Liquid-liquid phase separation (LLPS) of proteins regulated by small molecules, such as Adenosine triphosphate (ATP), ssDNA and RNA, has been observed. However, the physical mechanism of this regulation remains largely unknown. Here we develop a minimal multi-scale model to quantitatively study the influence of ATP on LLPS of Fused in Sarcoma (FUS). Based on Flory-Huggins theory, we explicitly include multivalent interactions instead of treating all interactions via simple Flory parameters. We find the nonlinear shift of phase diagrams with the increase of ATP concentration, which agrees with the first enhancement and then inhibition of LLPS observed in the experiment. The reason lies in the positive and negative cooperation between ATP-FUS and FUS-FUS interactions, where the existence of the ATP bridging plays a key role. Furthermore, we systematically study the phase behavior in a larger parameter space, and introduce a quantity to reflect different regulation degrees. The present model provides a clear physical picture relating microscopic interactions to macroscopic phase diagrams, as well as a good example for adapting a classical theory in polymer physics to understanding the new phenomena in intracellular phase separation.

On the other hand, Adenosine triphosphate (ATP) is known as an energy fuel for biological reactions typically with the concentration of micromolar. In cells, surprisingly, the concentration of ATP is at millimolar, much higher than that as fuel. To disclose the reason why there is a high concentration of ATP in cell, studies have shown that the high concentration of ATP is related to the dissolution of protein condensers. Moreover, RNA and ssDNA also exhibit qualitatively similar effect on intracellular LLPS: an enhancement of LLPS at low concentrations of small molecules and inhibiting LLPS at high concentrations. However, the mechanism of such small molecules regulating LLPS is still rather controversial. For example, it remains unclear whether ATP modulates LLPS via nonspecific effect (like hydrotrope) or specific interactions. Meanwhile, there is emerging evidence that abnormal protein aggregation is associated with many human diseases, including cancer, neurodegeneration, and infectious disease. To uncover the influence of small molecules on protein condensates is of both fundamental and physiological significance.

Here we aim to develop a multi-scale theory to build

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a direct relationship between microscopic driving force and macroscopic phase separation, to offer a quantitative study compared with experiments, to clarify the mechanism of the regulation of LLPS by ATP, and to expose the general factors regulating LLPS. We explicitly take multivalent interactions into account within the framework of the mean field theory, following ideas from the gelation of associating polymers [32–34], hydrogen bonding in aqueous polymer solutions [35–36], and cross-linking in salt-doped polymer blends [37].

For FUS solutions, multivalent interactions are specific binding between TYR and ARG residues. Each protein offers several TYR and ARG residues [23]. The number of TYR or ARG residues is named as the valency of the multivalent interaction. According to experimental data by Wang et al. [23], we model each FUS protein as a chain with length $N_1 = 526$. Each FUS protein has $m_1$ TYR residues and $m_2$ ARG residues, with $m_1 = m_2 = 34$. Solvents are modeled as chains with the length of $N_2 = 50$. The incompressible solution consists of $n_p$ FUS proteins and $n_s$ solvent molecules. The dimensionless free energy of the reference state, a solution without nonspecific interactions between FUS proteins and solvents, $\beta F_{\text{ref}}$, has purely entropic character and is written as: 

$$\beta F_{\text{ref}} = \phi \ln \phi + \frac{(1-\phi)}{N_2} \ln(1-\phi),$$

where $\phi = \frac{n_pN_1v}{N}$ is the volume fraction of FUS. $v$ is the volume of one residue of protein, which is taken as the unit volume, and $V = n_pN_1v + n_sN_2v$ is the volume of system. Next, we take the specific binding between TYR and ARG residues into account, assuming that each TYR/ARG has only one binding site. The partition function aroused by the formation of $k$ TYR-ARG bonds [32–37] is

$$Z_{\text{binding}} = C_{n_nm_1}^k C_{n_nm_2}^k k! \left(\frac{v}{N}\right)^k \exp(k\beta\epsilon_1).$$

$-\beta\epsilon_1$ is the free energy associated with the binding between a TYR and an ARG, which includes the affinity between TYR and ARG residues as well as the entropic loss during the formation of TYR-ARG bond [33–35]. More specifically, the specific chemical structures of TYR and ARG side chains appear to be important determinants of the formation of TYR-ARG bond [24]. Such reduction of conformational entropy is a common character for specific interactions, such as hydrogen bonds and cation-π interactions. According to $\beta F_{\text{binding}} = -\frac{v}{N} \ln Z_{\text{binding}}$, the dimensionless binding free energy can be written as

