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Regulation on liquid - liquid phase separation of Bovine γ-globulin by polyphenols

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Abstract: Liquid-liquid phase separation (LLPS) for proteins can provide specific local physiological environments for some crucial biological activities. Elucidating its regulation has a potential implication for curing the related diseases of LLPS. Our results show that epicatechin gallate (GCG), a polyphenol, can promote LLPS of bovine γ-globulin in the binary solution. Specifically, we found that a small amount of GCG (1 mM) induces LLPS in a homogeneous solution of 4% W/V PEG-1000 and 20 mg·mL-1 γ-globulin below the critical concentration for the phase transition. This suggests that adding polyphenols reduces the critical concentrations of PEG and proteins for LLPS, thereby expanding the LLPS region in the phase diagram. However, we observed that the polyphenol itself only induces γ-globulin solution to precipitate, rather than LLPS in the absence of PEG. Furthermore, when we fixed the concentration of polyphenol and protein, we observed the reentrant of liquid-liquid phase separation in the mixed solution with increasing PEG concentration. We concluded that the reentrant of phase separation is the result of the combined effects of the crowding effect of polyethylene glycol and the hydrophobic effect provided by GCG through the hydrophobic disruption regulation. We investigated the interaction between protein and polyphenol using fluorescence spectroscopy. We constructed a schematic model to elucidate the regulation mechanism of LLPS of polyphenol and PEG.

Keywords: liquid-liquid phase separation, Bovine γ-globulin, Hydrophobic effect, Electrostatic interaction

1.Introduction

The study of phase transitions in protein solutions is a flourishing research field1. On the one hand, it is a topic of fundamental interest as proteins represent the small size limit for colloids and feature peculiar effects due to shape and interaction anisotropy4. On the other hand, this research field is closely related to a wide range of applications, including formulation and stability of biopharmaceuticals, protein crystallization, biomaterials, and industrial food processing7. At the same time, the accumulation and deposition of the oligomers of the (Aβ, tau), α-synuclein, IAPP, and superoxide dismutase 1 proteins, which have been the mainstream concept underlying Alzheimer's disease (AD), Parkinson's disease (PD), type II diabetes (T2D), and amyotrophic lateral sclerosis (ALS) research, respectively, for many years8.

Liquid−liquid phase separation (LLPS) mediates the formation of many intracellular membrane-less organelles (MLOs), including the nucleolus, nuclear paraspeckles, stress granules, and many other ribonucleoprotein granules. Studies over the past several years have established that many cellular proteins, associated with MLOs[10, typically containing intrinsically disordered regions (IDRs) rich in charged residues, are able to phase separate into distinct liquid-like droplets in both the presence and absence of nucleic acids,which has provided a platform for investigating the

physical forces underlying LLPS12. Weak, multivalent homotypic protein−protein interactions are critical for the formation and dynamic nature of many phase-separated droplets. Such LLPS is driven by intermolecular interactions, including electrostatic, hydrophobic, π−πstacking, and hydrogen-bonding interactions14.

Polyethylene glycol (PEG1000) is a hydrophilic nonionic polymer used in many biochemical and industrial applications16. Due to its nontoxic character, this chemical can be found in cosmetics, food, and pharmaceutical products. In protein crystallography, PEG1000 is considered the most successful precipitating agent to produce protein crystals, the crucial step for the determination of the molecular structure of a protein18. All these applications make PEG1000 by far the most widely used polymer in aqueous solutions of biological molecules. These interactions are often described in terms of a depletion force, which arises because the polymer is depleted in the region between adjacent proteins19. Depletion force models have been successful in describing the effect of nonadsorbing polymers on colloidal suspensions20.

Polyphenol compounds are a class of natural products connected to the structure of polyhydroxy groups on benzene rings. It is abundant in the plant kingdom21. A variety of studies have shown that polyphenols have a series of physiological activities, such as antioxidant, anti-allergic, antibacterial, anti-inflammatory, anti-tumor, anti-diabetes, antiviral, etc.22 and have a variety of benefits to human health. It has been found that the interaction modes of polyphenols and proteins mainly include disulfide bond, hydrophobic interaction, hydrogen bond, etc.24 The interaction causes the structural changes of polyphenols and proteins to varying degrees, thus leading to the changes of phenol bioavailability, protein functional properties, etc., which provides a new idea and theoretical basis for their application in more fields26.

Green tea catechins (GTCs) are plant secondary metabolites that are believed to provide a variety of benefits for human health27. Its major components include (−)-epigallocatechin 3-gallate (EGCG) which is the most abundant and well-studied catechins in green tea, accounts for 65% of the total catechin content (Zaveri 2006), and others, (−)-epigallocatechin (EGC), (−)-epicatechin gallate (GCG), and (−)-epicatechin (EC), (-)-catechin (C), (−)-epicatechin-3-gallate (ECG), (+) catechin and (+) gall catechin (GC)28. Research shows that, the affinity of C with 2,3-trans structure (GCG) forγ--globulin was much lower than that of C with 2,3-cis structure (EGCG)22 Therefore we chose GCG as the research object to drive the LLPS of the Bovine γ-globulin.

LLPS can be used as a direct tool for probing protein–protein, protein–PEG and protein–GCG interactions. It has been shown that the position of the liquid–liquid boundaries provide insight about the nature and magnitude of the protein–protein interactions The advantage of LLPS analysis with respect to measurements of protein solubility is that data interpretation requires no assumptions regarding crystal phase properties.16 LLPS is important also for the analysis of nonequilibrium properties of the system. Because the nucleation of protein crystals is enhanced in proximity of the LLPS, this phase boundary is believed to be implicated in protein crystallization kinetics15.

2. Experimental

2.1 Materials and sample preparation

Bovineγ--globulin (Assay 96-100%,Lemeitian Medicine, 9007-83-4),GCG (2,3-trans)(HPLC≥98%，Lemeitian Medicine，1257-08-5),PEG1000 (Sigma-Aldrich, 81188), 1,6-hexanediol（Sigma-Aldrich, 629-11-8）,6-Aminocaproic (Sigma-Aldrich,A7824),NaCl (Merck 106404), HEPES(Sigma-Aldrich, HN78) and NaN3 (Sigma-Aldrich, S8032) were used as received. The solution of the GCG was prepared by dissolving polyphenol with methanol 5%(w/v). All solutions were prepared in a buffer of composition 20 mM HEPES pH = 7.0, 2 mM NaN3, using degassed Milli-Q water (Merck Millipore 18.2 M Ωcm). The solutions for the evaluation of phase behavior and the samples for UV experiments were mixed from buffer and stocks solutions (in buffer) of Bovine γ-globulin, PEG 1000 36% (w/v), GCG 30Mm and NaCl 4M, to a final NaCl concentration of 150 mM. All experiments were performed at room temperature (23±2℃).

