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Liquid-liquid phase separation (LLPS) in synthetic biosystems

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ABSTRACT

In living systems, there is emerging evidence that nature uses liquid-liquid phase separation (LLPS) to organize diverse cellular processes such as signal transduction, translation regulation, and gene expression among chemical chaos. Inspired by the naturally occurring LLPS, there is increasing interest in the deployment of LLPS in synthetic biosystems towards a wide range of applications. Although much progress has been made, there is still a limited understanding of LLPS in synthetic biosystems. Importantly, studies in LLPS in non-living systems (i.e., polymer systems) and in living systems have been progressed separately. There is an urgent need to summarize and integrate our current understanding of LLPS in different systems to inform the design of artificial LLPS in synthetic biosystems. In this review, we first summarize the development of theoretical modeling of LLPS in non-living systems and living systems. We then explore current approaches for the construction and functionalization of LLPS in synthetic biosystems. We finally review the state of the art of LLPS in synthetic biosystems towards applications in synthetic biology, cellular engineering and biotechnology.

1. Introduction

To spatiotemporally organize simultaneous biochemical reactions by compartmentalization in living cells is fundamental to life. One way to achieve this goal is to subdivide the cellular interior via membrane-bound vehicles. Intriguingly, such cellular compartmentalization sometimes is membrane-less, formed by liquid-liquid phase separation (LLPS). To date, LLPS has been widely observed and studied in various organelles, such as P bodies, germ granules, Cajal Bodies, and PML bodies, enabling space- and time- resolved regulation of a variety of cellular processes including RNA metabolism, ribosome biogenesis DNA damage response, signal transduction, gene expression, and stress response [1–4]. Besides regulation of many key biological reactions, LLPS were also found to be responsible for pathological protein aggregation [5]. LLPS not only creates spatiotemporal increase of concentration of certain proteins [6,7] but also entails localized increase in liquid viscosity, [8] both of which dramatically alter the reaction-diffusion system, thereby enabling/disabling the biological functionalities, stress response and gene expression pathways [9]. Moreover, reactions and functions enabled by LLPS can be switched on and off very rapidly via the formation and dissolution of the LLPS [10,11]. Importantly, in living cells, such LLPS is an actively regulated process accompanied with energy consumption, which could be controlled by transcription and various posttranslational modifications [12].

One prevalent driving force of LLPS in living systems is found to be multivalent interactions among proteins and RNA molecules, [13–15] which in contrast is rarely observed in complex coacervation in

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non-living system (e.g., polymer solution). To prevent gelation or pathological fibrillization formation by strong multivalent interactions, the multivalent interaction domain is usually linked to proteins containing intrinsically disordered regions (IDR). IDR is believed to be another key factor for LLPS formation [16–18]. In addition to IDRs, nature also employs proteins with multiple folded domains as building blocks to drive LLPS. These phase-forming proteins are referred to as scaffold proteins [15,19]. The scaffold proteins often preferentially recruit specific proteins and RNAs, namely client molecules, which define the function of the phase separated biomolecules [15,19]. The modularity of LLPS in living systems offers opportunity for bioengineering. Different biomacromolecules (e.g., naturally derived IDRs, repetitive folded interaction domains, and de novo designed protein polymers) have been utilized as phase-forming scaffolds [20–22]. Various biomolecules of interest have been enclosed within the phase-separated proteins by using IDR tags, interaction peptide tags, enabling LLPS with novo functions [23,24], leading to LLPS in synthetic biosystems.

Notably, LLPS in living systems (e.g., cells) is often referred to membrane-less organelles or biomolecular condensates, while LLPS in non-living systems (e.g., polymer solution) is often referred to as complex coacervation. A popular system for complex coacervation is a proteins-polysaccharide system such as whey protein and gum arabic [25,26]. Compared to membrane-less organelle, complex coacervation is mainly mediated by electrostatic interactions. It should be noted that LLPS which was termed as complex coacervation has been observed in food industry (e.g., proteins and anionic polysaccharides) decades ago, in which the factors that influence complex coacervation have been investigated extensively and systematically [27].

LLPS in both living and non-living systems share the same thermodynamic principles. Therefore, theoretical studies of LLPS in non-living systems could offer valuable insights for understanding LLPS in living cells, while the latter provide new ideas for designing LLPS in non-living systems. However, living and non-living systems exhibit several key differences in their LLPS: Firstly, the determinative driving force of complex coacervation is electrostatic attraction between opposite charges, which is significantly different from the dominate multivalent interactions in cells. Secondly, strong non-covalent interactions often result in gelation by supramolecular fiber formation in cells, while is often lead to large irregular aggregation in polymer solutions [28]. Thirdly, LLPS in living cells is actively regulated with energy consumption, allowing for LLPS to occur far from equilibrium state. In contrast, LLPS in polymer solutions is a passive process without energy consumption that occurs at thermodynamic equilibrium.

Importantly, the synthetic biosystem is an intermediate between living and non-living systems (Fig. 1). Therefore, the understanding and applications of LLPS in synthetic biosystems can be inspired by LLPS in both living and non-living systems. Conversely, biologically functional entities in living cells, such as RNA granules, can be synthetically reconstituted in a biosynthetic system to elucidate the biochemical and biophysical properties of LLPS in living cells [29]. Recent studies have attempted to design and construct tailor-made synthetic versions of biomolecular condensates for applications in various applications. Examples range from protein condensates designed to serve as microreactors by concentrating enzymes and substrates, [11,30] to condensates engineered to sequester and tailor cellular functions, [31] and condensates designed to probe cellular process in a spatial and temporal manner [32]. So far, LLPS in synthetic biosystems has been reproduced by tuning the interactions between diverse synthetic, multivalent macromolecules (e.g., multi-domain proteins and RNA) [33]. It is also noteworthy that there are phenomena that have only been observed in synthetic biosystems but not in the other two systems. For example, a transition from LLPS to micellization was achieved by changing the α-helical homo-polypeptides to co-polypeptides [34]. By focusing on LLPS in synthetic biosystems, we believe this review will provide a unique perspective for understanding LLPS in different systems (e.g., living, non-living and biosynthetic systems), and for applying LLPS for a much broader range of applications.

In this review, we focus on the current research progress of LLPS in synthetic biosystems. We first elaborate on theories that explain the fundamentals of LLPS in both living and non-living systems, limiting our focus on the factors that drive LLPS. We also discuss the major differences in theoretical modeling between the two systems. In the following, we summarize strategies and recent advances in constructing LLPS in biosynthetic systems. The potential applications of LLPS using

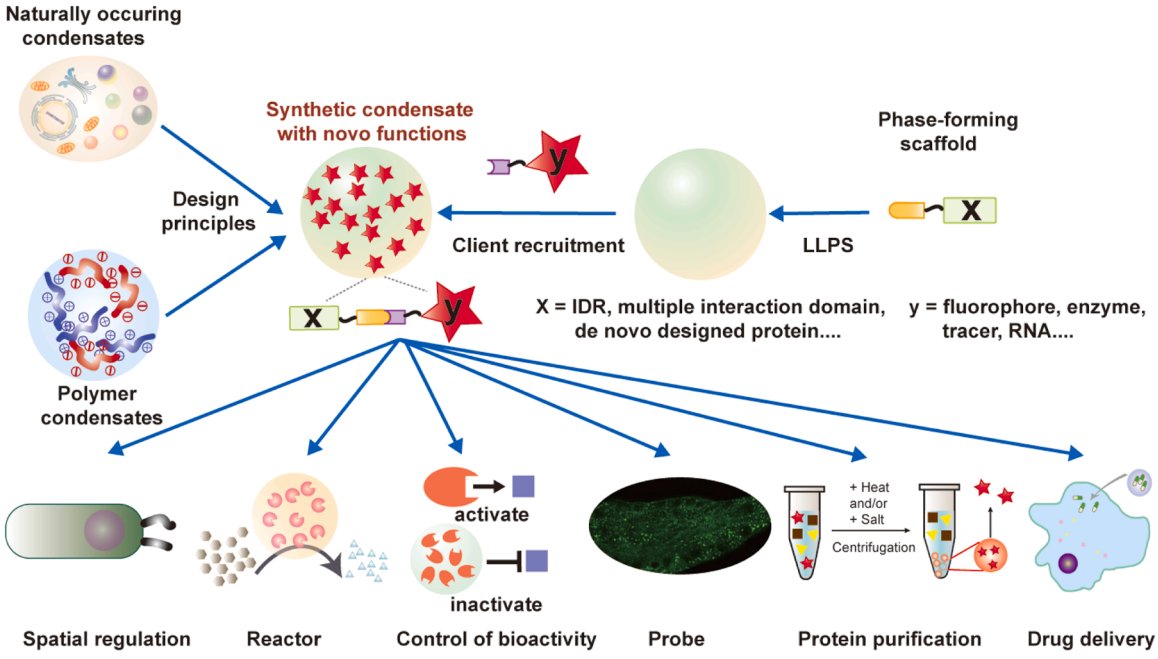

Fig. 1. Design and construction of biomolecular condensates in synthetic biosystems. Our understanding of LLPS both in living systems and non-living system guides the design of synthetic condensates with novo functions consisting of phase-forming scaffolds and specific biomolecules. Synthetic condensates formed through LLPS are promising next-generation smart materials towards applications in biotechnology, synthetic biology and cellular engineering.

biosynthetic molecules are discussed. Finally, we provide the remaining key questions that have yet to be addressed and potential future directions for LLPS in biosynthetic systems.

2. Theoretical framework for LLPS

2.1. Phase separation at equilibrium

Mechanisms that drive LLPS in biology are grounded in physical interactions. They can be theoretically modeled with less complex systems consisting only a few components. For example, components of cellular bodies such as P bodies and PML nuclear bodies can be divided into scaffolds and client molecules, in which only scaffold molecules are responsible for LLPS [15]. Unsurprisingly, LLPS follows the same solution thermodynamic principles with all the other types of phase separation. For a system composed of components with different phase transition temperature, there will inevitably be new phase(s) formed inside of existing phase, by decreasing the temperature. The simplest case is an ideal binary system (Phase diagram shown in Fig. 2a, with T_1 and T_2 as the melting point for A and B, respectively) for which both liquid and solid solution are assumed to be ideal solution.