$$\beta F_{\text{binding}} = \frac{m_1\phi}{N_1} [p \ln p + (1-p) \ln (1-p)] + \frac{m_2\phi}{N_1} \left(1 - \frac{m_1}{m_2}\right) \ln \left(1 - \frac{m_1}{m_2}\right) - \frac{m_1\phi}{N_1} \left(\ln \frac{m_2\phi}{N_1\epsilon} + \beta\epsilon_1\right),$$

where $p = \frac{k}{nm_1}$ is the fraction of TYR in the formation of TYR-ARG bonds. It reflects the extent of protein network crosslinked by TYR-ARG bonds. Thus, the total free energy of FUS solution is $F_{\text{tot}} = F_{\text{ref}} + F_{\text{binding}}$. After the minimization with respect to $p$ and $\phi$, we can construct the phase diagram. More details are given by Section I in the Supplemental Material (SM) [38].

It should be stressed that all the input parameters are obtained from experimental data except the free energy loss of TYR-ARG binding ($\beta\epsilon_1$). Theoretically predicted phase diagram as a function of $\beta\epsilon_1$ is shown in Fig. 1 (a). We confirm that although each microscopic specific interaction is in the order of $k_B T$, it is enough to cause macroscopic phase separation. The theoretical prediction that the binding free energy is of the order of thermal energy accords with the experimental definition of weak multivalent interaction. At $\beta\epsilon_1 = 2$, we find that the concentration of the condensed phase is about 100 times higher than that of the dilute phase, which agrees with experimental observations on condensers [22]. Therefore, $-2k_B T$ is chosen as TYR-ARG binding free energy for FUS solutions. The saturation concentration, which is the threshold of the phase separation, is defined as the the concentration of dilute phase when two phases coexist. Here we obtain the saturation concentration $\phi_{\text{sat}} = 0.006$ for FUS. If average protein radius in solvent is around 10 nm, the saturation concentration of FUS solution is several $\mu M$, quantitatively consistent with the experiments by Wang et al. [23]. At the molecular level, two coexisting phases show distinct TYR-ARG binding fractions, as shown in Fig. 1 (b). In the dilute phase, the fraction of TYR-ARG binding is almost zero, implying that FUS proteins do not interact each other. On the other hand, in the dense case, finite values of TYR-ARG binding fractions indicate that multivalent interactions between TYR and ARG residues are driving forces leading to the aggregation of FUS proteins via the formation of protein network. From the second derivative of the free energy (see Section I in SM [38]), we are able to extract the effective interaction $\chi'$ between proteins and solvent molecules. For the case of FUS with $m_1 = m_2$, $\chi'N_1 = \frac{m_p}{\phi(1+p)} > 0$ is obtained, indicating the effective repulsion between FUS and solvent depending on FUS concentration $\phi$ and the extent of TYR-ARG binding $p$. 

FIG. 1. Phase separation in FUS solutions without ATP. Phase diagrams as a function of $\beta\epsilon_1$ (a), the faction of TYR-ARG binding (b), and effective interaction for coexisting phases (c). The black curves represent the dilute phase, and the red ones are the condensed phase. Dash lines are tie-lines relating two coexisting phases. Blue dots are chosen as two coexisting phases of FUS solutions driven by TYR-ARG binding.
clearly shown in Fig. 1 (c). In polymer physics, the Flory parameter $\chi$ usually is assumed to be independent of the concentration, which typically represents the nonspecific interaction between different species. However, here we get the effective interaction is a complex function of the protein concentration. This result, to some extent, reflects the difference between nonspecific interaction and specific interaction at the mean-field level.

Next, we investigate the influence of ATP on the phase transition of FUS solutions. Now the reference system is a FUS solution with freely moving ATP molecules without any nonspecific interactions. For the purpose of determining the phase behavior, it is more convenient to introduce a free energy corresponding to a semiclosed system \[37\]. It means that ATP molecules are free to exchange with a reservoir at the chemical potential of $\mu_{\text{ATP}}$, which is determined by ATP concentration \( \phi \) in the reservoir (See Section II in SM \[38\]). Thus, the dimensionless free energy for the reference system becomes:

\[ \beta F_{\text{ref}} = \frac{\phi}{N} \ln \phi + \frac{(1-\phi-\phi)}{N} \ln (1-\phi-\phi) + \phi L \ln \phi - \phi L \mu_{\text{ATP}}, \]

where $\lambda = \frac{n_{\text{ATP}}}{n_{\text{ARG}}}$ is the ratio of the number of ATP molecules and that of FUS residues. According to the latest study by Kang et al \[31\], the main interaction between ATP and FUS is the binding between ATP and ARG residues. Therefore, there are two types of bonds in the solution: TRY-ARG and ATP-ARG bonds, which characterize the FUS-FUS and ATP-FUS interactions, respectively. Given the fact that ATPs are amphiphilic molecules, each ATP molecule can provide up to two sites for binding with ARG residues (i.e., $L = 2$). The partition function due to the formation of $k$ TRY-ARG bonds and $b$ ATP-ARG bonds can be written as:

\[ Z_{\text{binding}} = \sum_{n_{\text{ATP}}} C_{n_{\text{m1}}}^{k} C_{n_{\text{ATP}}}^{b} L C_{n_{\text{m2}}}^{k+b} \exp(k \beta_1 + b \beta_2). \]

$n_{\text{ATP}}$ is the number of ATP molecules in the solution.