2.2 Microscope images of droplets in vitro

Microscope images were captured using a fully equipped Nikon Ti-DH inverted microscope equipped with 48MP FHD Camera V8 and Instec MK2000B temperature controller, the temperature controller is connected to a industrial chiller c100W and semiconductor platform. After LLPS, microscopic examination was performed using Nikon100 oil-immersion objective（NA=1.30). The images were analysed with Fiji (ImageJ) for droplet size and density by analyzing particle function.

2.3 Turbidity assay

Proteins and Polyphenols were prepared as described above. Ultraviolet spectrophotometer is used to measure turbidity at 350nm wavelength.，No protein amino acid can absorb this wavelength of light. The turbidity(T) is defined by T=-ln（I/I0), with I0 incident light intensity and I intensity of light passed through the sample volume. All samples were examined in triplicate (n=3). We can use the degree of turbidity of the liquid as one of the bases to judge whether phase separation occurs.

2.4 The interaction was measured by fluorescence spectrometry

The final concentrations of polyphenol were 0, 2, 3, 4, 5, 6, 7, 8, 9 and 10μmol/L, and the final concentrations of Bovine γ-globulin were 20mg/ml, respectively. Prepare two sets of solution for each polyphenol and mix thoroughly. The second group was treated with 1%~8% PEG1000. The detection conditions of GCG and globulin were as follows: excitation wavelength was 295nm, slit width of excitation spectrum and emission spectrum was 10nm, and scanning range was 315-450nm emission spectrum. Slit widths, scan speed, and excitation voltage were kept constant within each data set and each spectrum was the average of three scans.

2.5 Determination of the interaction by UV–vis

A UV-vis spectrum was measured on a the Q5000 Ultra-micro ultraviolet spectrophotometer (Quawell, USA). Fixed the concentration of Bovine γ-globulin and PEG1000, (Bovine γ-globulin100mg/ml and PEG1000 3%(w/v) observed the ultraviolet absorption spectra of different concentrations of polyphenols. The final concentration of GCG was 0~5 m mol·L-1. The mixed solution was placed in a constant temperature water bath at 25 ℃ 20min, scanning range: 200~850nm.

3.Results and discussion

3.1 The phase behavior of Bovine γ-globulin and PEG1000

We first carried out many experiments using Bovine γ-globulin mother liquor with a concentration as high as 200mg·mL-1 and PEG1000 samples with a concentration as high as 36% (w/ V). The interaction between Bovine γ-globulin (Bovine γ-globulin) and polyethylene glycol 1000 (PEG1000) at room temperature was observed and the experimental phase diagram was drawn, as shown in figure (a). The image behavior is divided into three types: single phase, liquid-liquid phase separation (LLPS) and non-gel state, corresponding to regions Ⅰ, Ⅱ and Ⅲ in Fig.1(a), respectively. Figure 1e shows the turbidity of the solution after the reaction is stable with the increase of PEG1000 concentration.

When the PEG1000 concentration is less than 4%, when mixed with the protein, we observe that the two are fused into a clear and transparent stable phase in the centrifuge tube. In the inverted microscope, the line of sight is clear, and occasionally there is a small amount of protein polymer (see figure 4-2C). This performance is called single phase, and its range corresponds to region I in figure 4-1. However, when the concentration of PEG1000 is too high than 7%w/v (region Ⅲ), the loss interaction increases sharply, the non-equilibrium state appears in the centrifuge tube, and the viscous and inhomogeneous mixture appears in the centrifuge tube after vibration. the inverted microscope can distinguish the complex states such as suspension droplet aggregation and crystallization, as shown in figure 4-1a, droplets and crystals intermingle and scatter randomly.

During the phase transition of Bovine γ-globulin, we are most concerned about the liquid-liquid phase separation state of protein (region Ⅱ). It is known from the phase diagram that the protein concentration is fixed. When the concentration of PEG1000 reaches 4-6% (W/ V), liquid-liquid phase separation occurs in the solution. At this stage,when PEG1000 is dropped into a centrifuge tube containing Bovine γ-globulin solution and shakes well, the liquid becomes turbid but uniform, and the solution is balanced at 21 ℃ for about 12 hours, and then experienced brief centrifugation. Through visual observation, it can be found that the solution is divided into upper and lower layers, the upper layer is a pure transparent dilute egg white phase, the lower layer is yellowish transparent protein-rich phase, and the solution is formed by a sharp crescent-shaped separation of "rich egg white phase" and "dilute protein phase". At the same time, at the initial stage of the reaction, through careful examination by the optical microscope, many tiny droplets rich in protein can be observed (figure 4-2b), and the droplets collide with each other, resulting in rapid fusion and expansion (figure 1f). In the LLPS region, when the protein concentration is constant, the higher the PEG1000 concentration, the stronger the droplet fusion.