For a close system consists of n_A A and n_B B molecules, the free energy density for mixing of such ideal solution can be calculated with

$$\frac{\Delta G_m^{id}}{V} = \frac{kT}{v_0}(x_A \ln x_A + x_B \ln x_B)$$

where $x_A = \frac{n_A}{n_A+n_B}$ and $x_B = \frac{n_B}{n_A+n_B}$ is mole fraction of component A and B. In such ideal binary system, phase transition can be generated by reducing temperature or varying concentration of one component at certain temperature. However, in reality, ideal or near ideal solution rarely exists.

The interaction between two components needs to be considered, which yields a modified expression for free energy

$$\frac{\Delta G_m}{V} = \frac{kT}{v_0}(x_A \ln x_A + x_B \ln x_B + x_A x_B \chi)$$

where $\chi = \frac{1}{2kT}(2\epsilon_{AB} - \epsilon_{AA} - \epsilon_{BB})$ is the Flory-Huggins interaction parameter between A and B, where ϵ_{AB}, ϵ_{AA}, and $\epsilon_{BB}$are the pairwise interaction energy between adjacent lattice sites occupied by the two species [35]. When $\chi < 0$, the interaction is attractive, and $\chi > 0$ means that the interaction is repulsive, which may lead to phase separation at low temperature (e.g., $\chi > 0$ for solid solution and $\chi < 0$ for liquid solution shown in Fig. 1b). In a polymer solution, if we consider solvent (i.e., commonly small molecules) as component A and solute (i.e., (bio) polymers) as component B, then the degree of polymerization of B, N, need to be included to capture the volume difference between A and B. The free energy of such binary system (i.e., polymer solution) can be further described by Flory-Huggins theory [36].

$$\frac{\Delta G_m^p}{V} = \frac{kT}{v_0}(\phi_A \ln \phi_A + \frac{\phi_B}{N} \ln \phi_B + \phi_A \phi_B \chi)$$

where $\phi_A = \frac{n_A v_0}{n_A v_0 + n_B N v_0}$ and $\phi_B = \frac{n_B N v_0}{n_A v_0 + n_B N v_0}$ is volume fraction of A and B. Volume fraction is more convenient than the mole fraction because polymer has long chain length that makes the mole fraction of polymer tiny. It is generally agreed that $\chi < 1/2$means A is a good solvent for B, while $\chi > 1/2$ means A is a poor solvent, which leads to LLPS of two coexisting phases (polymer rich phase and polymer poor phase) at lower temperature, as shown in Fig. 2c. To examine the stability of a binary system, higher order derivatives of G need to be determined. In particular,

$$\left(\frac{d^2 G_m}{dx^2}\right)_{T,P} < 0$$

Above equation leads to amplification of any small fluctuations in thermal energy, and thus phase separation. To this end, all the points that satisfy $\left(\frac{d^2 G_m}{dx^2}\right)_{T,P} = 0$ is plotted as the boundary of spinodal decomposition (i.e., dash line in Fig. 2c), while the chemical potential of each phase at two different composition is identical, $\mu_i^\alpha = \mu_i^\beta$, (a common tangent line of $\left(\frac{dG_m}{dx}\right)_{T,P,\alpha} = \left(\frac{dG_m}{dx}\right)_{T,P,\beta}$) is plotted as the binodal line (i.e., solid line in Fig. 2c) [37]. Importantly, the area inside spinodal line is unstable where the phase separation happens spontaneously, while the area between binodal and spinodal line is metastable and a new phase is formed through the mechanism of nucleation and growth. It is noteworthy that Flory-Huggins theory fails to predict the lower critical solution temperature (LCST) observed in polymer blends, [38] such as polystyrene/acetone [39].

In a ternary system composed of one solvent (A) and two types of polymers (B and C), such as a classical complex coacervation system - proteins and polysaccharides in water - there will be three different Flory-Huggins interaction parameters: χ_{AB},χ_{AC} and χ_{BC}, which represent the two solvent-polymer interactions and one polymer-polymer interactions. Generally, if the overall destabilization force overcomes the stabilization force, phase transformation/separation will happen.

If the force between polymer B and C is repulsive (i.e., $\chi_{BC} > 0$) and the two solvent-polymer interactions are preferred over polymer-polymer interaction (i.e., $\chi_{AB} < \chi_{BC}$ and $\chi_{AC} < \chi_{BC}$), then segregative phase separation will form. In contrast, if the polymer-polymer interaction shows strongest attraction (i.e., $\chi_{BC} < 0$, and $\chi_{AB} > \chi_{BC}$, $\chi_{AC} > \chi_{BC}$), then associative phase separation will occur [27]. Different from segregative phase separation where two polymers concentrate in two phases separately, in associative phase separation, there is a polymer-rich phase that has both polymers and a dilute polymer-poor phase. Notably, complex coacervation is thermodynamically similar to

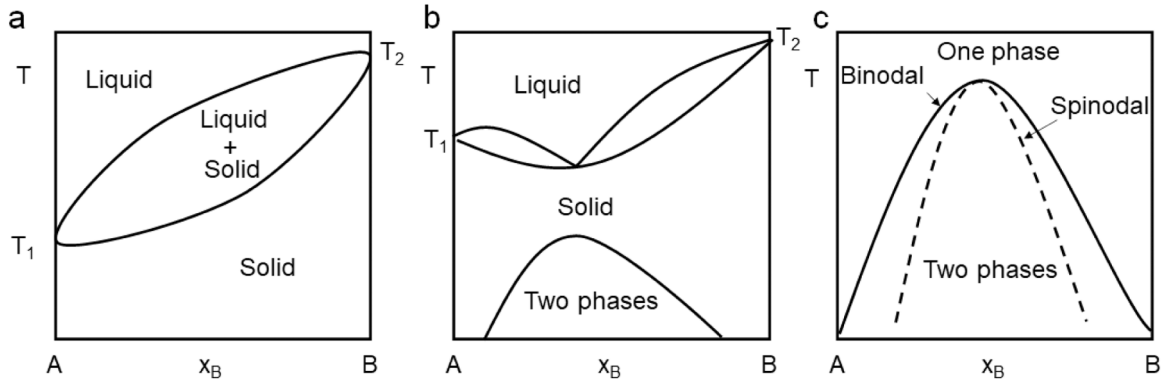

Fig. 2. Phase diagrams that are formed depending on component interactions. (a) Ideal binary solution; (b) Binary solution with $\chi_{AB} > 0$; (c) Spinodal decomposition under the dashed line.

a mixed solvent system (e.g., water and another alcohol) with one polymer, that is, a ternary system composed of solvent A, solvent B and polymer C, but with the same relationship, $\chi_{BC} < 0$, and $\chi_{AB} > \chi_{BC}$, $\chi_{AC} > \chi_{BC}$ [40]. The LLPS of such ternary system yields a polymer-poor phase with solvent A and a polymer-rich phase with solvent B.

Inspired by the complex coacervation in gum arabic and gelatin system, [41] theoretical model for such system composed of two oppositely charged polymers with one or more salts in water was developed by Voorn and Overbeek, [42] in which the free energy can be given by [37].

$$\Delta G_m^{VO} \sim kT\left(\sum_i \chi_i \phi_i + \sum_i \sum_{j>i} \chi_{ij}\phi_i\phi_j - \alpha\left[\sum_i \sigma_i \phi_i\right]^{3/2}\right)$$

The first two terms on the right represent Flory-Huggins interactions, which apply to polymer solution in general. The third term come from the theory of Debye and Hűckel, where $\alpha = 3.5$ for water [43]. As the third term include electrostatic interactions, the influence of salt concentration on phase separation (i.e., coacervation) can thus be evaluated (Fig. 3) [44].

Importantly, the phase separation in Voorn-Overbeek model is assumed to be LLPS, which forms by associating two oppositely charged polymers with solvent molecules trapping inside. The solvent molecules in conservation phase are essential, responsible for free arrangement of both polymers to increase the overall entropy, thereby resulting in liquid and enabling the reversibility of the coacervation. It is noteworthy that Debye and Hűckel model only considers weak interactions between electrolytes in dilute solution and is also limited by low salt concentrations [28,46,47]. Based on Voorn-Overbeek model, several models have been developed, such as Veis-Aranyi theory, [48] Nakajima-Sato theory [49] and Tainaka theory, [50,51] together with separately developed theory such as random phase approximation theory (RPA) [52–54]. After 1980, the theoretical efforts can be classified into three directions: scaling theories, field-theory-based analytical theories and liquid-state theories [55].

2.2. Factors that influence spinodal decomposition

Various models have been developed to overcome the limitations of previous general models by considering specific interactions using modified terms in equations. However, the more complex a system is, the less accuracy a theoretical model trying to quantitatively represent the system will have. Therefore, researchers should mainly utilize mathematical models as theoretical framework to shed light on experiment design and to help qualitatively understand the trend for each parameter that yields spinodal decomposition, rather than making quantitative prediction. For the simplest case for LLPS, the number of local minimum/maximums of $G(x)$ is 3 (as shown in Fig. 4 top panel).