The binding free energy is given by:

\[ \beta F_{\text{binding}} = \frac{m_{1} \phi}{N_{1}} \left[ p \ln p + (1-p) \ln (1-p) \right] + L \lambda \phi (q \ln q + (1-q) \ln (1-q)) \]

\[ + \frac{m_{2} \phi}{N_{1}} \left( 1 - \frac{pm_{1}}{m_{2}} - \frac{qL\lambda N_{1}}{m_{2}} \right) \]

\[ \ln \left( 1 - \frac{pm_{1}}{m_{2}} - \frac{qL\lambda N_{1}}{m_{2}} \right) \]

\[ - \frac{p \phi}{N_{1}} \left( \ln \frac{m_{1} \phi}{N_{1}} + \beta \epsilon_{1} \right) \]

\[ - qL \lambda \phi \left( \ln \frac{m_{2} \phi}{N_{1}} + \beta \epsilon_{2} \right), \]

where $q = \frac{b}{L \lambda \phi N_{1}}$ is the fraction of ATP participating in ATP-ARG binding. Therefore, the total free energy becomes $F_{\text{tot}} = F_{\text{ref}} + F_{\text{binding}}$. It can be seen that $p$ and $q$ are coupled in Eq.(2), indicating the correlation between ATP-FUS and FUS-FUS interactions. To obtain phase diagrams, more details can be found from Section II in SM \[38\].

When ATPs are introduced into the FUS solution, the inhomogeneous distribution of ATP happens when phase separation occurs. Theoretical calculation shows that more ATP molecules prefer to stay in the dense phase (see Figure S1 \[38\]), in agreement with experimental observations \[28\]. Figure 2 (a) shows the change of the saturation concentration $\phi_1$ with the increase of ATP concentration. The solid black curve shows that $\phi_1$ decreases first and then increases. This indicates that a small amount of ATP leads to the occurrence of phase transition in the solution with less FUS concentration; but a large amount of ATP results in the phase separation in the solution with more FUS proteins. The inset of Fig. 2 (a) presents the volume fraction of dense phase $\phi_2$, which shows the increase followed by the decrease. The phase-separated region represented by $\Delta \phi = \phi_2 - \phi_1$ increases first and then decreases, indicating less ATP enhancing LLPS and more ATP inhibiting LLPS. To explore the mechanism, we compare ATP with the other case, in which all parameters are the same to ATP except for $L = 1$. Under the condition of $L = 1$, $\phi_1$ increases and $\phi_2$ decreases monotonously in Fig. 2 (a). It is obvious that LLPS will be always inhibited when small molecules with $L = 1$ are added. This result implies the importance of two binding sites provided by ATP molecules. More direct evidence based on the molecular level is given in Fig. 2 (b). The inset of Fig. 2 (b) shows the variation of the ratio of the number of ATP-ARG bonds to that of ATP with the increase of ATP concentration. By comparing the two cases, we find that the ability of ATP to form bonds with ARG residues is almost twice that of small molecules with $L = 1$. It implies that a reasonable amount of ATP must
bind with two ARG residues in the formation of dense phase, which is named as the bridge effect of ATP. Such effect, in turn, effectively decreases the TYR-ARG binding as a driving force leading to the phase separation, as shown in the main graph of Fig. 2 (b). Thus, the behavior of ATP binding with two ARG residues provides the positive cooperation with TYR-ARG binding in the formation of protein network. However, the bridge effect depends on the ATP concentration, which disappears with the inclusion of large amount of ATP due to the limited number of ARG residues. Hence, in the case of large ATP concentration, ATP molecules start to bind with one ARG residue or stay free, similar to small molecules with $L = 1$. Under this circumstance, the formation of the protein network via TYR-ARG binding is inhibited, in which the influence of large amount of ATP is called as the negative cooperation with TYR-ARG binding. To vividly reflect the influence of ATP depending on its concentration, a schematic representation is given in Fig. 3.

