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The Influence of Surfactants on Liquid-Liquid Phase Separation of Globular Protein

Xiaoting Wang, Guangcan Yang* and Yanwei Wang*

College of Mathmatics and Physics, Wenzhou University, China

yanggc@wzu.edu.cn

Abstract: Liquid-liquid phase separation (LLPS) holds a crucial role in various biological processes, encompassing protein folding, assembly, and functioning. In the present study, we studied the impact of the anionic surfactant sodium dodecyl sulfate (SDS) and neutral surfactant dodecylpyranosyl glucoside (DG) on the LLPS of bovine serum albumin (BSA) in a yttrium (III) chloride hexahydrate (YCl_3) solution. The LLPS phenomenon occurs at a low critical concentration (LCC) of YCl_3 and dissolves at a high critical concentration (HCC). We observed that SDS augments the LLPS region when introduced into the solution. In contrast, DG primarily reduces the YCl_3's HCC while having a slight effect on the LCC. Specifically, with a fixed BSA concentration of 40 mg/mL, the LCC rises from 6 mM to 19 mM with the addition of SDS, and the HCC leaps from 8 mM to beyond 20 mM. On the contrary, with a mild DG concentration (5-7 mM), the HCC drops until the LLPS disappears around 8 mM, implying the surfactant's inhibition of phase separation. Based on the analysis of spectroscopy and dynamic light scattering, we deduce that SDS primarily modulates BSA LLPS via electrostatic interactions, whereas the suppression of LLPS by DG is largely attributable to hydrophobic associations.

Keywords: liquid-liquid phase separation, bovine serum albumin, dodecyl dimethyl amine oxide, trivalent metal salts

1. Introduction

Liquid-liquid phase separation (LLPS) is instrumental in numerous biological processes, including protein folding, assembly, and function. It occurs when proteins or similar macromolecules experience environmental variations, leading to a condensation process where proteins form compact phases resembling liquid droplets1. Several factors can instigate LLPS, including temperature, pH, ionic strength, and the presence of specific chemical ligands4.

Globular proteins, such as bovine serum albumin (BSA), commonly feature in studies examining protein interactions with various chemical agents8. The phase behavior of BSA solutions, primarily in trivalent yttrium (III) chloride hexahydrate (YCl_3) solutions, has undergone considerable investigation10. Within YCl_3 solutions, BSA's LLPS showcases a low critical concentration (LCC) and a high critical concentration (HCC), contingent on YCl_3 ion concentration and solution pH13. In acidic solutions, the cationic surfactant dodecyl dimethyl amine oxide (DDAO) can effectively modulate BSA and YCl_3's LLPS, with DDAO lowering the HCC incidence while keeping the LCC stable15.

The influence of other types of surfactants, such as non-ionic and anionic surfactants, on phase behavior remains an area for further exploration. Nonionic surfactants, for instance, can shift protein aggregation states, thereby affecting their solubility and fluidity, or attach to proteins to alter their biological activity17. In this study, we aim to investigate the impact of adding anionic and nonionic surfactants on the critical salt concentration in BSA LLPS. Ionic surfactants like sodium dodecyl sulfate (SDS) are renowned for their strong interactions with proteins, possibly leading to protein denaturation due to electrostatic interactions19. In contrast, nonionic surfactants, identified by lower hydrophilicity and enhanced hydrophobicity, generally demonstrate lesser reactivity with proteins but can still influence protein stability and function. They can modify aggregation states, solubility, and fluidity, or bind to proteins, consequently impacting biological activity.

In this present course, our quest lies in determining how the anionic surfactant SDS and the neutral surfactant DG influence phase behavior and scrutinizing the driving mechanism of this regulation through microscopy, spectroscopy, and dynamic light scattering.

2. Material and Methods

Sodium dodecyl sulfate (SDS), dodecylpyranyl glucoside (DG), bovine serum albumin (BSA), yttrium (III) chloride hexahydrate (YCl_3), PEG-1000, and 1,6-hexanediol were procured from Sigma Aldrich. We prepared distinct solutions of 100 mM SDS, DG, and YCl_3, and a separate 200 mg/mL BSA solution. Each solution was prepared and diluted using deionized water purified through a Milli-Q system. All experimental procedures were consistently conducted at a precisely maintained temperature of 45℃.

Droplet observations were facilitated through a Nikon ECLIPSE Ti-S inverted optical microscope, with images captured at 60x magnification. In BSA-YCl_3 systems, we introduced varying levels of SDS or DG to ascertain their particular effects. To ensure the validity and replicability of our results, each experimental set was repeated at least three times.

The dynamic light scattering instrument (Malvern Zetasizer Nano ZS90, UK) were used for zeta potential measurements of all solutions. Specifically, we introduced 900 μL aliquots of mixed solutions into cells (Zetasizer nano series, order: DTS1070). Each measurement was repeated at least three times for consistency and to maintain accuracy, after which an average value was computed.