However, such number could be larger than 3 in many living and non-living systems, yielding a different free energy curve, which in turn makes LLPS a metastable state or even a non-equilibrium state. The non-equilibrium state has been widely noticed in living cells, with intracellular condensates subjected to various posttranslational regulation and

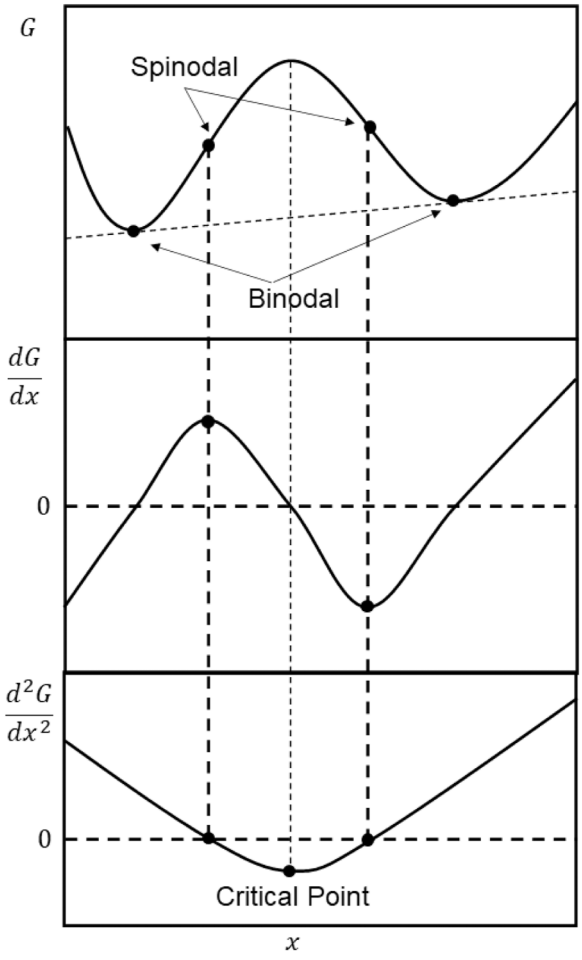

Fig. 4. Free energy versus concentrations in a binary system. The second and third paragraphs should be the second and third derivative.

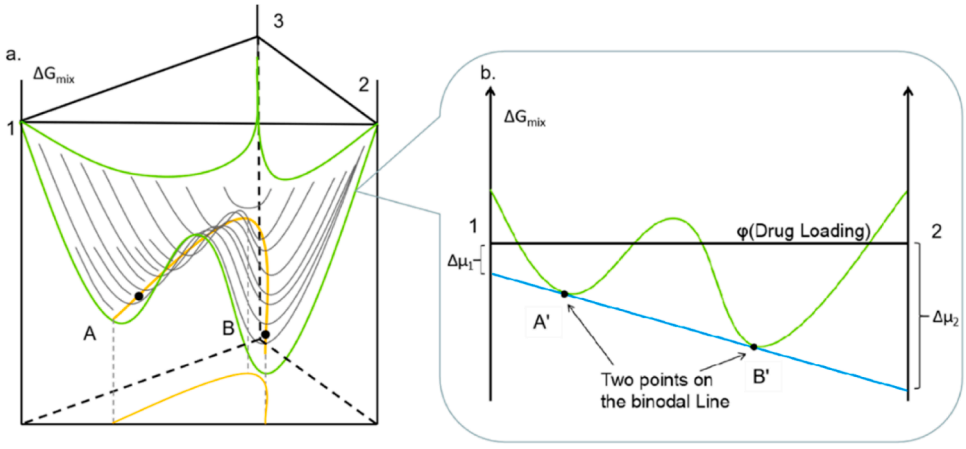

Fig. 3. (a) Free energy in a ternary system (two oppositely charged polymers and one solvent). (b) Free energy profile of a binary system at constant temperature. (a) Adapted from ref. [45] with permission from MDPI, copy right 2019.

adenosine triphosphate-dependent biological activity [12].

In scenario shown in Fig. 4, spinodal decomposition requires free energy to "concave up" between the two concentrations that allows $\left(\frac{d^2G_m}{dx^2}\right)_{T,P}=0$, (i.e., two spinodal points), and $\left(\frac{d^2G_m}{dx^2}\right)_{T,P}<0$ between these two concentrations. To achieve this, it is essential that factors should be delicately balanced at two spinodal concentrations, while factors that promotes demixing (aggregation) will dominate when polymer concentration lies between the two spinodal concentrations. On one side of the balance, entropy always favors mixing, which can be estimated equation (1) [45,56]. On the other side, there are several options for factors that favors demixing, depending on the system. Common factors to be tuned are, but not limited to, polymer concentration, charge density, molecular weight, polymer configuration and topological structure, salting concentration, stoichiometry and temperature [55].

In a system composed of oppositely charged polymers, the demixing driving force is electrostatic attraction, which can be calculated by the Debye-Hűckel term in Voorn-Overbeek model, which always gives $\left(\frac{d^2G_m}{dx^2}\right)_{T,P}<0$ at different concentration [57]. The electrostatic attraction is determined by charge density (i.e., spacing between two adjacent charged sites) in a straightforward manner. An increase in the charge spacing will decrease the demixing driving force, thereby greatly suppressing the tendency of coacervation formation [58]. Notably, intermolecular electrostatic interactions along polymer influence the coacervation rather than intrachain charge pairing [40]. For biopolymers, charges are heterogeneously distributed along backbone, which is determined by its sequence [59]. Even though charge heterogeneity could hardly be calculated using theoretical model, [60] it plays a key role in coacervation [61]. Such charge heterogeneity effect on coacervation behavior was investigated using polypeptide solution in which charge monomer sequence was precisely controlled via solid-phase synthesis. [62] It was found that the sequence effect yielded differences in entropic confinement of condensed counterions along the polymer, and entropy dominated coacervation while enthalpic contributions were negligible.

The effect of salt in charged polymers solution can be complicated, because three additional interactions are introduced into the system (i.e., cation-anion, cation-polyanion, anion-polycation). In general, increase in salt concentration will bring the composition of the two coexistent phases close together (Fig. 3). This was due to the decrease of gain in free energy for demixing when salt is present [42]. The addition of trace amount of salt will promote coacervation formation [63]. But at sufficient high salt concentration, coacervation will even be completely suppressed [64]. Salt resistance was found to increase as polymer molecular weight increased. Moreover, the effect of pH on LLPS is usually discussed together with pKa of each polymer, so that polymers are full charged [65]. There exist specific pHs where polymer complex formation is initiated (pH_c) and where coacervation occurs (pH_φ). [66] In addition, pH_c is independent of polymer/polymer concentration ratio while pH_φ varies inversely with it.

In short summary, because entropic force always favors mixing and yields $\left(\frac{d^2G_{mix}}{dx^2}\right)_{T,P}>0$, to create the "concave up" in $G(x)$ as shown in Fig. 4, the general strategy of generating LLPS is to introduce forces that favors demixing, which results in $\left(\frac{d^2G_{demix}}{dx^2}\right)_{T,P}<0$ for certain range of concentration. Such demixing force should also be large enough to overcome the entropic force, so that the overall $\left(\frac{d^2G_m}{dx^2}\right)_{T,P}=\left(\frac{d^2G_{mix}}{dx^2}\right)_{T,P}+\left(\frac{d^2G_{demix}}{dx^2}\right)_{T,P}=0$ at two separated spinodal concentrations. In a system composed of oppositely charged polymers, the electrostatic attraction is the major demixing force and can be tuned by various factors such as molecular length, charge density, salt concentration, stoichiometry and pH. In other system in which the demixing force is determined by nonelectrostatic interactions, including H-bonding, hydrophobic-hydrophobic interaction, coordination interaction or other types of specific bonding, the demixing force can be estimated based on Flory-Huggins interaction.

2.3. Liquid-liquid vs liquid-solid (or liquid-gel) phase separation

It should be noted that LLPS is fundamentally distinct from liquid-solid phase separation (LSPS), even though both could form in a similar phase separation process. In LLPS, the attractive interactions between the molecules are relatively weak and transient, thereby allowing dynamic and continuous exchange of the molecules in LLPS with its surroundings within seconds to minutes [67,68]. The LLPS also exhibit liquid-like behavior such as fusion upon contact [8,69,70]. In contrast, LSPS is entailed by strong directional non-covalent bonding, which yields the solution gelation. The liquid to gel phase transition is also called maturation or hardening [71–75]. The molecules are immobilized within the assembled structure. Such rheological difference between LLPS and LSPS is often characterized by particle-based microrheology [76].

Membrane-less organelles are known to be formed by LLPS. An increasing number of reports suggest that membrane-less organelles have viscous liquid-like features, such as spherical shapes, coalescence into large droplets upon contact with one another, flowing in response to applied shear stress, wetting and dripping [77]. It is the liquid state that plays a central role in the diverse structural and functional properties in living cells, entailing coalescence of nucleoli, reversible assembly of granules and enhancement of enzymatic performance [4,14,72,78]. However, all the theoretical models mentioned above are only responsible for spinodal decomposition. In other words, the free energy calculation could suggest the occurrence of phase separation but cannot inform whether LLPS or LSPS will form. How to induce LLPS while avoiding LSPS is still challenging.

LLPS to LSPS transition is often recognized as pathological protein aggregation in cells [79]. For example, a neuropathological hallmark of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Alzheimer disease (AD), frontotemporal degeneration (FTD) and Parkinson's disease (PD) is the appearance of poorly soluble protein aggregates and the level of these protein deposits often correlates with degree of neurovegetative diseases [80,81]. Microtubule-associated protein Tau (also known as MAPT) is aggregated as neurofibrillary tangles in the affected neurons of patients with AD or FTD [82,83]. In addition to neurodegenerative diseases, LLPS to LSPS transition also play a vital role in cancer. Recent study has shown that formation of AKAP95 condensates with proper material properties is essential for the activity of AKAP95 in splice regulation, which ensures sufficient production of key transcripts for cancer and supports cancer cell growth [84]. Particularly, the formation of liquid-like AKAP95 condensates concentrate the factors involved in RNA metabolisms in high local concentration and allow for optima activity for splice regulation of cancer-related targets, leading to tumorigenesis. In comparison, hardened condensates retard the reactions by restricting the movement and interactions of the factors as well as RNA substrates. In addition to supporting tumorigenesis, LLPS is also essential for ensuring the activity of cancer suppressor proteins. SPOP (speckle-type BTB/POZ protein) is a tumor suppressor serving as substrate adaptor of a cullin3-RING ubiquitin ligase (CRL3) which ubiquitinates various pro-oncogenic proteins for the following proteasomal degradation [85–88]. It has been shown that the substrate binding triggered LLPS of SPOP is essential for the colocalization of SPOP and its substrates in membraneless organelles in cells [89]. Mutations of SPOP have been found in solid tumors including prostate, gastric and colorectal cancers [90,91]. Cancer-associated SPOP mutations which disrupt the interactions between substrate and SPOP also abolish the SPOP phase separation, resulting in a failure of the SPOP to co-localize with substrates and consequently reduced protein ubiquitination of substrates. Accumulation of proto-oncogenic substrates can then promote cancer cell growth. Recent studies have also revealed that

LLPS is involved in infectious diseases. The COVID-19 pandemic is caused by a novel coronavirus, namely severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [92]. There is emerging evidence that multiple functions of N (nucleocapsid) protein of SARS-CoV-2 such as facilitation of genome processing, driving of virion assembly, and suppression of host immune responses is achieved through the LLPS [93–96]. Unphosphorylated N protein has been shown to phase separate with viral RNA into gel-like condensates, which is suited for nucleocapsid assembly [93,96]. In comparison, phosphorylation of N protein converts the gel-like condensates to highly dynamic, liquid-like condensates, which can facilitate viral genome processing [93,96].