We note that not only ATP, but also RNA and ssDNA have the similar effect on LLPS [30][31]. It is thus useful to study a larger parameter space to explore more general conclusions. First, from the perspective of protein-protein interaction, a phase diagram in the $\phi-\beta\epsilon_1$ plane at various $\varphi$ is given in Fig. 4 (a). The larger binding energy between protein residues, the wider the phase-separated regime occurs. Thus, the phase diagram has a lower critical point. When a small amount of small molecules is added, the binodal curve moves downward, implying the enlarged phase-separated region. With the increase of small molecules, the binodal curve goes up, and the phase-separated region narrows down. The nonlinear shift of the binodal curve at different $\varphi$ clearly indicates that the enhancement and inhibition of LLPS depend on the concentration of small molecules. To further quantify such an influence, we introduce $S = \Delta \phi - \Delta \phi_0$, where $\Delta \phi$ and $\Delta \phi_0$ are the volume fraction difference between dense and dilute phases for conditions with and without small molecules respectively. $S > 0$ indicates that the influence of small molecule is enhancing LLPS, while $S < 0$ reflects the inhibition. Furthermore, the value of $S$ reflects the extent of enhancement or inhibition. Figure 4 (d) shows the change of $S$ in different conditions corresponding to Fig. 4 (a). For two cases of $\varphi = 0.002$ and 0.02, $S$ shows an increase before $\beta\epsilon_1 = 1.6$ then decreases. As shown in Fig. 4(a), when $\beta\epsilon_1 < 1.6$, $\Delta \phi_0 = 0$ (i.e., there is no phase separation) in the pure protein solution. However, when $\beta\epsilon_1$ is larger than 1.6, the value of $\Delta \phi_0$ will increase with increasing $\beta\epsilon_1$, and there may exist a change from the enhancement ($S > 0$) to inhibition ($S < 0$), depending on the interplay between small molecule-protein interaction and protein-protein interaction. For the condition with a large amount of small molecules, phase separation is always inhibited, because the crosslinks of protein network via TRY-ARG bonds are weakened owing to the bond formation between one small molecule and one ARG residue. Second, with the continuous variation of the concentration of small molecules, we obtain a loop-like phase diagram in Fig. 4 (b). For the case of $\beta\epsilon_1 = 1.2$, a closed-loop comes into being, since no phase separation occurs at $\varphi = 0$. With the increase of small molecules, the phase-separated region first becomes larger and then shrinks in cases of $\beta\epsilon_1 = 1.2$, 1.6 and 2.0. For $\beta\epsilon_1 = 2.4$, the phase-separated regions always reduce with increasing $\varphi$. Accordingly, Fig. 4 (e) exhibits a nonmonotonic variation with the ATP concentration in the former three cases, namely $S$ first increases and then decreases. The maximum $S$ implies the existence of an optimal concentration of small molecules characterizing the strongest enhancement. It can be seen
that the effect of small molecule on LLPS is always enhancement for small $\beta\epsilon_1$, such as $\beta\epsilon_1 = 1.2$, while inhibition for large $\beta\epsilon_1$, like $\beta\epsilon_1 = 2.4$. Only when $\beta\epsilon_1$ is medium, its influence includes both enhancement and inhibition. Last, from the view of the interaction between small molecule and protein, Fig. 4 (c) shows the diagram as a function of $\beta\epsilon_2$. At $L = 1$, the phase separation is inhibited with the increase of $\beta\epsilon_2$. This is because small molecule–protein binding from the beginning competes with protein–protein binding to become the dominate interaction due to the increase of $\beta\epsilon_2$, causing that protein network becomes more and more difficult to form since ARG residues are occupied by small molecules with $L = 1$. Once small molecules can bind with two or more protein residues, the case is completely opposite. As the cases of $L = 2$ and 3 show, the phase separation is enhanced with the increase of $\beta\epsilon_2$. Under those cases, small molecule can bind with more than one ARG residues, which plays the bridge effect and works as the additional driving force leading to the formation of protein network. Figure 4 (f) further verifies the complete different influences depending on whether the bridging effect of small molecules exists or not.

In conclusion, we develop a minimal multi-scale theory, which links the molecular interaction and macroscopic phase diagram directly. We confirm a feasibility scheme of regulating LLPS by introducing small molecules. The regulation can be achieved by the introduction of additional specific interaction comparable to protein–protein interaction, where the positive or negative cooperation may lead to the enhancement or inhibition of LLPS. The bridging effect resulting from small molecules binding with several protein residues is the key to the regulation. Although it is a classic mean field theory in polymer physics, it still can be used to explain the complex mechanism resulting from the competition between different multivalent interactions, to obtain phase diagrams in a simple and clear way, and to show its strong vitality in the field of intercellular phase separation. By the virtue of the simplicity and instructiveness, it is an effective way to adapt classical theories to new fields.

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