radius, polarization and hydration[28].

4. Materials and Methods

4.1. Materials and sample preparation

Ovalbumin(OVA, 98%) was purchased from Shanghai Yuanye Biotechnology Co. PEG-4000, PEG-8000, $NaH_2PO_4 \cdot H_2O$, Na_2HPO_4, NaOH, HCl, Tris-hydroxymethyl-aminomethane(Tris), 1,6-hexanediol (1,6-HD), 6-Aminocaproic acid (6-A) and various types of salts (NaCl, KCl, $MgCl_2$, $CaCl_2$, and YCl_3). The medicines used were used directly without further purification. The experimental water was prepared by Milli-Q ultrapure water system (Millipore, Billerica, Massachusetts, USA).

The experiments were prepared using a 10 mM Tris buffer system (pH = 7) by dissolving Tris in ultrapure water and adjusting the pH using NaOH or HCl. To configure the phosphate buffer (PB), take 39 mL of the configured 0.2 M $NaH_2PO_4 \cdot H_2O$ and 61 mL of the configured 0.2 M Na_2HPO_4 solution and mix them completely to obtain 0.2 M PB solution, adjust the pH with NaOH or HCl to 7. Each stock solution was prepared using the Tris buffer system, except for the reagent solutions required when probing the PB: the OVA solution (200 mg/mL), PEG-4000 (50% m/v); PEG-8000 (50% m/v), 1,6-HD (50% m/v), 6-A (50% m/v), and the different salt solutions (2 M for NaCl, KCl, $MgCl_2$, $CaCl_2$, and YCl_3). All experiments were carried out under room temperature conditions (25±2℃), and each stock solution was diluted with Tris buffer to the desired concentration and then mixed proportionally to a final volume of 100 μL of the reaction system.

4.2. Optical microscopy observation

The LLPS process was observed using an optical inverted microscope (Nikon, Tokyo, Japan) at 60X oil-mirror magnification by capturing microscopic images of droplet formation in solution through a CCD camera equipped with the microscope. The experiment can be divided into three observation stages. First, the phase separation behavior of mixed solutions of OVA and PEG-8000 with different concentration gradients was determined by observing the microscopic images. Second, 1,6-HD and 6-A solutions were added to examine the effects of hydrophobic and electrostatic interactions on phase separation. Finally, salt solutions with different valence states (Na^{+}, K^{+}, Mg^{2+}, Ca^{2+}, and Y^{3+}) and concentration gradients were sequentially introduced to observe the effect of ionic strength on the LLPS process. The oil mirrors were cleaned before and after each experiment using

professional lens cleaner and dust-free wipes, and the experiments were repeated several times to ensure the reproducibility of the experiments.

4.3. Turbidity Quantification Using UV-Vis Spectrophotometer

Q5000 UV-Vis Spectrophotometer (Quawell, San Jose, CA, USA) was used to measure the absorbance of a mixture of solutions at 600 nm. 600 nm is not typically the absorption peak for proteins, PEG, or salts, which reduces the direct interference of these substances during the measurement. This is important for studying the interaction of components in a mixture or specific reaction processes. According to the Lambert-Beer law, when parallel light passes through a solution containing a homogeneous light-absorbing substance, the absorbance of the solution is proportional to the product of the solution concentration and the thickness of the solution layer. Solutions in which LLPS occurs become turbid due to the formation of droplet-shaped macromolecular condensates that scatter and absorb light, thereby reducing the transmitted intensity of the sample. Measurement of absorbance therefore provides an indirect indication of the turbidity of a solution. The more droplets are distributed, the higher the turbidity. Remove the prepared protein solution from the 4℃ freezer and allow it to return to room temperature before diluting and mixing with the other component solutions to ensure that the final concentration is consistent with the measured value. After incubating the sample solution for 5 minutes, 2 μL of sample was dropped onto the base of the Q5000 Micro UV-Vis Spectrophotometer for measurement. A blank control was established using 2 μL of 10 mM Tris buffer (pH = 7).

5. Conclusions

In this study, the regulation of LLPS in OVA/PEG-8000 system by different valence cations was elucidated experimentally and analytically, and the main conclusion is that both monovalent and divalent cations inhibit the phase separation to a certain extent at the mild salt concentration, and the inhibition brought by monovalent is stronger than that brought by divalent. This is due to the fact that at medium salt concentration, the dielectric constant of the solution decreases and the inter-ionic interactions are enhanced due to the presence of PEG. Monovalent cations (Na^+, K^+) are better able to maintain the protein hydration layer (Layers of water molecules ordered by hydrogen bonding on the surface of ions or proteins) due to the weaker water molecule displacement capacity, producing a stronger short-range repulsive effect and causing the system to require a higher critical protein concentration for phase separation to occur. In contrast, divalent cations (Ca^{2+}, Mg^{2+}) significantly disrupt the protein hydration layer, weakening this stabilizing effect. In addition, the molecular weight size of PEG does not affect the qualitative law of salt regulation, but only the value of the critical concentration for the trend of salt concentration change. In addition, the molecular weight size of PEG does not affect the qualitative law of salt regulation. At the same time, the trends of PB and Tris buffer systems on ion characterization affecting phase separation were not different. This provides a new perspective for understanding the ionic regulation laws of phase separation of biological macromolecules.