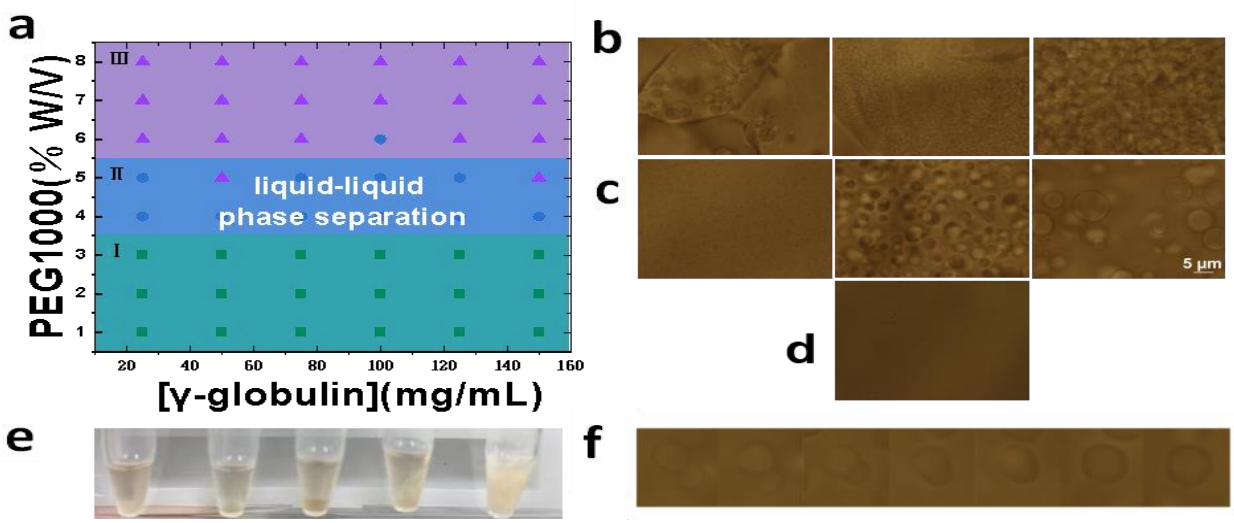

Figure1: (a) the state diagram of γ--globulin in the presence of polyethylene glycol 1000, room temperature 21 ℃. represent non-equilibrium,represent LLPS and represent single phase. The images observed under the inverted microscope are shown in (b) (c) (d), respectively. (e) droplet fusion from single phase to stratification to latex texture (f) under inverted microscope with the increase of polyethylene glycol 100 concentration.

3.2 The regulation of GCG on the liquid-liquid phase separation of bovineγ- globulin and PEG1000

In the process of exploring the regulation of bovine γ- globulin by GCG, one of the major interference factors is that polyphenols must be dissolved in methanol, and the IDRs of the protein binds to methanol on the basis of electrostatic interaction, thus driving the occurrence of liquid-liquid phase separation. Therefore, when studying the regulatory mechanism of GCG on bovine γ- globulin. The interference of methanol must be overcome first. In view of this, we chose the intermediate critical single-phase position of liquid-liquid phase separation of Bovine γ-globulin and PEG1000. Under the condition that the concentration of protein solution was 25mg·mL-1 and the concentration of PEG1000 was 3% (w / v), the methanol concentration range needed for liquid-liquid phase separation was tested. As shown in figure 2 (a), when the methanol concentration reached 4% (w / v), droplets began to appear in the originally calm line of sight. It means that the concentration of methanol must be at least 100% to have an effect. In the follow-up experiment, we used 5% (w / w) methanol to dissolve GCG to get polyphenol mother liquor, so that the final concentration of methanol

was less than 2% (w / v) in the experiment of Bovine γ-globulin reaction, which was basically negligible and minimized the interference of methanol to the experiment.

As shown in figure 4-5, when 1mM GCG is added to Bovine γ-globulin, the liquid-liquid phase separation region is basically unchanged. As the concentration of GCG increases to 3mM, the phase separation region begins to extend downwards. As can be seen in figure 4-5b, the exhaustion at 3% PEG1000 concentration is not enough to promote the occurrence of LLPS, but the state can be changed after the addition of 3mM, and the top of the phase separation region also moves down slightly. Until the concentration of GCG reaches 5mM, even if it does not exist in the crowded environment, liquid-liquid phase separation can still occur, and the LLPS region almost completely covers the lower half of the phase diagram, and the phase behavior of protein changes obviously.

Interestingly, when 5mMGCG was added, a single-phase point appeared in the liquid-liquid phase separation region, and LLPS suddenly disappeared when the concentration of Bovine γ-globulin was 100mg·mL-1 and PEG1000 was 4% (w / v). Through many experiments, it can be determined that under the composition of the solution, the protein delamination phenomenon is interrupted, which has attracted our attention.

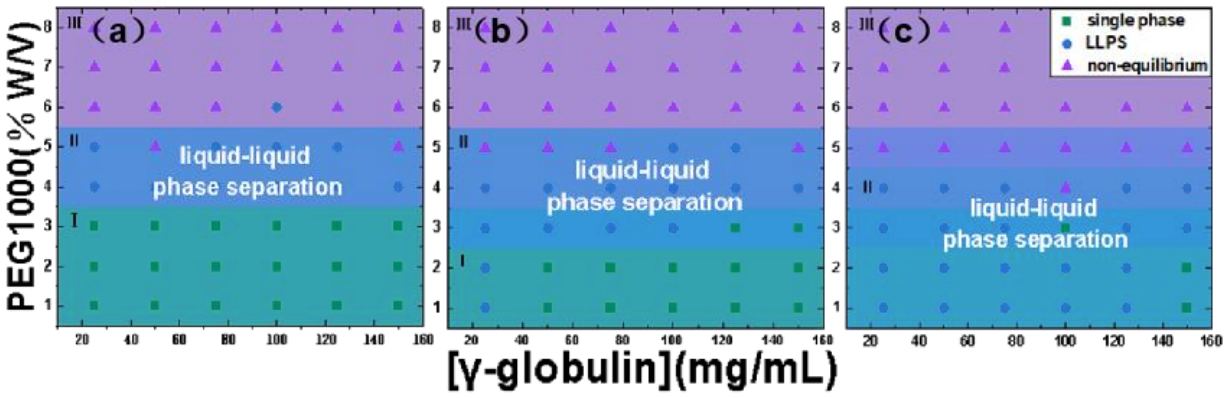

Figure2: (a) Critical region [CP=25 mg/ml, CPEG1000=3% (W/v)], the effect of methanol on LLPS. (b) two-dimensional concentration phase diagrams of γ-globulin, polyethylene glycol 1000 and GCG, in which (b1) contains1mM, （b2)contains 3 mM, and (b3) contains 5 mM GCG. After adding 5 mM GCG to (b3), when the concentration of PEG1000 is 4% (w / v), LLPS suddenly disappears and appears as a single phase.

3.3 The interaction between GCG and Bovine γ-globulin was observed and recorded.

It was found that in the process of mixing Bovine γ-globulin of 75mg·mL-1 with GCG of 0.6mM-5mM, there was only weak droplet aggregation behavior, no droplet fusion was found, and there was no solution delamination in the centrifuge tube after the reaction. As shown in figure 3a, only three states can be observed under an inverted microscope: single-phase, "grape cluster" gel beads and crystals. When the concentration of polyphenols is lower than 0.8 mM, it is single phase, when the concentration of GCG increases, it shows the shape of "grape string" of droplets, but no fusion is observed, and when the concentration of polyphenols is higher than 3mM, it shows a crystalline state. On this basis, adding PEG1000 to provide crowding conditions can promote the occurrence of phase separation and produce fused droplets, and if the role of GCG is lost, the exhaustion is not strong enough, and phase separation will not occur, indicating that the protein phase separation region has expanded under the synergistic regulation of PEG1000 exhaustion and GCG affinity for Bovine γ-globulin.