To determine solution absorbance, we used the Q5000 ultra- micro UV spectrophotometer (Quawell, San Jose, CA, USA) - an accurate measuring device for the concentration or reaction rate of tiny samples via UV absorption. Solutions were assessed at an absorption peak of 280 nm, using water as a base for comparisons. To certify precision and repeatability, each measurement was performed five times, post which an average value was calculated.

3. Results and Discussion

3.1. Effect of SDS on BSA-YCl_3 binary system

The incorporation of the anionic surfactant SDS provokes alterations in the phase behavior of BSA-YCl3 binary systems, as shown in Figure 1. The diagram's colored region signifies where LLPS takes place, while the uncolored region marks the uniform area. The diagram's varying colors correspond to different SDS concentrations. Prior to the introduction of SDS, the initial LLPS range is represented by the green areas. Yet, with the integration of varying SDS concentrations (3 mM, 5 mM, 7 mM), there is a shift in the LLPS region.

Incorporating 3 mM SDS, in particular, markedly reshapes the BSA and YCl3 systems' LLPS area (Figure 1a). At this measure, there is a substantial upward shift in the LLPS region boundary within the BSA solution. This 3 mM SDS dosage stimulates a transformation in the LLPS region, transitioning its color from green to blue.

The addition of 5 mM SDS results in the green LLPS region shifting towards a purple hue (Figure 1b). Similarly, incorporating 7 mM SDS causes the LLPS zone to change its color from green to yellow (Figure 1c). Throughout these tests, the BSA concentration varied from 30 to 80 mg/mL, while YCl3 concentrations ranged between 4 mM and 20 mM. Overall, any SDS incusion typically prompts an upward repositioning of the LLPS area.

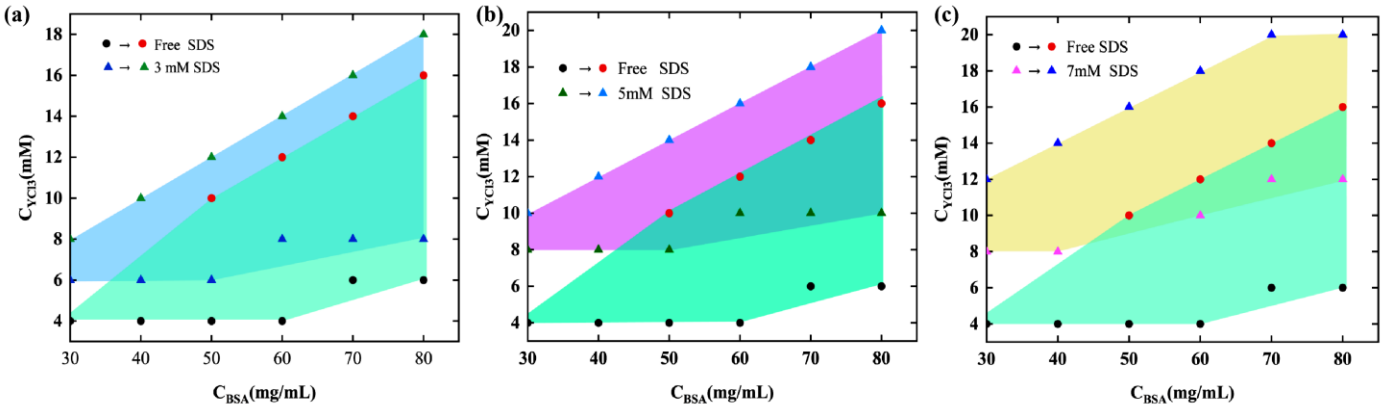

Figure 1. Phase diagram of LLPS of BSA after addition of different concentrations of SDS. (**a**) The addition of 3 mM

SDS resulted in the LLPS region changing from green to blue, (**b**) The addition of 5 mM SDS resulted in the LLPS region changing from green to purple, and (**c**) The addition of 7 mM SDS resulted in the LLPS region changing from green to yellow. (The BSA concentration ranges from 30 to 80 mg/mL, and the YCl_3 concentration ranges from 4 mM to 20 mM).

We observed that the addition of SDS exerted a suppressive effect on the LLPS of BSA. To further validate our findings, we conducted additional experiments aimed at elucidating the impact of varying SDS concentrations on LLPS, while maintaining a constant concentration of BSA. In Figure 2, the concentration of BSA was consistently maintained at 40 mg/mL throughout the experiments. As observed in Figure 1, YCl_3 can induce LLPS in BSA at concentrations ranging from 4 mM to 6 mM. In Figure 2a presents microscopic images of BSA-YCl_3 solution in both the absence and presence of SDS. In which, we can see that the LLPS of BSA undergoes a significant shift when 3~7 mM SDS is added in the presence of 4 mM YCl_3. Without the presence of 3~7 mM SDS, 4 mM YCl_3 can induce LLPS in BSA. However, once SDS is added, 4 mM YCl_3 is unable to initiate LLPS in BSA.