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References

1. Banani, S.F., et al., *Biomolecular condensates: organizers of cellular biochemistry.* Nature reviews Molecular cell biology, 2017. **18**(5): pp. 285-298.
2. O'Flynn, B.G. and T. Mittag, *The role of liquid–liquid phase separation in regulating enzyme activity.* Current opinion in cell biology, 2021. **69**: pp. 70-79.
3. Su, Q., S. Mehta, and J. Zhang, *Liquid-liquid phase separation: Orchestrating cell signaling through time and space.* Molecular cell, 2021. **81**(20): pp. 4137-4146.
4. Peng, P.-H., K.-W. Hsu, and K.-J. Wu, *Liquid-liquid phase separation (LLPS) in cellular physiology and tumor biology.* American journal of cancer research, 2021. **11**(8): p. 3766.
5. Hašek, J., *Poly (ethylene glycol) interactions with proteins.* Z. Kristallogr. Suppl, 2006. **23**: pp. 613-618.
6. Wu, J., et al., *Investigation of the interaction between poly (ethylene glycol) and protein molecules using low field nuclear magnetic resonance.* Acta biomaterialia, 2013. **9**(5): pp. 6414-6420.
7. Ge, W.-Y., et al., *Screening of liquid–liquid phase separation conditions for proteins with a mixed solution kit of biomacromolecular crowding agents.* Journal of Molecular Liquids, 2024. **406**: 125038.
8. MacAinsh, M., S. Dey, and H.-X. Zhou, *Direct and indirect salt effects on homotypic phase separation.* eLife, 2024. **13**: RP100282.
9. Maldonado, S., et al., *Salt-induced stabilization of apoflavodoxin at neutral pH is mediated through cation-specific effects.* Protein Science, 2002. **11**(5): pp.1260-1273.
10. Surfaro, F., et al., *An alternative approach to the osmotic second virial coefficient of protein solutions and its application to liquid-liquid phase separation.* The Journal of chemical physics, 2023. **158**(16): 124902.
11. Ivanov, I.B., et al., *Hofmeister effect on micellization, thin films and emulsion stability.* Advances in colloid and interface science, 2011. **168**(1-2): pp. 93-104.
12. Hofmeister.F, *Zur Lehre von der Wirkung der Salze: Dritte Mittheilung.* Archiv für experimentelle Pathologie und Pharmakologie, 1888. **25**: pp. 1-30.
13. Nostro, P.L., et al., *Hofmeister specific ion effects in two biological systems.* Current opinion in colloid & interface science, 2004. **9**(1-2): pp. 97-101.
14. Choi, S.J., et al., *Molecular characteristics of ovalbumin–dextran conjugates formed through the Maillard reaction.* Food Chemistry, 2005. **92**(1): pp. 93-99.
15. Rostamabadi, H., et al., *Ovalbumin, an outstanding food hydrocolloid: Applications, technofunctional attributes, and nutritional facts, A systematic review.* Food Hydrocolloids, 2023. **139**: 108514.
16. Zheng, T., et al., *Development of ovalbumin implants with different spatial configurations for treatment of peripheral nerve injury.* Bioactive materials, 2024. **35**: p. 401.
17. Arora, S., et al., *Phase Separation and Aggregation of a Globular Folded Protein Small Ubiquitin-like Modifier 1 (SUMO1).* The Journal of Physical Chemistry Letters, 2023. **14**(40): pp. 9060-9068.
18. Kaur, T., et al., *Molecular crowding tunes material states of ribonucleoprotein condensates.* Biomolecules, 2019. **9**(2): p. 71.
19. Wang, Y., et al., *Comprehensive regulation of liquid–liquid phase separation of polypeptides.* Molecules, 2023. **28**(18): p. 6707.
20. Lima, E.R., et al., *Ion-specific thermodynamical properties of aqueous proteins.* Anais da Academia Brasileira de Ciências, 2010. **82**: pp. 109-126.
21. Lone, M., *Development of nanoscale screening technology for the detection and quantification of aggregation in protein therapeutics*. 2013, University of Nottingham.
22. Yang, F., et al., *Regulation on liquid-liquid phase separation of Bovine γ-glob-ulin by polyphenols.* Journal of STEM, 2024. **1**: pp. 1-00004.
23. Zhang, Y. and P.S. Cremer, *Interactions between macromolecules and ions: the Hofmeister series.* Current opinion in chemical biology, 2006. **10**(6): pp. 658-663.
24. Zhu, L., et al., *Ionic effect on the microenvironment of biomolecular condensates.* Journal of the American Chemical Society, 2024. **146**(20): pp. 14307-14317.
25. Slejko, E. and B. Hribar-Lee, *The effect of macromolecular crowders on dielecric constant of aqueous electrolyte solutions.* Journal of Molecular Liquids, 2021. **336**: 116295.
26. Arakawa, T. and S.N. Timasheff, *Mechanism of protein salting in and salting out by divalent cation salts: balance between hydration and salt binding.* Biochemistry, 1984. **23**(25): pp. 5912-5923.
27. Wang, X., G. Yang, and Y. Wang, *The Influence of Surfactants on Liquid-Liquid Phase Separation of Globular Protein.* Journal of STEM, 2024. **1**: pp. 1-00002.
28. Saha, R. and R.K. Mitra, *Trivalent cation-induced phase separation in proteins: ion specific contribution in hydration also counts.* Physical Chemistry Chemical Physics, 2022. **24**(38): pp. 23661-23668.