At the same time, when the concentration of GCG was 5mM, the addition of PEG1000 in figure 3C eliminated the aggregation and precipitation caused by the reaction of Bovine γ-globulin with polyphenols and reversed to LLPS. Both

crowding effect and hydrophobic effect can promote protein binding, phase separation and crystallization, but the superposition of each other has the opposite effect. We speculate that the crowding effect may be due to the destruction of the binding of proteins and polyphenols, weakening the hydrophobic effect and weakening the interaction between proteins. This also reminds us again that the different ratio of GCG to PEG1000 will affect the state of protein.

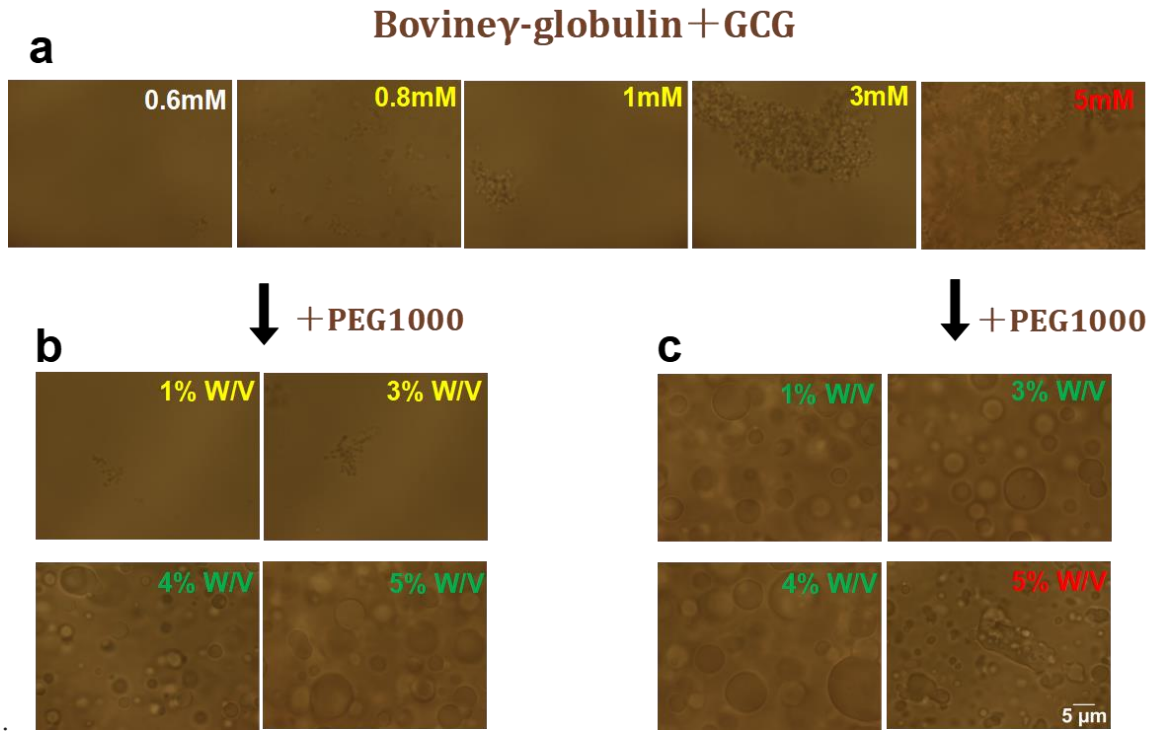

Figure 3: (a) Imaging of 75mg·mL-1 γ- globulin and GCG at different concentrations under an inverted microscope. When the concentration of Polyphenol was lower than 0.8 mM, it was single-phase; when the concentration of GCG increased, it showed a "grape cluster" shape; when the concentration was greater than 3 mM, it crystallized. (b) PEG1000 was added to 75mg·mL-1 protein and 0.8 mM GCG for regulation. (c) LLPS appeared when PEG1000 was added to the crystals of protein and 5 mM polyphenol production. The white label in the upper right corner represents the single phase, yellow represents the "grape cluster" form, green represents phase separation, and red represents crystallization.

3.4 Reentrant condensation of bovine gamma globulin

Based on the previous work, we found that in the process of GCG regulating the liquid-liquid phase separation of Bovine γ-globulin, single-phase data appeared briefly in the extended region of phase separation. at the same time, the crystallization region of Bovine γ-globulin and GCG was transformed into single-phase with the addition of PEG1000. These two "strange" phenomena led us to do more collection of the three-phase reaction of Bovine γ-globulin, PEG1000 and GCG.

The effect of 4mMGCG on the liquid-liquid phase separation of Bovine γ-globulin was studied. The blue circle indicates the area where the phase separation of Bovine γ-globulin occurs, and the green square is the area where there is no phase separation. The reentrant phase separation mechanism of protein can be observed, and the solution changes from phase separation to single phase and phase separation occurs again. From a coordinate point of view, the observed phase diagrams of (3p3), (4mag2), (5mag2) in figure 4a and (4pen3) in figure 2b (3) are consistent with this phenomenon, indicating that phase separation has a process of re-enhancement from strong to weak.

Visually, the state of the initial stage of protein mixing can also indicate whether phase separation will eventually occur. The turbidity of the solution at the initial stage of mixing means that the solution will be separated or precipitated, and the clarification of the solution shows that it is still in a single-phase state after the completion of the reaction. The higher the absorbance, the lower the transmittance and turbidity of the solution; the lower the absorbance, the higher the transmittance, the clearer the solution. That is, the area with high absorbance accords with the expectation of liquid-liquid phase separation. Therefore, the ultra-micro ultraviolet spectrophotometer Q5000 can be used to measure the absorbance of protein to observe whether phase separation occurs. We collected the absorbance of protein mixed solution at 500nm, and chose the wavelength of this range to avoid the interference of characteristic peaks of aromatic amino acid groups such as tyrosine and tryptophan on the experimental data, so as to judge the turbidity change of the solution. As shown in figure 4b, the black broken line is the absorbance of the binary system of Bovine γ-globulin and PEG1000 of 75mg·mL-1 without GCG. The absorbance of the solution is low in single phase. When the concentration of PEG1000 is more than 5%, due to the enhancement of crowding, LLPS occurs, and the absorbance increases significantly. The red break line is bovine gamma globulin concentration 75mg·mL-1, and the absorbance of bovine gamma globulin mixture at 1% (W/ V) and 2% (W/ V) PEG1000 is about 0.7, indicating that GCG can induce liquid-liquid phase separation under weak crowding effect, while when the concentration of polyethylene glycol 1000 increases to 3% (W/ V), the absorbance decreases, almost the same as when GCG is not added. The value of Z is consistent with other experimental phenomena, which can determine the nature of its single phase and prove that the protein does exist reentrant condensation.