The LLPS region of the BSA-YCl_3 system (Figure 2b) illustrates how the addition of varying concentrations of SDS can modify its characteristics. The tendency illustrated that as SDS is added, a higher concentration of YCl_3 is required to trigger the LLPS of BSA. The addition of SDS increases the lower critical concentration from 6 mM to 19 mM, while the higher critical concentration rises from 8 mM to over 20 mM. With the increase in SDS concentration, the LLPS region exhibited an overall upward shift in trend. This shift indicates that the addition of SDS effectively suppressed the LLPS process in BSA.

To validate the suppressive effect of SDS on the LLPS of BSA-YCl_3, we introduce Polyethylene Glycol (PEG) into the solution. In Figure 3a, we additionally prepared the sample by setting the BSA concentration to 40 mg/mL, fixing the YCl_3 concentration to 16 mM, and then adding 40% W/V PEG-1000. Upon the addition of PEG-1000, the system transitions from a non-LLPS state to an LLPS state. Based on our former experiments, we further tested the effect of SDS concentration from 1 mM to 30 mM on the LLPS of BSA (Figure 3b). Upon the addition of 5 mM SDS, the initially moderately dense droplets gradually became increasingly sparse. Upon adding 10 mM SDS, the solution contains both droplets and gel consisting of the same gredients. As the SDS concentration increased from 20 mM to 30 mM, the droplets evolved into a gel form and finally became precipitation.

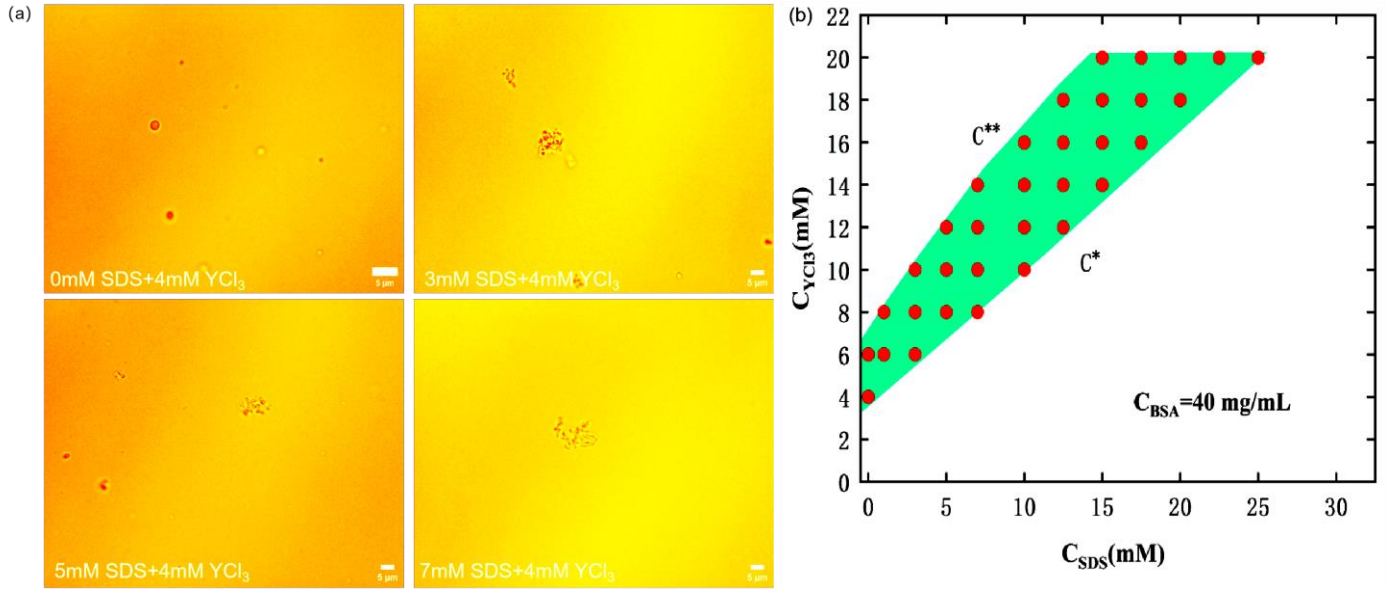

Figure 2. Effect of SDS on BSA-YCl_3 binary system. (**a**) optical inverted microscopy plots of 40 mg/mL of BSA with 4 mM YCl_3 in the absence or presence of 3~7 mM SDS (scale bar is 5 μm), (**b**) phase diagram of the BSA-YCl_3 binary system regulated from 1~30 mM SDS. The concentration of BSA is 40 mg/mL.