The key question to ask is: what are the driving forces that only yield LLPS, but not LSPS? It is also reported that high biopolymer concentration leads to insoluble amyloid fibrils (LSPS), while low biopolymer concentration results in liquid-like condense (LLPS) [97,98]. For example, when the concentration of polyQ peptides increases above a first threshold value, spherical liquid-like assemblies of 10–50 nm in diameter condense and LLPS forms [97]. When the concentration increases above a second threshold value, LSPS forms with generation of amyloid fibrils. However, in our recent work, we noticed that, when strong non-covalent interactions are presented between oligopeptides, the increase of oligopeptide concentration will result in intermolecular pi-pi stacking first, then β sheet to fibril formation, and finally sol-gel transition of the whole solution, without having any LLPS throughout the whole process [99]. To this end, we believe that weak, transient non-covalent interactions may lead to LLPS, while strong non-covalent interactions yields LSPS or even global sol-gel transition (Fig. 5).

In contrast to H-bonding or other multivalent interactions in biosystems that are stable regardless of solution conditions, electrostatic attraction could be largely tuned or weakened by screening of potentially interacting of charged polymers though ion pairing with slat ions [100]. When salt concentration is very low in solution, precipitate (solid phase) will be generated due to the strong electrostatic attraction. With sufficiently high salt concentration, coacervation (i.e., LLPS) will form instead of precipitate (Ternary phase diagram shown in Fig. 6) [101]. Therefore, by varying the salt concentration and pH, LLPS can be easily formed in oppositely charged polymers solution without LSPS formation. To the best of our knowledge, unlike biosystems, there is no report claiming that the observed LLPS in oppositely charged polymers solution is a non-equilibrium state that will eventually turn in to more stable LSPS as time increases.

2.4. Beyond equilibrium system

Equilibrium or non-equilibrium state. First of all, it is critical to determine whether the LLPS in synthetic biosystem forms under equilibrium or non-equilibrium state. The mathematical models summarized in Sections 2.1–2.3 are all based on thermodynamic equilibrium condition, which can be applied to most LLPS *in polymeric systems*. However, in contrast, it is hypothesized that LLPS may not even be an equilibrium state *in living systems*. [77] The fluidity of liquid phase is actively kept out of equilibrium by consumption of energy in form of ATP, such as nucleoli, [8] P granules [102] and stress granules. [103] Instead, the equilibrium state is pathological fibril formation and gelation, that is, LSPS. This might help to rationalize why many of the sequence of intrinsically disordered peptides (IDPs) that drive LLPS are also commonly observed in proteins that cause pathological protein aggregation. For example, ALS-associated protein FUS assembles into a liquid-like compartment convert with time into an aberrant aggregated state. [71] Disease-related RBP hnRNPA1 undergoes LLPS into protein-rich droplets, but accompanied by gradual accumulation of insoluble fibrillization. [72] A series of engineered and natural RNA-binding proteins with intrinsically disordered regions can yield LLPS, but become less dynamic over time through LSPS. [74] All above examples point to a speculation that LLPS might merely be a non-equilibrium state in cells, and LLPS to LSPS transition will inevitably transpire if LLPS is not actively and energetically sustained. In short, if LLPS is formed under non-equilibrium condition, factors that affect both thermodynamic equilibrium and kinetical pathways should be carefully tailored concurrently, in order to manipulate the LLPS formation in synthetic biosystems, which could be practically challenging. Moreover, any conclusion on how solution conditions (e.g., concentration, temperature, non-covalent interactions) influence LLPS formation by assuming the system at equilibrium state could be substantially inaccurate if the actual investigated system is at non-equilibrium state.

Mathematical models for kinetic pathways under non-equilibrium state. We keep the focus of Section 2 on equilibrium state because the studies of kinetic pathways under non-equilibrium state are still limited, because the factors that drive the phase

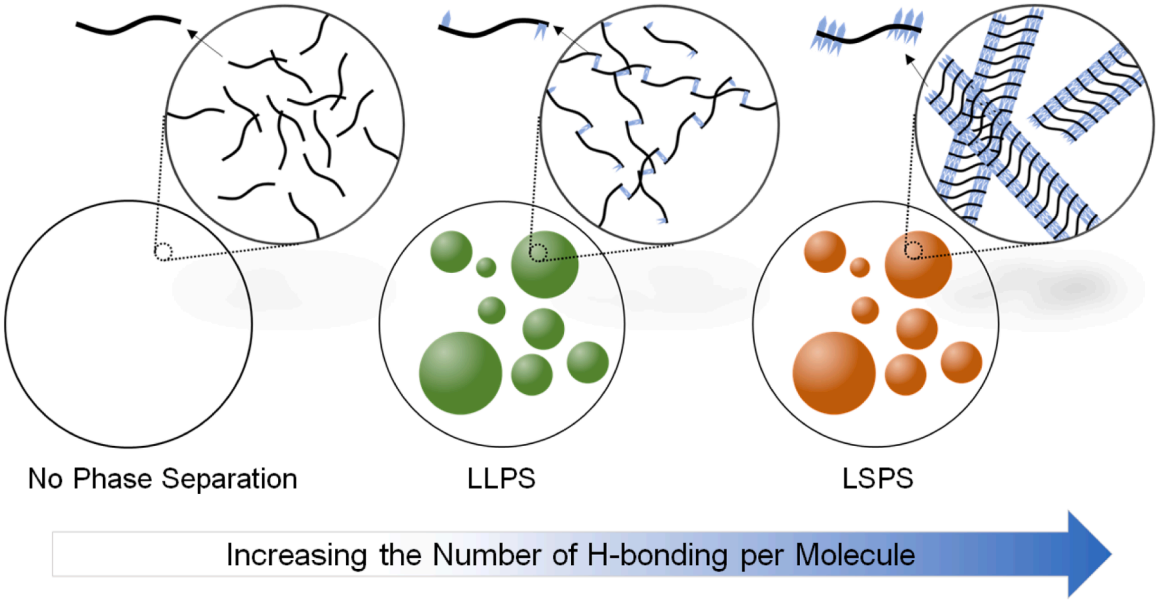

Fig. 5. Spinodal decomposition may lead to LLPS or LSPS, depending on the interaction strength between molecules (intermolecular attractive interaction strength increases from left to right, while concentration stays unchanged).

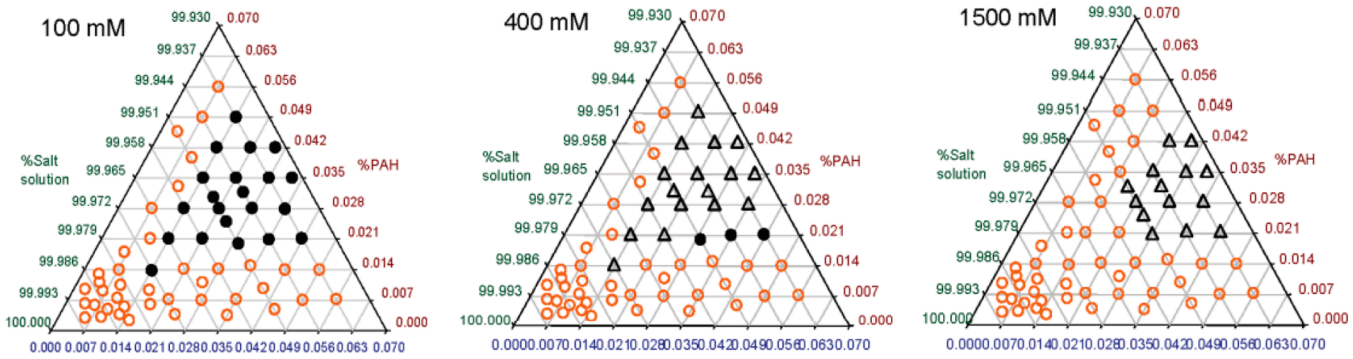

Fig. 6. Ternary phase diagrams of fully neutralized PAA/PAH mixtures (0.05 wt% total polymer concentration) in aqueous with different NaCl salt concentration (100, 400, and 1500 mM) at pH= 8.6.
(a) Adapted from ref. [50] with permission from American Chemical Society, copy right 2010.

separation at equilibrium could also significantly alter the kinetics at non-equilibrium state. [104] For example, in our previous work, we observed that concentration was the cause for phase separation in a synthetic biosystem composed of π-conjugated oligopeptides using a combination of microrheology, confocal fluorescence microscopy, optical spectroscopy, and electron microscopy. [99] However, we have