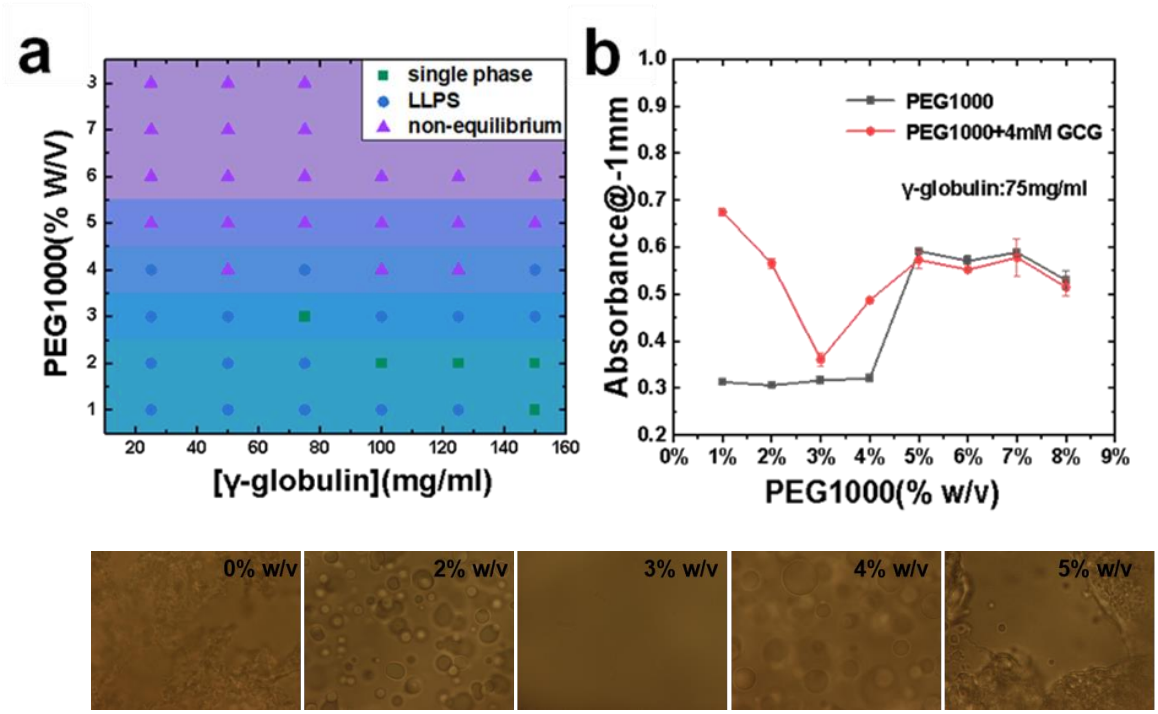

Figure 4: (a)Phase diagram of γ- globulin reaction with PEG1000 in the presence of 4 mM GCG at 21℃. (b) Absorbance of γ- globulin under UV-vis, 4 mM GCG affects the progress of LLPS occurring as concentrations of PEG1000 increasing.

3.5 1,6-hexanediol effects on the phase behavior of LLPS

Hydrophobic interaction in the phase separation condensate. By adding fatty alcohol 1 mL 6-hexanediol to treat the pre-formed droplets, we found that when 1-6 hexanediol of 100mM was added to the liquid-liquid separation system controlled by 5mMGCG, the number of droplets decreased significantly under the microscope and completely

disappeared when it increased to 200mM (figure 5a). This shows that GCG mainly regulates the interaction between proteins through hydrophobic interaction, and the hydrophobic interaction is weakened. The degree of liquid-liquid phase separation is also weakened. To further verify, we directly applied 1-6 hexanediol to the binary system containing only 5mMGCGBovine γ-globulin and found that it could also shield the hydrophobic interaction and make the crystallization of protein weaken and twist to a single-phase state, which further verified the conjecture that GCG provided hydrophobicity in this system (Fig. 5b), and further studied the interaction between Bovine γ-globulin and GCG.

Furthermore, the turbidity of protein solution was determined by ultraviolet absorbance method. The comparison between the blue broken line and the red broken line in figure 5c shows that the absorbance of PEG1000 with concentration less than 4% (w / w) decreased under the action of 200mm M1 6-hexanediol, and the turbidity in the initial stage of the reaction changed from high to low, indicating that the original turbidity of the solution became clear after shielding and hydrophobic action, realizing the process from condensation to densification.

From these observations, it can be concluded that the process of molecular separation and condensation formed at the concentration of 4mM and 5mMGCG may be related to the enhancement of hydrophobic effect, and the electrostatic interaction between protein and protein is ignored. We think that the coordination of hydrophobic interaction and crowding effect may be the key driving factors for the separation of polyphenols and protein re-entry.