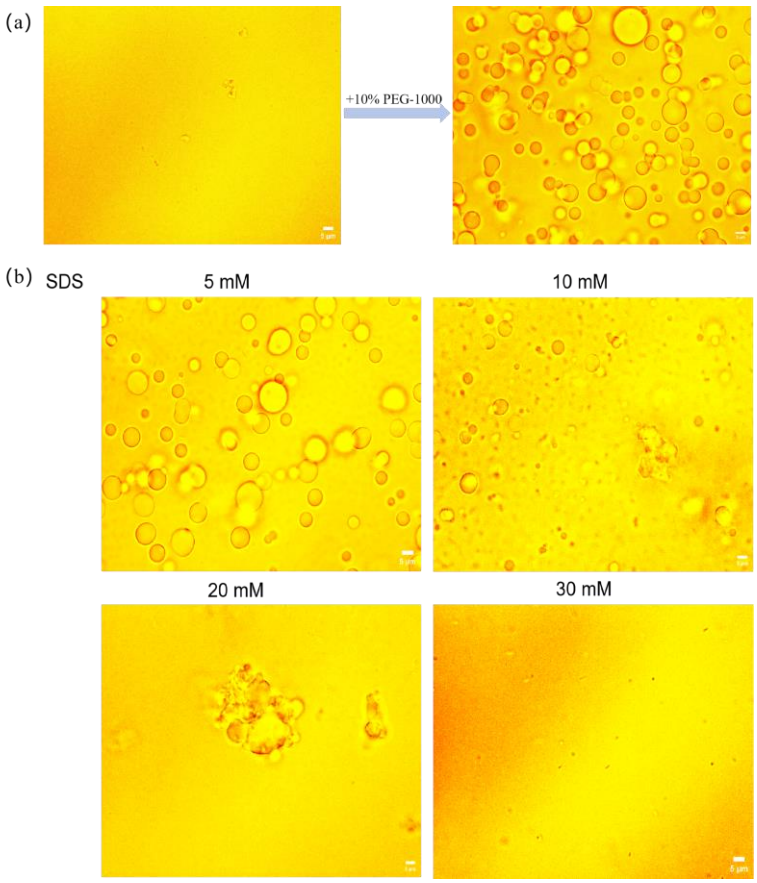

Figure 3. The influence of varying concentrations of SDS on the liquid-liquid phase separation of BSA in the presence of PEG-1000. (**a**)Optical microscopy map of LLPS in BSA induced by PEG-1000. (**b**)Effect of 1~30 mM SDS on LLPS of BSA in the presence of PEG-1000, based on (a), scale bar is 5 μm. (40% W/V PEG-1000, 40 mg/mL BSA, 16 mM YCl_3).

To understand the mechanism of the LLPS regulation of the BSA-YCl_3 binary system by SDS, we investigate the process by dynamic light scattering (Figure 4a) and Q5000 ultra-micro ultraviolet spectrophotometer (Figure 4b). In Figure 4a, the electrophoretic mobility of BSA was assessed and plotted as a function of SDS concentration, demonstrating the effects of different SDS concentrations on BSA mobility at a particular concentration of 40 mg/mL. The black curve depicts the electrophoretic mobility of BSA-YCl_3 without the addition of SDS. The diagram illustrates that as the concentration of YCl_3 is elevated, the surface charge transitions from negative to positive. Specifically, the concentration of YCl_3 at the critical point of charge reversal is 6 mM. However, the inclusion of various SDS concentrations can alter the critical point at which the charge reversal occurs. At the charge reversal critical point, YCl_3 concentration is 9 mM, 10 mM, and 12 mM in the presence of 3 mM, 5 mM, or 7 mM SDS respectively. Notably, it is evident that the critical salt concentrations inducing LLPS in 40 mg/mL BSA, triggered by YCl_3 in the presence of varying SDS concentrations, align closely with the YCl_3 concentrations corresponding to the charge reversal critical point. With the addition of SDS, the electrophoretic mobility of BSA gradually decreases, suggesting that the binding of SDS to BSA enhances the negative charge on its surface.

The changes in the absorbance of BSA in the presence of 0 mM, 3 mM, 5 mM, and 7 mM SDS were shown in Figure 4b. The absorption of the BSA mixture was measured at 280 nm. The black line represents the absorbance of the BSA-YCl_3 system in the absence of SDS. The red line, blue line, and green line depict the absorbance of the BSA-YCl_3 binary system in the presence of 3 mM, 5 mM, and 7 mM SDS, respectively. The high light absorption suggests a high turbidity of the solution, resulting in a cloudy appearance.