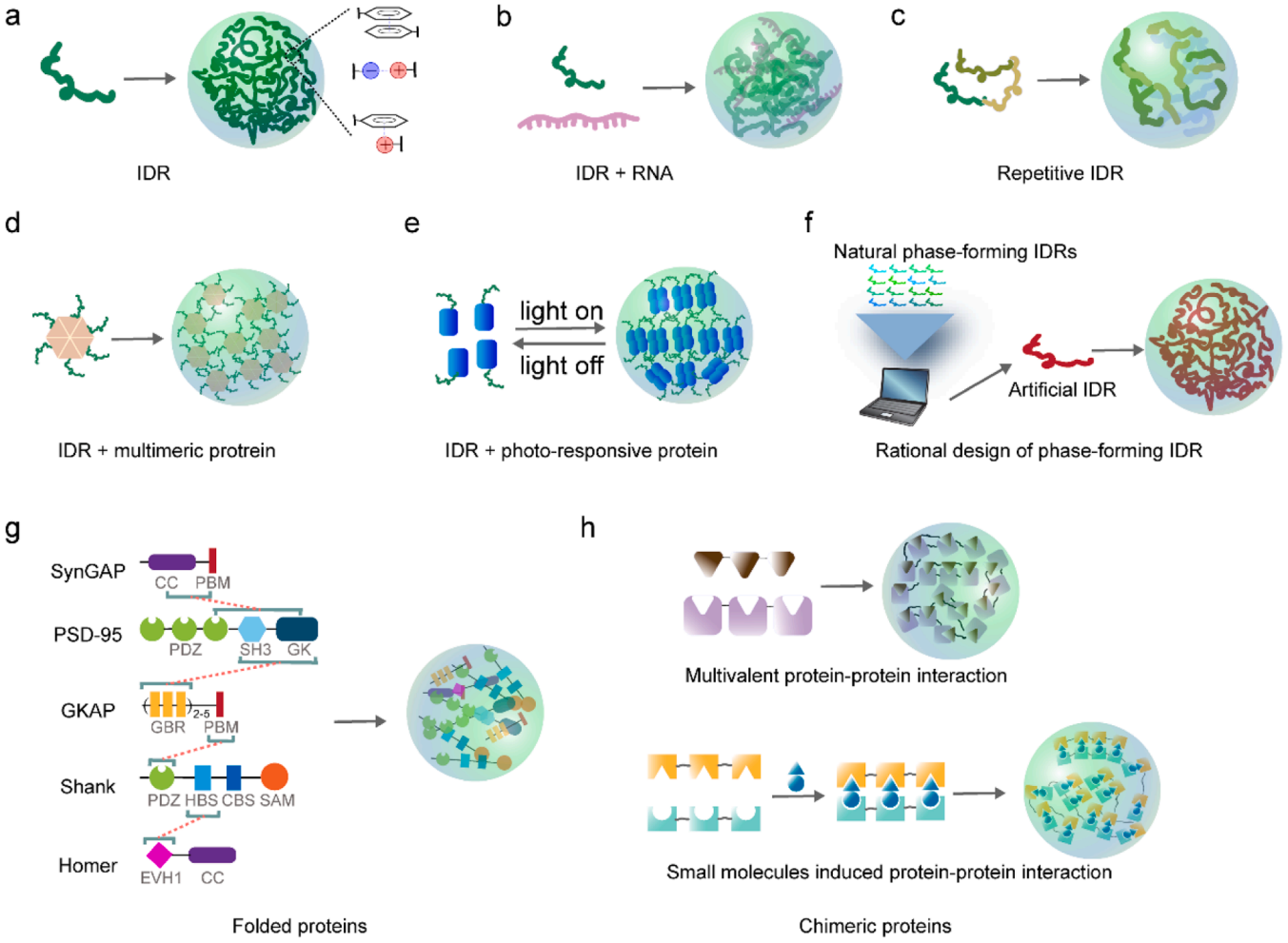

Fig. 7. Formation of LLPS in synthetic biosystems can be achieved using a variety of phase-forming scaffolds. a, IDRs drive phase separation through weak molecular interactions. The zoomed-in portion shows examples of transient molecular interaction (dashed gray) such as pi-pi interaction, electrostatic interaction, and cation-pi interaction. b, In addition to interactions between IDRs, heterotypic interactions between RNA and proteins promote LLPS. c, Tandemly repeated IDR proteins increase the valency of phase-forming scaffolds, enhancing phase separation. d, Valency amplification of IDR scaffold can also be achieved by fusing IDR with multimeric protein. e, A combination of photo-responsive protein and IDR enables a light-switchable phase separation. f, A better understanding of sequence encoded phase behavior of naturally occurring IDRs enables a rational design of artificial phase-forming IDRs. g, Multiple interactions between folded protein drive the formation of biomolecular condensates. Red dashed lines indicate protein-protein interactions. h, Chimeric proteins phase separate through multi-valent interactions between repetitive interaction domains (top) or small molecules dependent protein-protein interactions (bottom).

shown that the balance between reaction and diffusion greatly affected the kinetics of the phase separation process, in which the diffusion flux is also determined by concentration. [105] In addition, Heo et al. discovered that diffusion rate alone may be sufficient to yield the phase separation at non-equilibrium state, by visualizing the kinetics of histone spatial reorganization in tenocytes and mesenchymal stromal cells. [106] In short, the developed mathematical models are mainly slightly modified diffusion equations, which might be over simplified for presenting the biological system.

3. Design and construction of LLPS in synthetic biosystems

Over the past few years, tremendous progress has been made in the development of phase separation in synthetic biosystems, due to a better understanding of the composition, formation, and modulation of LLPS in both living and non-living systems. To simplify the phase separation systems, the components of biomolecular condensates are typically divided into two classes: scaffolds and clients, depending on their contributions to the phase separation [15]. Scaffolds, acting as network hubs, lead the formation of biomolecular condensates through multivalent interactions, while clients are recruited into the condensates via interactions with scaffolds. This modularity enables the design and construction of customized synthetic condensates with new functions.

3.1. IDRs as phase-forming scaffold

3.1.1. IDRs

Native phase-separating proteins often contain IDRs. IDR mediated phase separation can be found throughout the cell such as nucleolus, [107] P-bodies, [108] and stress granules [109]. In contrast to folded proteins, IDRs do not adopt a defined 3D structure and often are enriched in a subset of amino acids, namely low-complexity sequences (LCD). IDRs serve as stickers to drive LLPS through a myriad of molecular interaction types such as pi-pi, cation-pi, dipole-dipole, hydrophobic, charge-charge interactions and hydrogen bonding, [10,110–113] many of which are weak and promiscuous interactions (Fig. 7a). Naturally occurring phase-separating IDRs can be classified into different types according to the sequence composition. Prion-like IDRs such as the IDRs of protein fused in sarcoma (FUS) and TAR DNA-binding protein 43 (TDP-43) compose one archetype of phase-separating IDRs [114–116]. Prion-like IDRs are named because of the sequence similarity to yeast prions and often have a limited number of amino acids types mostly including polar amino acids, such as glycine, glutamine, tyrosine and serine [117]. FUS IDR is probably the most well-characterized prion-like IDR in the phase separation studies. Previous studies have shown that the multivalent cation-π interactions between the tyrosine residues within prion-like IDRs and arginine residues within the FUS RNA-binding domains determines the saturation concentration of FUS phase separation [118,119]. It has also been shown that the material properties of the phase separated FUS is governed by glycine (maintains liquidity) as well as serine and glutamine (promote hardening) [118]. Non-prion-like IDRs such as IDRs of DEAD-box helicase 4 (DDX4) and RNA helicase LAF-1 (LAF-1) represent another class of IDRs [118]. In addition to the polar and aromatic residues, these IDRs are also enriched in charged residues [1]. Recent studies have demonstrated the important roles of the charged residues in driving phase separation [10,118,120]. Not only the presence of charged residues but also the their patterning influence the phase behavior of IDRs: IDRs with evenly distributed charged residues have low propensity to phase separate whereas IDRs containing more or denser clusters of charged residues promotes phase separation [118]. It is now increasingly recognized that the phase-forming properties of IDRs are encoded in their sequences (i.e., composition, patterning, and spacing of amino acids sequence), which dictate the phase behavior and material properties of the resulting biomolecular condensates [59,121].

Importantly, the weak interactions between IDRs alone is often insufficient to drive LLPS under physiological conditions [122]. For example, although the N-terminal IDR region of the RNA-binding FUS is necessary and sufficient for LLPS, the C-terminal of FUS can further promote phase separation by providing the heterotypic interactions between IDRs and RNAs [123]. Indeed, many naturally occurring biomolecular condensates are RNA/protein (RNP) bodies, in which RNAs also act as a phase-forming scaffold to provide additional binding sites for proteins (Fig. 7b) [9,124,125]. Inspired by the native RNP bodies, Simon and coworkers developed a genetically engineered phase-forming protein scaffold in protocell, which consists of a N-terminal disordered elastin-like polypeptides (ELPs) and a C-terminal RNA-binding domain from PGL-1 of *Caenorhabditis elegant* [126]. ELPs are synthetic polymers of repetitive pentapeptide VPGXG, in which X denotes any amino acid sequence except proline [127]. ELPs underwent a low critical solution temperature (LCST) phase transition (phase separation upon increasing temperature) [128]. Addition of RNA was observed to lower the critical temperature. Alternatively, by mimicking the non-specific electrostatic interactions between RNAs and RNA binding proteins, supercharged GFPs and RNAs have been explored for phase separation under biomimetic conditions [129]. However, supplementation of RNAs is not cost-effective and may hinder the use of synthetic biomolecular condensates, which is particularly true for in vitro applications since the RNA is prone to degradation because of the ubiquity of RNase.