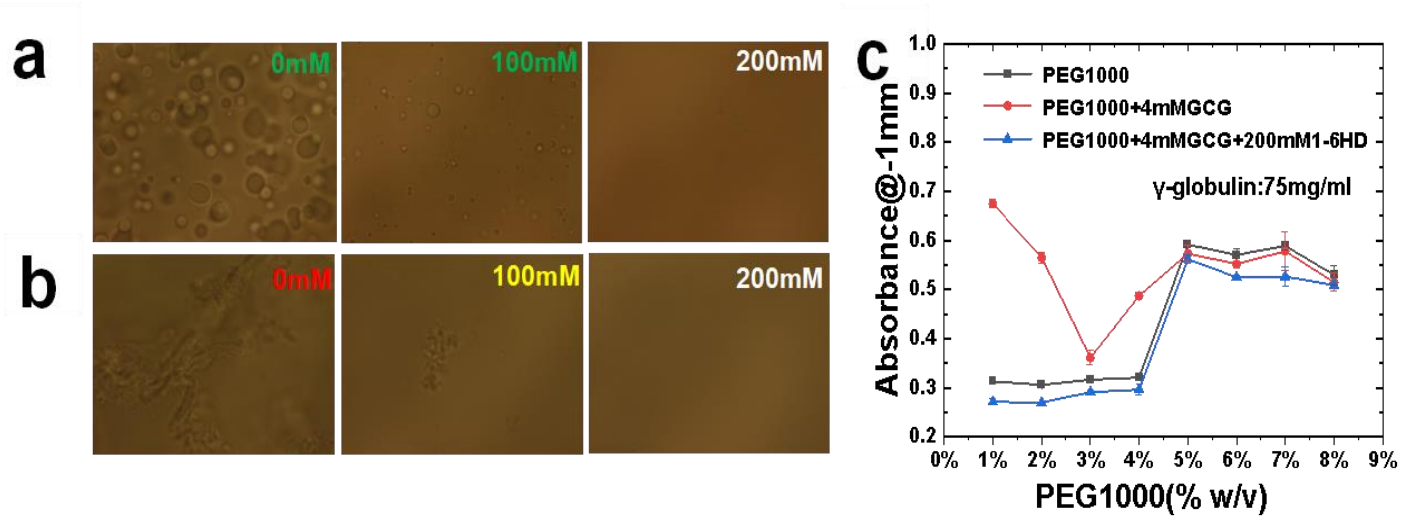

Figure 5: (a) The mixed solution of 75mg·mL-1 γ- globulin and 4% (w/v) PEG1000 was regulated by 4 mM GCG, and after adding 0 mM,100 mM, 200 mM 1-6 hexathiol, the droplets were screened due to the hydrophobic effect, and gradually decreased and completely disappeared;(b) When a mixture of 4 mM GCG and 75 mg·mL-1 globulin was added with the same concentration of 1-6 hexanediol as above, the hydrophobic effect was reduced, and the crystallization was reduced to disappear.（b） After shielding the hydrophobic effect, the absorbance of bovine γ- globulin solution is restored to that before GCG regulation, and the turbidity decreases.

3.6 6-Aminocaproic effects on the phase behavior of LLPS

Based on previous studies, we know that 6-aminoacetic acid (6-Mel A) can change the electrostatic interaction to weaken the LLPS of biological macromolecules. Therefore, in this experiment, we added 6-aminoacetic acid to the liq-

uid-liquid separation system regulated by 4mMGCG (CP= 100mg·mL-1) to change the electrostatic interaction and observed the effect of electrostatic interaction on LLPS. For example, in 6a, it was found that the number of droplets observed under inverted microscope decreased obviously with the increase of 6Mui A concentration from 0mM to 400mM, but did not disappear completely, indicating that there was an electrostatic force between Bovine γ-globulin and GCG, but this effect was weaker than that of hydrophobic interaction. Even if shielded by high concentration of 6mura, there were still droplets driven by hydrophobic interaction.

Similarly, we used Q5000-ultramicro ultraviolet spectrophotometer to test the absorbance of the solution under this series of data. As shown in figure 4-11, in the presence of 400mM 6-Aminocaproic, with the change of PEG1000 concentration from 0% to 9%, compared with the absorbance (red line) regulated by 5mMGCG, the absorbance of the solution after shielding electrostatic interaction is close to that before unregulated. The change of absorbance curve is consistent with the imaging results under optical inverted microscope. It can be proved that the electrostatic interaction also contributes to the regulation of GCG on the liquid-liquid phase separation of Bovine γ-globulin.

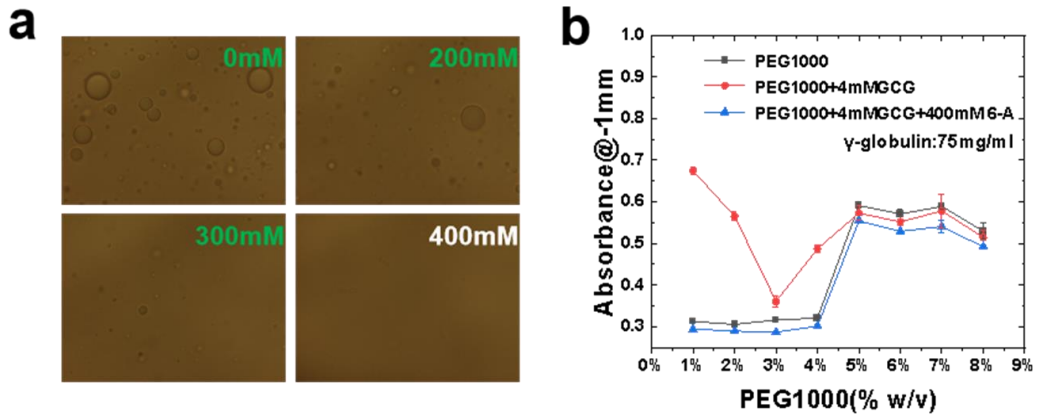

Figure 6: (a)75 mg·mL-1 γ-globulin and 4% (w / w) PEG1000 mixed solution adjusted by 4mM GCG, after adding 200 mM, 300 mM, 400 mM 6-aminoacetic acid, the liquid-liquid phase separation was weakened due to shielding electrostatic interaction, and the droplets gradually decreased and disappeared completely.(b)After shielding electrostatic interaction, the absorbance of Bovine γ-globulin solution returned to that before GCG regulation, and the turbidity decreased.

3.7 Interactions between Bovine γ-globulin and GCG

Tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) are three aromatic amino acid residues that absorb UV and fluorescence in protein molecules. When molecular interaction, excited state reaction, molecular rearrangement, energy transfer, ground state complex formation and collision quenching occurred, the fluorescence intensity of fluorescence chromophore decreased. Therefore, fluorescence spectra are often used to study the interaction between small molecules and proteins. In this study, a series of solutions containing the same concentration of γ- globulin and different concentrations of polyphenols were determined by fluorescence spectrometry, and the results were shown in Figure 7a. γ- globulin solution has an emission peak at 340nm, and the fluorescence intensity of γ- globulin emission peak decreases with the increase of polyphenol concentration. The fluorescence intensity decreased faster when PEG1000 was added (Fig.7b). This fluorescence quenching phenomenon indicates that the interaction between GCG, γ- globulin and PEG1000 leads to the formation of weak fluorescent polyphenol-γ- globulin complex.