In Figure 4, we can see that the addition of SDS leads to a gradual decrease in electron mobility, clearly indicating an augmentation of negative charges of BSA. Specifically, we can divide the process into three stages. In the first stage, when the concentration of YCl_3 is lower than 3 mM, Y^{3+} can more effectively bind to the negatively charged side chain exposed by BSA. However, it is insufficient to counterbalance the overall negative charge on the BSA surface. During the second stage, when the YCl_3 concentration ranges from 3 mM to 7 mM, Y^{3+} effectively neutralizes the negative charge

carried by BSA, forming a cation bridge that initiates the aggregation of BSA and ultimately results in the formation of LLPS. In the third stage, the concentration of YCl_3 exceeds 7 mM, and the overcompensation from excessive Y^{3+} allows for a charge reversal on the BSA surface. The balance of short-range attraction and long-range repulsion results in the redissolution of aggregates marking the end of LLPS. As SDS is introduced into the solution, the electrophretic mobility of BSA is gradually decreased. This is attributed to the dissociation of SDS into negatively charged ions in the solution, which subsequently interact with negatively charged regions of BSA and Y^{3+} ions. Thus, the LLPS of BSA is primarily suppressed by the addition of SDS.

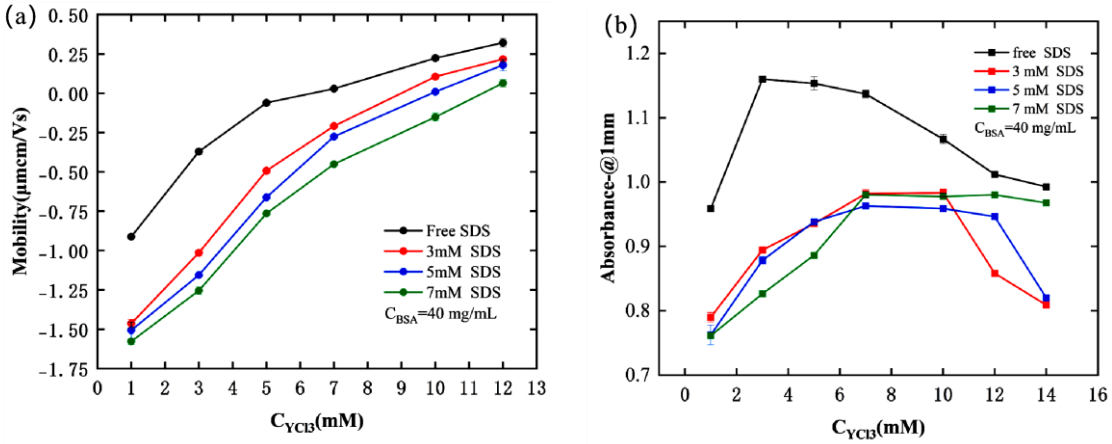

Figure 4. (**a**)Electrophoretic mapping of BSA-YCl_3 in the presence of SDS, (**b**) Changes of BSA with YCl_3 concentration in the presence of different concentrations of SDS.

3.2. Effect of DG on the BSA-YCl_3 binary system

In order to gain a deeper understanding of the impact of surfactants on BSA liquid-liquid phase separation, we selected a non-ionic surfactant - Dodecyl Betain (DG) as a regulator. We observed that the introduction of DG also impacts the phase behavior of the BSA-YCl3 system (as shown in Figure 5), however, this effect differs markedly from the impact of anionic surfactants on BSA liquid-liquid phase separation. In the experiment, the concentration range of BSA was between 30 to 80 mg/mL, and that of YCl3 was between 4 to 20 mM. The colored area correspons to the range of LLPS. With increasing concentrations of DG (3 mM, 5mM, and 7 mM), the LLPS area for BSA changes: the HCC of the LLPS area decreases from the black square to the blue triangle, then to the green triangle, eventually to the purple rhombus. Thus, the addition of DG reduces the LLPS region of BSA. On the other hand, upon introducing DG into the BSA-YCl3 system, the LCC (red dots) at which BSA undergoes LLPS induced by YCl3 remains nearly constant.

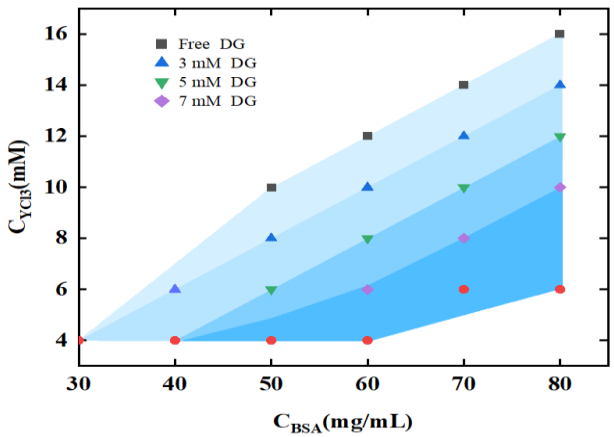

Figure 5. Phase diagram for 30~80 mg/mL BSA at various concentrations DG. (The concentrations of DG are 3 mM, 5 mM, and 7 mM, respectively.)