3.1.2. Amplifying IDR scaffold valency

Multivalent scaffolds are widely used in the construction of synthetic biomolecular condensates due to the crucial role of multivalent interactions in LLPS. One popular strategy to increase the scaffold valency is to use tandemly repeated IDR proteins (Fig. 7c) [20,23]. *Caenorhabditis elegans* protein LAF-1 is a constituent protein of P-granules, which is comprised of an N-terminal disordered domain enriched in arginine and glycine (RGG domain), a catalytic domain, and a C-terminal disordered domain [130]. It has been shown that RGG domain is necessary and sufficient for phase separation in the absence of RNA but only leads to a weak phase separation [120]. Schuster et al. developed a robust phase-forming system in which LLPS can be achieved under physiologically relevant protein concentration and temperature using repetitive RGG [20]. The degree of phase separation scales with the number of RGG repeats. To control phase separation, protease recognition site or photocleavage cite were incorporated into the RGG phase-forming scaffold [20,131]. In particular, cleavage of a tandem RGG fusion to a single RGG domain led to the dissolution of the biomolecular condensates with the presence of protease or light. Furthermore, FUS is a another widely used phase-forming IDR [17,118]. The dependence of its phase-forming propensity on IDRs' valency is evidenced in a study in which the LLPS of different FUS variants were examined [23]. It was shown that the degree of LLPS scaled with the length of FUS. The tandem FUS protein are much more prone to LLPS whereas the single FUS and truncated FUS variants only showed weak LLPS. These studies demonstrate that the valency of phase-forming IDRs is a key parameter to construct LLPS in synthetic biosystems.

Valency amplification of IDR scaffold has also been achieved by fusing IDR with homo- or hetero-multimerization domains (Fig. 7d) [107,132,133]. For instance, NPMI is a pentameric protein that engenders the formation of the liquid-like granular component (GC) of nucleolus [134]. NPMI consists of an N-terminal oligomerization domain, a central disordered center, and a C-terminal RNA binding domain [135]. The oligomerization domain of NPMI is essential for the phase separation of NPMI [107,135]. Researchers have utilized oligomerization to promote phase-separation. For example, a self-assembled protein cage, human ferritin, which is assembled from 24 copies of FTH1 subunit, has been used to server as a well-defined nucleation "core" to locally concentrate IDRs to promote phase separation (referred to as Corelet system) [136]. Notably, based on the rapid and quantitative tunability, a mechanism was proposed for how cells organize dilute intracellular proteins into condensates without globally up-regulating

protein synthesis: the protein cage mediated multivalent complexes with slow diffusion rates can capture and amply the concentration of IDR binding partners, resulting in locally concentrated IDRs sufficient for condensate formation [136].

In addition to stable multimerization, light switchable multimerization has been introduced into LLPS system to enable light-switchable condensates (Fig. 7e). Cry2 is a light-responsive protein which self-associates upon blue light illumination and dissociate in the dark [137]. In contrast, PixD/PixE assembles into a multi-subunit complex with a 10:4 or 10:5 ratio in the dark and dissociate into PixD dimer and PixE monomer under blue light illumination [138]. Fusing IDRs with PixD/PixE or Cry2 enables the generation of light-dissociable condensates or light-associable condensates, respectively [30,139,140]. The advantage of photo-controllable LLPS is that light can be applied and withdrawn spatiotemporally. In addition, light-induced phase separation is well suited for living biosystems, in which other means to control protein-phase behavior such as changing temperature, pH, and/or ionic strength, remains challenging in hemostatic living cells. Thus, the optogenetic tools holds promise in constructing switchable biomolecular condensate in which the assembly/disassembly state of protein condensates can be tuned by light, allowing control over their functionalities.

3.1.3. Rational design of phase-forming IDRs

In contrast to naturally derived IDR-scaffold which may cause undesired crosstalk with endogenous entities, artificial IDRs are ideal candidates for serving as phase-forming scaffolds because they are orthogonal to living systems. However, although IDR in proteins can be identified using a variety of algorithms [141], the ability to predict their phase behavior is still deficient. Much effort has been devoted to relating sequence of IDRs to their phase behavior and to guiding the de novo design of phase-forming IDRs (Fig. 7f). One common way to decipher the sequence codes is by mutagenesis strategy [75,118,142]. For example, Wang et al. have elucidated how specific amino acids affect the phase behavior of IDRs proteins in the FUS family [118]. They found that the interactions between tyrosine and arginine are the driving force of phase separation, while glycine, serine, and glutamine entail the material property of FUS condensate. This finding enables modulation of phase behavior of FUS proteins both in vitro and in cells. Furthermore, the authors proposed a model to predict the phase behavior of FUS family proteins.

Based on the understanding of how the sequence determines LLPS, de novo design of phase-forming IDR is an emerging area. Simon et al. used ELP as the starting building blocks to construct uniform condensates with controlled size and architecture in vitro [143]. The ease of production, amenability to modification, and well-defined phase behavior from material science makes ELP as an ideal candidate for construction of synthetic condensates in a biological context [144–146]. In parallel, a principle to encode phase behavior of IDRs has been proposed by using a comprehensive analysis of the feature sequence of Pro- and Gly-rich IDRs, [61] allowing the de novo designed IDR scaffolds with defined LCST or UCST phase behavior over a wide range of temperatures in vitro. In addition, these design rules also enable identification of proteins with a high phase separation propensity from a given proteome.

Recently, Dzurick et al. have shown an exciting example in which rationally designed artificial IDPs (A-IDPs) exhibits predictable phase behavior both in vitro and in cells [16]. Using a combination of previously obtained feature sequence and proteomic analysis, an octapeptide (GRGDSPYS) was designed to serve as the initial building blocks of A-IDPs. The in vitro phase behavior of A-IDPs, such as condensate saturation concentration, critical temperature, and permeability is highly dependent on the molecular weight and aromatic: aliphatic ratio. Notably, these in vitro obtained rules can be applied to the design of condensates in living cells, enabling prediction and control of A-IDP phase behavior in complex intracellular environments. Taken together, a combination of computational and experimental approaches will facilitate the design of tailor-made phase forming scaffolds.

3.2. Folded proteins as phase forming scaffold

3.2.1. Folded proteins

In living cells, folded proteins can also serve as the phase-forming scaffolds. Examples can be found in signaling pathways such as T cell activation, actin-regulatory pathways, and synapses organization [147–149]. Unlike IDRs scaffolds which drive phase separation via temporary and promiscuous interactions, folded proteins phase-separate through stereospecific interactions between interaction motifs/domains. Folded protein-based scaffolds have several advantages compared to IDR scaffolds. Frist, most of the protein-protein interaction pairs have been extensively studied with atomic resolution structures available online, which facilitates the modification and adaptation of the protein scaffolds. Second, folded proteins can be easily synthesized through heterologous expression system in a soluble form whereas IDRs are known to be liable to aggregate [150]. Third, folded protein can phase-separate without undergoing phase transition from liquid, to gel, and to solid, avoiding unwanted changes of the material properties of the resultant condensates [151]. Finally, many IDRs are known to be related to pathogenic progress which may trigger unwanted cellular responses [152].

Folded proteins derived from postsynaptic densities (PSDs) have been used for the LLPS in synthetic biological systems. PSDs are protein rich compartments located at the intracellular side of the postsynaptic membrane, which is implicated in the processing of signaling molecules from presynaptic axonal termini. PSD-95 is a postsynaptic adaptor protein consisting of multiple interaction domains, which is responsible for orchestration of multiple signaling cascade [153]. SynGAP is negative regulator of synapse activation and exists as a trimer which can bind to PSD-95 in a 3:2 stoichiometry [149]. Zeng et al. have successfully reconstituted a PSD-like assemblies harnessing SynGAP/PSD-95 complexes induced phase separation [149]. Later, the same group successfully reconstituted PSD analogous by using PSD-95 and PSD-95-asscociated proteins, including GKAP, Shank, and Homer. The multivalent interactions between adaptor PSD-95 and PSD-95 associated proteins, such as PSD-95 and GKAP, GKAP and Shank3, and Shank3 and Homer, lead to the formation of PSD-like assemblies via phase separation (Fig. 7g) [154]. Moreover, Liu et al. further simplified the above phase separation systems, generating a phase forming system consisting of three scaffold-like components, including only GKAP, Shank, and homer [21]. Multivalent interactions between scaffold-like components promote the formation of LLPS without the presence of PSD-95. In addition, the three-component phase-forming system is robust as evidenced that the modification of the scaffold proteins with a client recruitment tag did not influence the integrity of the condensates [155]. These studies demonstrate the great potential of folded proteins containing multiple interaction domains for serving as phase-forming scaffolds.

3.2.2. Chimeric protein scaffolds

Chimeric proteins containing multiple folded proteins have also been used as phase-separating scaffolds. Phox and Bem1p (PB1) domain of p62 has acidic surface on one side and basic surface on the opposite sides. The electrostatic interactions between each PB1 molecules result in a formation of high-molecular-weight homo-oligomers in a front-to-back manner [156]. Azami-Green (AG) is a fluorescent protein that exits as a tetramer [157]. The fusion of PB1 and AG (AG-PB1) form LLPS in different cell types [158]. Alternatively, two target domains with potential interactions were fused to AG and PB1, respectively, to examine if there is protein-protein interaction between the targets by detecting the formation of fluorescent puncta via LLPS of the fusions [159]. In another study, a pair of multivalent scaffolds, multidomain protein fusion consisting of repetitive SH3 domains ($SH3_m$) and protein

fusion containing repetitive cognate ligands (PRM_n), were used as phase-forming scaffolds (Fig. 7h, top) [15,33]. Notably, the level of phase separation scales with the repeat number of interaction domain, which is consistent with the observations in IDR-based condensates. Similar to the multi-valent IDRs which promote phase separation, increased valency in the protein-ligand interaction pair drives more robust phase separation under lower protein concentration [15,33].

Stimuli triggered protein-protein interaction have been used for the construction of inducible protein condensates. FK506 binding proteins (FKBP) can bind to FKBP-rapamycin binding proteins (FRB) with high specificity and fast kinetic in a rapamycin dependent manner [160]. Based on this chemical inducible dimerization, Nakamura et al. reported a novel strategy, iPOLYMER, for formation of small chemical induced synthetic condensate (Fig. 7h, bottom) [29]. iPOLYMER systems contains two scaffold-like components: 1) polypeptide containing tandem repeats of an FKBP; 2) polypeptide containing tandem repeats of an FRB. Mixing above two polypeptides leads to the readily formation of protein condensates in the presence of rapamycin. They further constructed a light-inducible system, iPOLYMER-LI, which contains two parts: 1) polypeptide containing tandem repeats of a SspB; 2) polypeptide containing tandem repeats of an iLID. SspB can bind to iLID upon blue light illumination [161]. As a result, the mixture of the multivalent polypeptides forms LLPS upon blue light illumination reversibly. The inducible iPOYMER condensate featuring molecular sieving properties enables the sequestration of mRNAs by mimicking the stress-granule in living cells.