The fluorescence quenching mechanism of polyphenols and other small molecules and proteins is analysed by Stern-Volmer equation:

$$F_0/F = 1 + K_{SV}Q = 1 + K_q\tau_0 Q \quad (1)$$

In the formula, F0 and F represent the fluorescence intensity of γ- globulin when GCG are absent and the concentration is Q; Ksv, dynamic quenching constant; Q, concentration of GCG; Kq, quenching rate constant; τ_0,the average lifetime of biomacromolecules without quencher, about 10-8s. Figure 7c showed the Stern–Volmer plots for g-globulin fluorescence quenching by PEG1000 and GCG. As seen from Fig.7c, the Stern–Volmer plots for GCG are linear. It was found that both dynamic and static quenching were involved for GCG on γ-globulin fluorescence, which demonstrated by the fact that the Stern–Volmer plots slightly deviated from linearity toward the y-axis at higher polyphenols concentrations.

Quenching can be further analysed by using the double logarithm formula:

$$\lg\left[\frac{F_0-F}{F}\right] = \lg K_a + n\lg Q \quad (2)$$

Where: K_a, binding constant, n combining number of bits. According to the data, a double logarithmic graph is drawn, as shown in Figure 7d. Ka and n values were calculated by the intercept and slope of the curve in Fig. 7d respectively. Through calculation, it can be seen from calculation that ka increases after the addition of PEG1000, while n basically does not change. The results showed that the binding strength of proteins was enhanced under the crowding effect of low concentration polyphenols, the regulation of phase separation is mainly related to the interaction between small molecules and proteins.

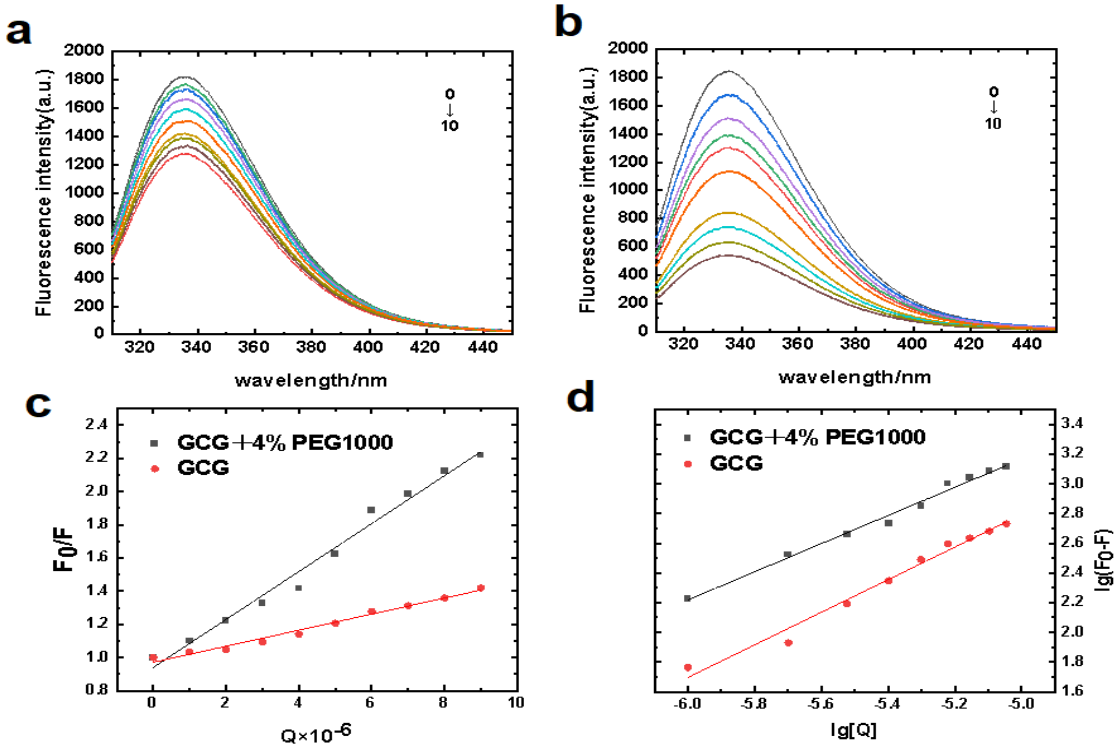

Figure 7: (a) Fluorescence spectra of GCG binding to γ- globulin; (b) Fluorescence spectra of GCG binding to γ- globulin after the addition of 4%PEG1000;(c) The Stern-Volmer plots for interaction of GCG,PEG 1000 and γ- globulin ;(d) GCG,PEG1000 and γ- globulin Double log graph.

3.8 GCG on Bovine γ-globulin structure

The UV–Vis absorption spectroscopy is widely exploited to study the protein-drug complexation and protein conformational changes (Chi and Liu, 2010). This technique is dependent on degree of absorption of UV–vis light by the protein molecule. The absorption of UV light occurs due to the presence of aromatic amino acids mostly tryptophan,

tyrosine and phenylalanine. The absorbance of tryptophan, tyrosine and phenylalanine depends on the microenvironment of their chromophores, and their wavelength shift either red or blue depends upon the polarity of surrounding environment.

In this experiment, we have collected the absorption spectra Bovine γ-globulin, the concentrations of protein and PEG were fixed, and different concentrations of polyphenols were observed. Its ultraviolet absorption spectrum, the results are shown in the figure. With GCG increase of element concentration, the absorption peak of protein shifted from 281nm to 286 and then to 291nm, showing a weak red shift. Absorption spectrum of Bovine γ-globulin alone shows maximum absorption around 280 nm because all aromatic amino acids absorb light maximally at 280 nm. Upon addition of different concentrations of GCG, significant increase in the absorbance of Bovine γ-globulin with red shift in the wavelength maxima was recorded. The increase in absorption and shift in wavelength maximum shows that there is complex formation between Bovine γ-globulin and catechin and polarity of microenvironment is also changed. Because dynamic quenching does not change the absorption spectrum of protein, but static quenching can change it, the results show that a new complex is formed after the interaction between GCG and Bovine γ-globulin, which changes the absorption spectrum of protein, and the fluorescence quenching of Bovine γ-globulin by GCG is static quenching It is also reported that the mechanism behind increase in absorption and shift in wavelength maximum towards higher wavelength is due to the exposure of aromatic amino acids to the external environment.