If we fixed BSA concentration at 40 mg/mL while varying concentrations of DG, a similar suppression of LLPS can be observed, as shown in Figure 6. In Figure 6(a), we can see that 3 mM DG has also no effect on LLPS , while 5 mM and 7 mM DG transform the state of LLPS to gel state. The phase diagram is presented in Figure 6(b) for DG concentrations spanning from 1 mM to 30 mM. We noted that LLPS was only observed at 4 mM YCl_3 with 7 mM DG. Highr DG concentration leads to LLPS vanished.

The influence of various DG concentrations on the mobility of BSA at a fixed concentration of 40 mg/mL is shown in Figure 7a. The black line depicts the electrophoretic mobility of BSA-YCl_3 in the absence of DG addition. At the charge reversal critical point, YCl_3 concentration is 5 mM, 4 mM, and 4 mM in the presence of 3 mM, 5 mM, or 7 mM DG respectively. Upon the addition of DG, the electrophoretic mobility of BSA gradually decreases, indicating that the binding of DG to BSA results in an increase in the negative charge on its surface. The addition of DG facilitated the reversal of the BSA surface charge and led to a decrease in the high critical salt concentration of the original system. In Figure 7b, the black line represents the absorbance of the BSA-YCl_3 binary system in the absence of DG. The red line, blue line, and green line depict the absorbance of the BSA-YCl_3 binary system, with 3 mM, 5 mM, and 7 mM of DG added, respectively.

To find out the driving force of the inhibition mechanism of DG on BSA-YCl_3 LLPS, we added a hydrophobicity disruptor 1,6-hexanediol into the solution. The result is shown in Figure 8. was comprehensively discussed in conjunction with Figures 6 to 8. We can see that the addition 30 mg/mL of 1,6-hexanediol led to the transformation of the gels into droplets. Thus we can infer that the DG molecule primarily interacts with BSA-YCl_3 through hydrophobic interaction.

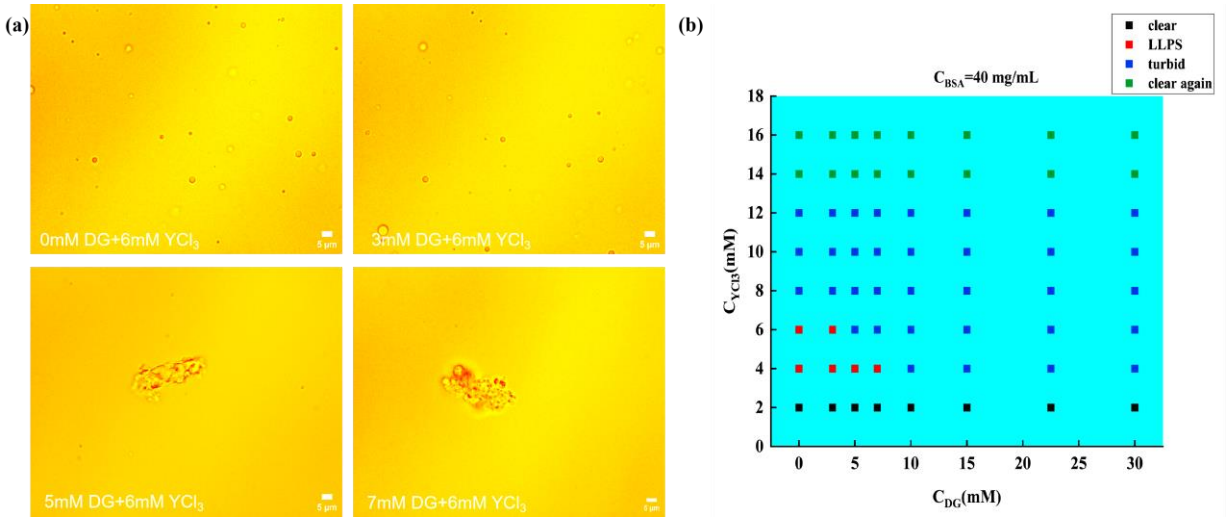

Figure 6. Effect of different concentrations of DG on the LLPS of BSA at 40 mg/mL. (**a**) Optical inverted microscopy of 3~7 mM DG interacting with 40 mg/mL BSA in the presence of 6 mM YCl_3, (**b**) Effect of Different Concentrations of DG on BSA-YCl_3 System (scale bar: 5 μm).