One advantage of folded protein is that the molecular basis of protein-protein interaction is well studied, providing the opportunity to relate nanoscale interactions to mesoscale phase behaviors. Recently, Heidenreich et al. reported a two-component phase separation system: 1) a multidomain protein consisting of a homodimerization domain, a red fluorescent protein and Im2; 2) a multidomain protein consisting of a homotetramerization domain, a yellow fluorescent domain and E9 [162]. Im2 binds to E9 specifically and the binding affinity can be finely tuned by point mutation of the protein [162]. Moreover, the distance between each Im2 fused to the termini of dimer is ~18 nm whereas the distance is only ~4 nm between each E9 [163,164]. As a result of the incompatibility between the distances, the intramolecular interactions are abolished. The phase behavior of this system is merely dependent on the specific interaction between E9 and Im2 intermolecularly, thereby allowing precise characterization of how tunable interaction affinity impacts LLPS and material state of the condensate. By mapping the high-resolution phase diagram in vivo, it was shown that increased binding affinity enhances phase separation until the system becomes kinetically trapped when E9 and Im2 with relatively high binding affinities were applied. In line with this study, Araki et al. showed that phosphorylation of SynGAP which decreases the binding affinity between SynGAP and PSD-95, leads to the dispersion of SynGAP from PSD [165]. These studies demonstrate that the modulation of the interaction affinity between scaffold proteins represents a powerful strategy for tuning the propensity of phase-forming scaffolds to phase separate.

3.3. Recruiting client proteins

Client-like components are dispensable for the formation of condensates, but recruitment of client biomolecules endows the biomolecular condensates with the desired functions. In living systems, client molecules are recruited into the condensates through the interactions between the clients and the scaffolds depending on the cellular needs. Inspired by nature, different client recruitment approaches have been developed and a variety of client molecules such as fluorescent proteins, protein factors, mRNAs, and enzymes have been successfully recruited into synthetic condensates, endowing LLPS in synthetic biosystems with desired functions.

3.3.1. Stable client recruitment

One straightforward way to recruit client protein into condensates is tagging the client protein with the same phase-separating IDRs (Fig. 8a). IDRs are fused to the client proteins at the N or C terminus. Phase-forming scaffold bearing the same IDR sequences can recruit IDR-tagged clients through the interactions between IDRs. However, one potential limitation of this strategy is that the IDRs are often very large which may interfere with the client protein. This issue can be partially overcome by using truncated IDR as the recruitment tag. However, shortening the IDR may compromise the recruitment efficiency. In a synthetic LLPS, half-truncated FUS was tested to place the client proteins into the condensate made of longer FUS [23]. Although half FUS is too short to drive LLPS, half-truncated FUS-tagged client proteins (GFP or homo-molecular fluorescence complementation probe) were recruited into the synthetic condensates. It is noteworthy that the recruitment efficiency is scaled with the length of IDR tags. Clients bearing full length FUS were recruited into FUS condensate more efficiently than that of half-truncated FUS-tagged client proteins. Notwithstanding the success in IDR-based client recruiting tags, IDR-tagging strategies have several limitations. First, they are inherent cumbersome even after truncating, often containing several hundred amino acids, which may impose adverse effect on the protein folding and activities of client proteins [30]. Second, the large molecular sizes of client protein-IDR fusion may hinder their expression in living cells, leading to a low protein expression that is not enough for phase separation [166]. Third, tagging the client proteins with the same IDRs used in IDR-based scaffold may compete for scaffold-scaffold interaction and destabilize the condensates, or even dissolving the formed condensates [167].

Short interaction domains provide potential solution to the above issues (Fig. 8b). Several sets of interaction peptide pairs have been developed over the past years, providing a library of peptide pairs with varied binding affinity such as coiled-coil, cohesion and dockerin, and SH3 domain and PRM ligand [168–170]. For example, we recently have developed a high-affinity peptide-peptide interacting pair, referred to as RIAD and RIDD, derived from peptide pair involved in protein kinase activity regulation [171]. RIAD (2.3 kDa) and RIDD (6.7 kDa) both are small. RIAD binds to RIDD dimer tightly both in vitro and inside cells. RIAD and RIDD have been used as recruitment tags for the employment of enzymes into synthetic condensates [21,172]. Compared to untagged protein cargoes, it was shown that RIDD-tagged proteins can be recruited into RIAD-tagged synthetic condensate more efficiently through RIAD-RIDD interaction, leading to an improved cargo protein enrichment (2-fold vs. 50-fold) [21]. In a study which compared the recruitment efficiency of different cargo recruitment strategies, it was shown that the fusion of a single IDR domain to client protein only mediated weak cargo protein incorporation [20]. In comparison, recruitment of clients by using small interaction peptide SYNZP1 and complementary SYNZIP2 peptide was significantly more effective through the peptide-peptide interaction. To achieve a comparable cargo protein recruitment efficiency as the interaction peptide-tagged system, it was shown that a fusion to a tandem repeat of IDR was required, however, which may interfere with the client protein activities. As the advancement in developing peptide-peptide interaction pairs, we envision that the control of orthogonality, stoichiometry and density of client proteins will be achieved by using novo peptide pairs.

3.3.2. Dynamic client protein recruitment

Besides stable client recruitment, dynamic client recruitment and release has also been achieved using stimuli-responsive client protein recruitment strategies. For example, to allow for an inducible release of client proteins from synthetic condensates, a TEV protease cut site was inserted between client protein and recruitment tag (Fig. 8c) [20]. Expression of TEV protease led to readily release of the client proteins from synthetic condensates. Further, an additional thrombin site was introduced into another client protein. A sequence of TEV treatment and thrombin treatment leads to a multi-step, multi-cargo release [20].

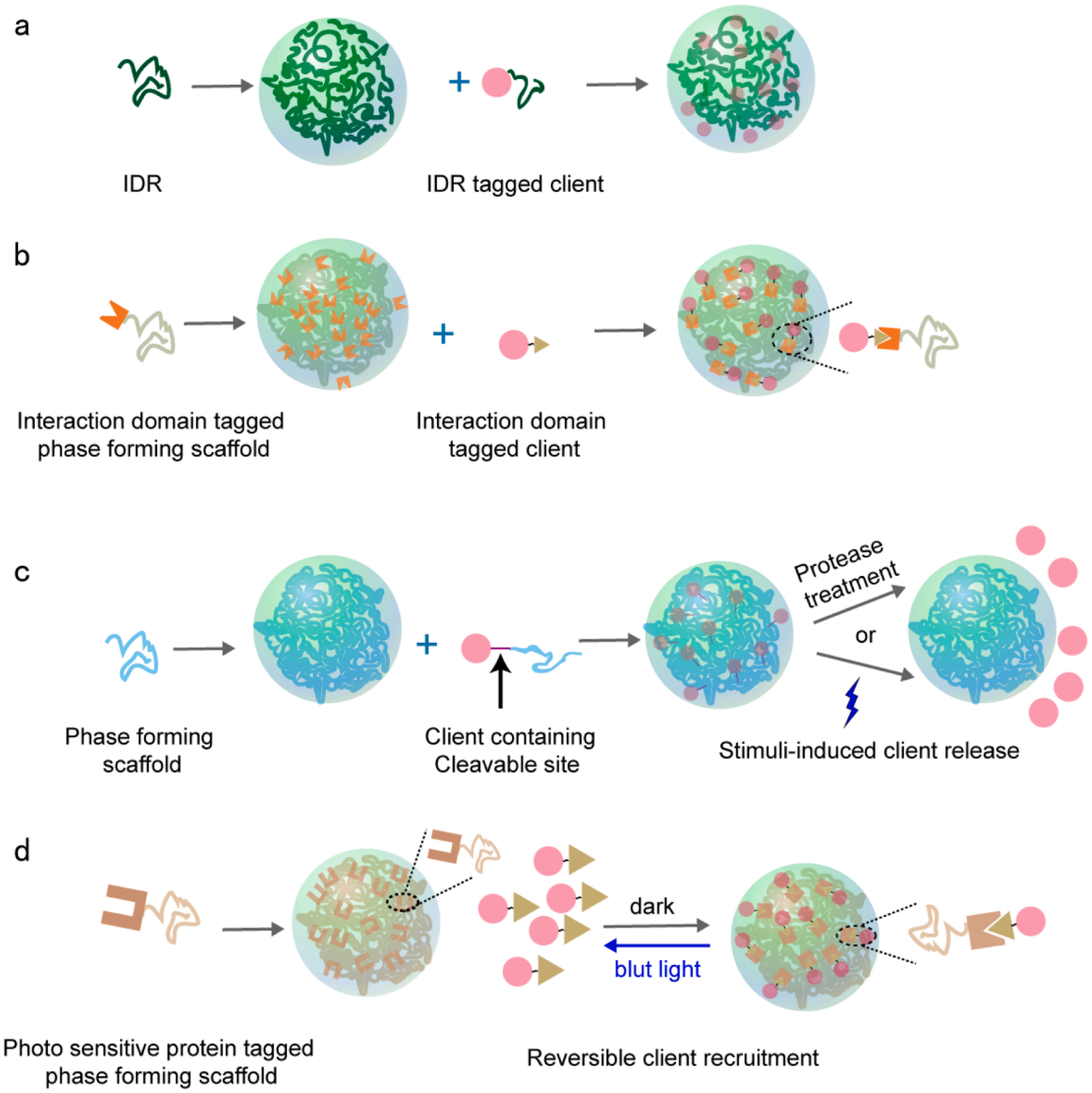

Fig. 8. Targeted recruitment of client proteins in synthetic condensates. a, Client protein fused with phase-forming IDR can be recruited into the synthetic condensates comprised of the same IDR. b, Efficient and specific client recruitment can be achieved by tagging the client and phase-forming scaffold with short interaction domains/motifs. c, Incorporation of cleavable site between client protein and recruitment tag enables stimuli-induced client release from synthetic condensates. Black arrow points to the cleavable site (e.g., protease cut site or photocleavable site). d, Light-switchable protein-protein interaction enables reversible client recruitment. The conformational change of optogenetic protein is dependent on light, resulting in a reversible association between client proteins and synthetic condensates.