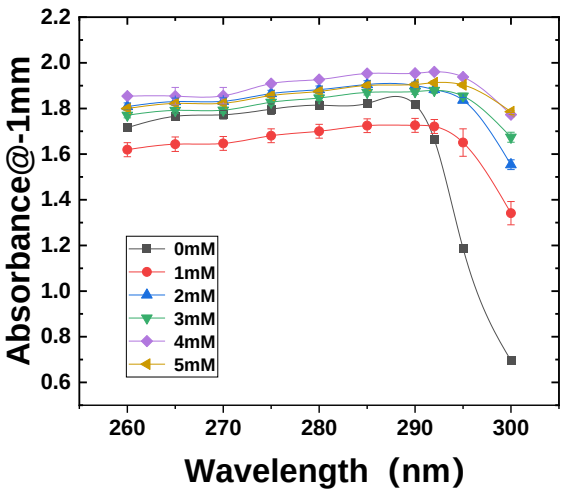

Figure8: Effect of GCG concentration on Bovine γ-globulin absorption peak.

4. Conclusions

Based on the above data, the regulation process of GCG on the LLPS phase behavior of Bovine γ-globulin and PEG1000 binary system was summarized. GCG can promote the phase separation of proteins, and when the concentration of GCG increases from 1mM to 5mM, the required PEG1000 concentration decreases gradually. However, PEG1000 is essential in the regulation of liquid-liquid phase separation by GCG, and phase separation cannot occur without this element. In addition, it can be seen from the phase diagram that the addition of GCG does not gradually enhance the phase separation in one direction but is accompanied by the process of weakening at first and then strengthening. This means that the addition of polyphenols adds more mysterious power to the crowding effect originally provided by PEG1000.

The non-covalent interactions between polyphenols and proteins mainly occur under warm conditions, such as natural physiological conditions, including hydrogen bonding, electrostatic adsorption, hydrophobic interaction and

van der Waals force. Hydrogen bonds are formed by the binding of hydroxyl groups on polyphenols to hydrophilic groups of proteins (including N, O or S side chain groups). Electrostatic adsorption is that polyphenols are adsorbed on the surface of protein molecules by positive and negative ion exchange. Hydrophobic interaction is a strong interaction between non-polar groups and non-polar groups. Hydrophobicity is the main force of non-covalent binding between polyphenols and proteins. In order to understand the interaction between proteins in this process, hydrophobic shielding agent 6-hexanediol and electrostatic shielding dose 6-aminoacetic acid were added. The morphology under the inverted microscope and the transmittance under the UV spectrophotometer showed that the phase separation was effectively inhibited after adding the shielding dose. It is preliminarily judged that the enhancement of hydrophobic effect and electrostatic interaction changes the phase separation region. The interaction between polyphenols and protein was studied by fluorescence spectroscopy. The binding constant of GCG to Bovine γ-globulin and the change of absorption peak at 500nm were determined by ultra-micro ultraviolet spectrophotometer Q5000. It was found that the interaction between polyphenols and protein expanded the range of LLPS.

As shown in figure 9, combined with the theoretical knowledge of the interaction between GCG and polyphenols and the analysis of experimental data, the mechanism of bi-directional regulation of γ-globulin liquid-liquid separation by GCG was described. (a PEG1000 d) describes the protein-protein interaction under the exhaustion action of hydrophilic Nonionic polymer PEG1000. With the increase of PEG1000 concentration, the exhaustion increases, and the binding between proteins occurs more closely. (e) show the combination of polyphenols with proteins to form precipitates. (F) when a small amount of PEG1000 was added, the hydrophobic and electrostatic interactions between polyphenols and proteins were enhanced, accompanied by crowding effect, resulting in the occurrence of LLPS. (G) the results showed that the binding of polyphenols to protein was relatively weakened with the increase of PEG1000 concentration. When a threshold is reached, the hydrophobic and electrostatic effects are not strong enough, and the interaction intensity between proteins can not be coordinated with the crowding effect, and LLPS just does not occur. (h) with the continuous increase of PEG1000 concentration, the binding of polyphenols to protein has been greatly weakened, mainly due to crowding effect, resulting in the turbidity of the solution and the formation of small droplets.

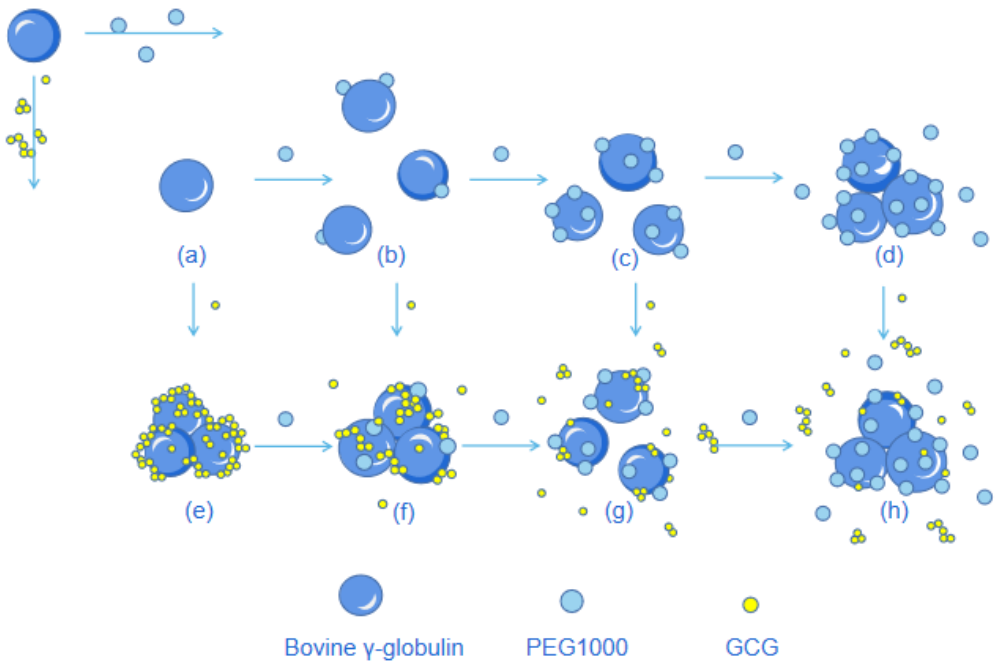

Figure 9: Mechanism diagram of GCG regulation on liquid-liquid separation of Bovine γ-globulin. (a-d) with the increase of PEG1000 concentration, the crowding effect was enhanced, and phase separation occurred. (e-h) The binding strength and number of GCG to globulin decreased with the increase of PEG1000 concentration. Under the combined action of hydrophobic effect and crowding effect, the binding between globulin was weakened first and then strengthened.

Conflicts of interes: The authors declare no conflict of interest.

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