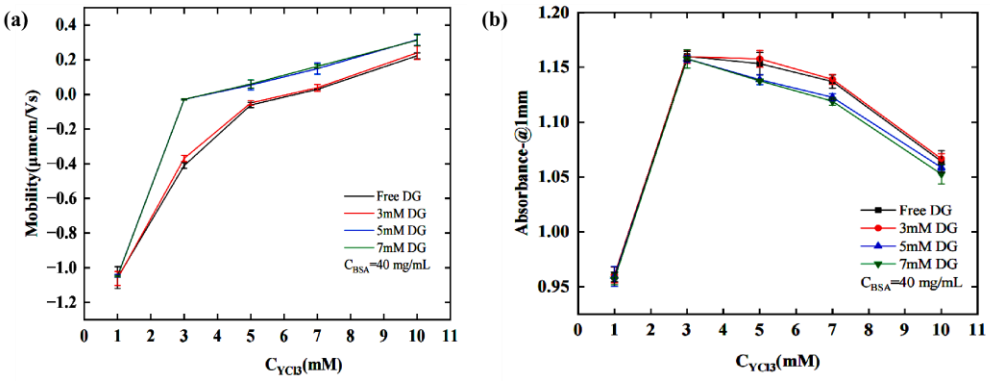

Figure 7. Effect of different concentrations of DG on BSA LLPS. (**a**) Electrophoretic mapping of BSA-YCl_3 in the presence of DG, (**b**)The effect of DG concentration on the absorbance of BSA-YCl_3.

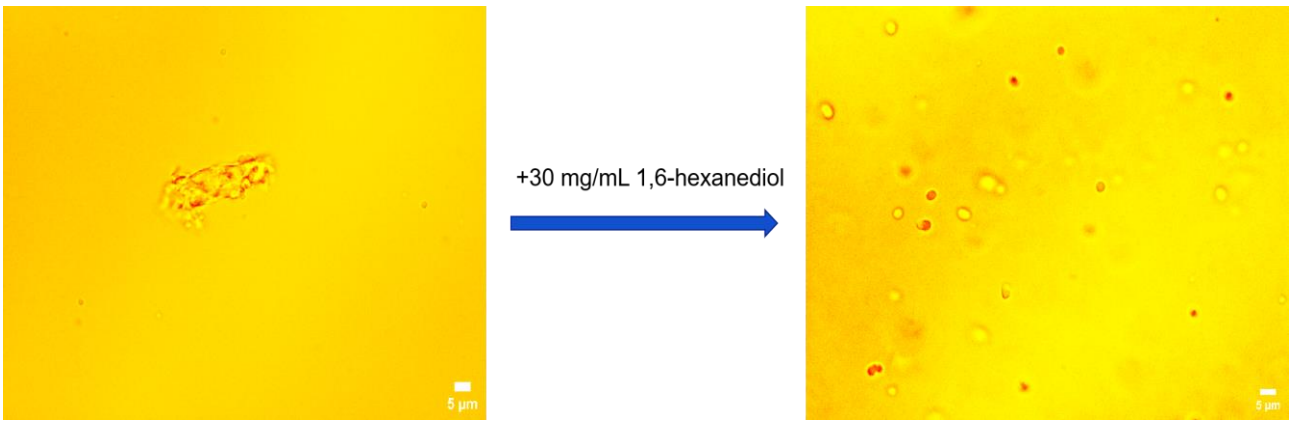

Figure 8. Effect of 1,6-hexanediol 30 mg/mL on BSA-YCl_3 system in the presence of 5 mM DG (BSA concentration is 40 mg/mL, YCl_3 concentration was 6 mM).

3.3 Discussion

In Figure 9, we present a schematic diagram for the plausible microscopic mechanism of the regulation of SDS and DG on LLPS of BSA-YCl_3 system. Figure 9(a)~ (d) shows the case in the absence of SDS and DG. (a) Corresponding to the concentration of YCl_3 being lower than the low critical salt concentration, BSA is negatively charged in aqueous solution, and the surface of BSA attracts a small amount of Y^{3+} through electrostatic interaction to form a complex of BSA and YCl_3. At this time, the electrostatic repulsion between the complexes is still dominant, and the solution is clear and transparent. (b) and (c) corresponding to the concentration of YCl_3 between the low critical salt concentration and the high critical salt concentration, Y^{3+} can properly compensate for the net negative charge carried by BSA, and Y^{3+} forms a cation bridge by associating with BSA, causing the original mutually exclusive BSA to cluster together to form a protein-rich lower concentrated phase, and the solution becomes cloudy. (d) The corresponding concentration of YCl_3 is higher than the high critical salt concentration. Due to the excessive concentration of YCl_3, the surface net negative charge of BSA is overcompensated, which in turn leads to the transformation of BSA from negative to positive, and the solution becomes clear again. Figure (e) to (h) correspond to the case of adding SDS. e: When Sodium Dodecyl Sulfate (SDS) is introduced at a salt concentration lower than the Low Critical Concentration (LCC) of Liquid-Liquid Phase Separation (LLPS), it binds with BSA in both monomer and micelle forms. Given that the BSA surface harbors numerous unneutralized negative charges, the addition of SDS does not change the original state of the system.

f: At the LCC of LLPS for YCl3, the addition of SDS weakens the mutual interactions of the original system.

g: At the High Critical Concentration (HCC) of LLPS, the introduction of SDS inhibits the charge reversal of BSA; nevertheless, due to the high concentration of YCl3, phase separation still occurs.

h: Above the HCC of LLPS, SDS binds separately with BSA and YCl3, continuing to maintain the LLPS of the system.

Figure (i) to (l) correspond to the case of adding dodecylpyranosyl glucoside (DG). i: When DG is introduced at a salt concentration lower than the LCC of LLPS, it binds with BSA in both monomer and micelle forms, but does not change the original state of the system.

j: At the LCC of LLPS for YCl3, the system undergoes LLPS, but the addition of DG does not alter the original state of the system.

k: At the HCC of LLPS, the addition of DG weakens the original state of the system.

l: Above the HCC of LLPS, the number of DG molecules binding with the BSA surface increases, leading to the disappearance of BSA's liquid-liquid phase separation.

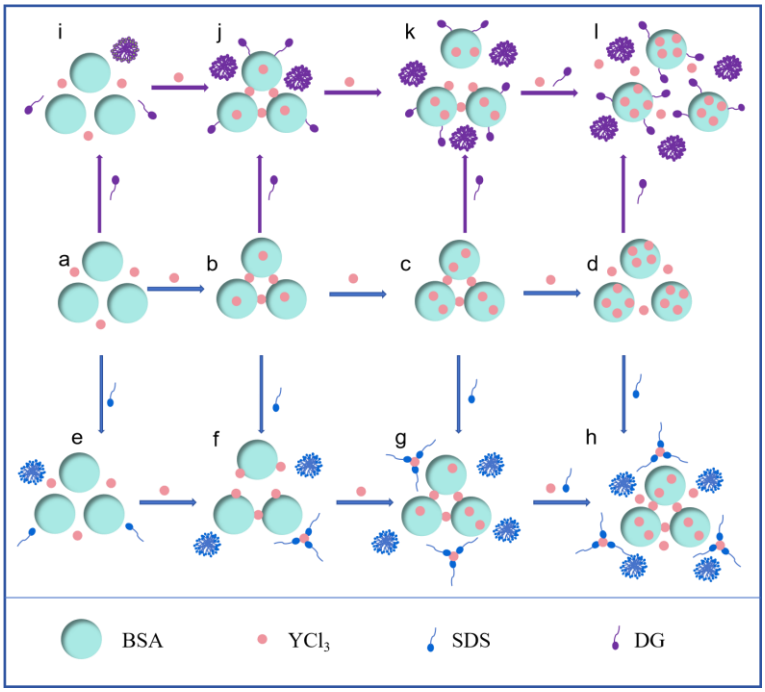

Figure 9. Schematic diagram of the regulatory mechanism of SDS and DG on the LLPS of BSA. (**a**) The interaction between BSA and YCl_3 at a concentration below the low critical salt concentration; (**b**) The LLPS of BSA and YCl_3 at low critical salt concentration; (**c**) BSA interacts with YCl_3 at high critical salt concentration; (**d**) BSA interacts with YCl_3 at a concentration higher than high critical salt concentration; (**e**) The effect of SDS on the interaction between BSA and YCl_3 with a concentration below the low critical salt concentration; (**f**) The effect of SDS on the LLPS of BSA and YCl_3 at low critical salt concentration; (**g**) The effect of SDS on the LLPS of BSA and YCl_3 at high critical salt concentration; (**h**) The effect of SDS on the interaction between BSA and YCl_3 at a concentration higher than high critical salt concentration; (**i**)The effect of DG on the interaction between BSA and YCl_3 with a concentration below the low critical salt concentration; (**j**) The effect of DG on the LLPS of BSA and YCl_3 at low critical salt concentration; (**k**) The effect of DG on the LLPS of BSA and YCl_3 at high critical salt concentration; (**l**) The effect of DG on the interaction between BSA and YCl_3 at a concentration higher than the high critical salt concentration.

4. Conclusions

In summary, our study highlights the significant role of surfactants, specifically sodium dodecyl sulfate (SDS) and dodecylpyranosyl glucoside (DG), in modulating the liquid-liquid phase separation (LLPS) of bovine serum albumin (BSA) in yttrium (III) chloride hexahydrate (YCl_3) solutions. The observations underscore a decisive influencing factor in the phase behavior of BSA solutions, dictated by the critical concentrations of YCl_3. Particularly, SDS enhances the phase separation effect, increasing the YCl_3's low critical concentration (LCC) and high critical concentration (HCC) range. Conversely, DG deemphasizes phase separation, significantly reducing the HCC while having negligible impact on the LCC. The study, therefore, provides some insights into the interaction mechanisms between surfactants and proteins, with SDS impacting through electrostatic forces and DG via hydrophobic interactions. These findings might provide some hints for deeper understanding of phase behavior modulation in biological systems. Further exploration of this direction is necessary to comprehend how these substances regulate LLPS and potentially develop new therapeutic strategies for various medical conditions.

Author Contributions: G.Y. conceived the project. G.Y. and Y.W. designed the experiments. X.T. performed the experiments. G.Y., Y.W., and X.W. analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Sample Availability: Samples of the compounds are available from the authors.

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