Although protease triggered cargo release enables the logic-gate cargo release, co-expression of proteases may exert additional burden to the host cells. Moreover, this protease-based strategy lacks temporal control. Besides protease induced cargo release, chemogenetic tools have been leveraged for inducible client protein recruitment. For example, FKBP and FRB were fused to phase-forming scaffold and client proteins, respectively [158,173]. Addition of the dimerizer, rapamycin, results in employment of client proteins into synthetic condensates via rapamycin mediated dimerization between FKBP and FRB [20].

However, all the above strategies only yield single cycle of clients release or recruitment. Repeatable client recruitment and release is much more favored towards the goal of regulating protein activities at will. Yoshikawa et al. reported an impressive work in which an optogenetic tool, referred to as LOVTRAP system, were used to achieve reversible recruitment and release of cargo proteins for several cycles (Fig. 8d) [158]. LOVTRAP is comprised of a photoreceptor LOV2 domain and a small protein Zdk1, which binds to each other in the dark and dissociate upon blue light illumination [174]. LOV2 and Zdk1 were fused to phase-separating scaffold and client protein, respectively, generating an optogenetic synthetic protein-recruiting/releasing condensate (optoSPREC). Blue light illumination and removal allows repeatable client protein release and recruitment on the time scale of seconds. The reversibility of this system is robust as evidenced by several rounds of cargo protein release/recruitment. Alternatively, reversable protein recruitment/release can be achieved using thermal-sensitive coiled-coil interaction pair, TsCC(A) amd TsCC(B) [175]. It was shown that TsCC(B) containing protein cargoes can be recruited into TsCC (A)-tagged synthetic condensates in a temperature dependent manner [173]. Taken together, reverse recruitment and release of client proteins from synthetic condensates in a controlled manner opens the pathway to giving more insight into the understanding of the functions of native biomolecular condensates and to generating smart soft biomaterials which enables the control of the target bioactivities at will by recruitment and release of the target molecules.

4. Applications of LLPS in synthetic biosystems

It is increasing appreciated that biomolecular condensates play critical roles in a variety of cellular functions, such as signaling transduction, RNA processing, translation regulation and transcription regulation. Biomolecular condensates insulate specific biochemical activities from surrounding environment, provide unique conditions and concentrate certain biomolecules, contributing to the orchestrated metabolism. Our knowledge on the physiochemical properties and functions of LLPS in both non-living systems and living systems inspired biologists and chemists to design artificial LLPS with new functions in synthetic biosystems. Synthetic LLPS with engineered novo functions have been proven to be a useful tool in a variety of applications in the biological systems. An overview of the synthetic condensates presented within this review are summarized in Table1.

4.1. Conferring bacterial with spatial organization

Besides membrane-bound organelles, eukaryotic cells use LLPS to insulate bioactivities from complex intracellular environment, ensuring the coexistence of competing or even contradict reactions. In contrast, bacteria were once thought of 'bags of enzymes' with little or no spatial organization [190]. Yet, in the past few years, due to the advancement in imaging and proteomics, there is emerging evidence that prokaryotic cells also possess phase-defined membranelles organelles, providing a means for subcellular spatial organization beyond protein shells-bound organelles (e.g., carboxysomes). There is increasing body of evidence that the prokaryotic LLPS are implicated in diverse biological processes such as transcription, cell division, CO_2 fixation and RNA decay [191–195]. These initial observations are probably exemplary of a more general mechanism in the subcellular spatial organization of bacterial and set stage for researchers to design and construct custom biomolecular condensate in prokaryotes.

In an initial work, Ge et al. have utilized ELP tag to construct a synthetic LLPS in *E. coli* [196]. Expression of ELP-GFP fusion led to the formation of protein condensates in living *E. coli*. It is noteworthy that the machinery of protein biosynthesis (i.e., nucleic acids and ribosomes) were excluded from the synthetic protein condensates enriched in ELP-GFP fusions (Fig. 9a). This study demonstrates the potential of biomolecular condensate in functional partitioning of intracellular milieu into separate spaces without the aid of complex membrane systems, enabling distinct biological processes to take place without interfering with each other (e.g. protein synthesis and protein storage). Similarly, Huber et al. have developed a unique method for the formation of organelle-like condensates in *E.coli* using amphiphilic scaffold proteins consisting of a hydrophobic protein domain and a hydrophilic protein domain adapted from ELP (Fig. 9b) [197]. The artificial amphiphilic protein resembles phospholipids which are the main constituents of membrane. Site-selective incorporation of unnatural amino acid into the genetically encoded phase-forming scaffold allows introduction of functional groups into the synthetic organelles [197,198]. These studies pave the way for construction of LLPS in prokaryotes.

Besides ELP-based LLPS in prokaryotes, IDRs from eukaryotes have been used to confer prokaryotes with spatial organization [166,172]. In a study using unfolded and structural proteins, all tested proteins, including spider silk and resilin, exhibited the ability to serve as building blocks for phase separation in prokaryotes. Fusion of IDRs to proteins such as fluorescent proteins and enzymes had no adverse effect on protein activities as evidenced either by the fluorescence or catalytic activities. Spatial organization of metallothionein (MT, a protein can bind and reduce selenite into selenium) in synthetic condensate resulted in a compartmentalized reactor, generating homogenous and well dispersed Se nanoparticles. Although this is a promising proof-of-principle evidence supporting the LLPS mediated spatial organization in prokaryotes, the research has also encountered shortcomings that need to be addressed: no significant yield improvement was observed when colocalizing cascade enzymes within synthetic protein condensates compared with freely floating enzymes. The author suggested that this observation is likely due to that the eukaryotes derived silk protein is heterologous to the prokaryotic expression system, which may interfere with its expression level. The recently observed phase-forming proteins from prokaryotes may prove useful to overcome this limitation.

4.2. Biomolecular condensate-based reactors

In nature, compartmentalization of metabolic enzymes in biomolecular condensates is a common strategy for cells to regulate biochemical reactions, orchestrating the metabolism to meet cellular needs [199, 200]. Two well-known examples are purinosomes involved in purine de novo biosynthesis and glucosomes involved in glucose metabolism [201–204]. Moreover, enhanced reaction rates within biomolecular condensate can be found in many biological processes including protein filament nucleation, innate immune response, CO_2 fixation and mRNA silencing [11,13,147,192,205–208]. Peeples et al. suggested that the enhanced reaction rate in phase-separated compartment is likely due to increased concentration of reactants and improved K_M [176]. Compared to the membrane-bound and protein-bound synthetic versions of reactors [209,210], LLPS are ideally suited for serving as reaction vessels for synthesizing value-added products in synthetic biology and metabolic engineering owing to the unique features. First, while biomolecular condensates have restricted permeability, they are also highly porous and exchange molecules with surroundings constantly, imposing no diffusion barrier to reactants duo to the avoid of physical barrier such as bilayer lipid membrane. Second, the diameter of biomolecular condensates is in the range of hundreds of nanometers to several micrometers, allowing accommodation of large quantities of enzymes and reactants. Third, the assembly states of biomolecular condensates can be tuned by changing the environmental conditions, which enables the ease of modulation of reaction rate through assembly and dissolution of the reactors.

Zhao et al. reported a LLPS-based reaction vessel through which the metabolic flux of an engineered pathway was tuned in a light-dependent manner [30] (Fig. 10). In this study, IDRs (FUS^N) tagged optogenetic protein (Cry2 or PixE/PixD) were fused to two sequential enzymes (i.e., VioE and VioC) related to violacein biosynthesis, respectively. Switching blue light on/off altered the oligomerization sate of the optogenetic proteins, leading to the assembly/disassembly of the catalytic condensates. In respect of metabolic node, light-responsive condensates enabled control over metabolic flux by colocalizing cascade enzymes within protein condensates, converting more intermediates to the desired products and reducing the flux of intermediates towards unwanted, non-enzymatic pathways. Impressively, strain containing LLPS-based reactors exhibited 6.1 ± 0.9-fold improvement in the level of the final product (deoxyviolacein) compared to the control strain expressing only freely floating enzymes. This improvement matches the theoretical maximum enhancement for a two-enzyme cascade reaction by a means of enzyme colocalization [211]. However, some of the IDR-tagged enzyme lost catalytic activity. This adverse effect is likely due to the relatively large size of the phase-forming scaffold. Thus, there is a compelling need to identify the minimal scaffold of for phase separation.

More recently, Dzuricky et al. reported a study in which the activity of enzyme was manipulated by modulating the LLPS of an A-IDP [16]. The authors found that the propensity of A-IDP to phase separate is scaled with A-IDP molecular weight, which in turn influence the activity of the encapsulated enzymes. A β-galactosidase catalyzed reaction was chosen as a model to investigate how encapsulation of enzymes within protein condensates influence the enzymatic reaction rate. Red-fluorescent protein tagged A-IDP was fused with so-called alpha peptide (αp), giving αp–A-IDP–mRuby3 fusion. αp is a peptide that can bind and recruit the inactive fragment of β-galactosidase (LacZΔM15) to

Table 1
Synthetic LLPS for applications in synthetic biology, chemical biology, cellular engineering and biotechnology.

Scaffold-like component	Client-like component	Client recruitment strategy	System	Application	References
ELPs and RNAs	mRNA	RNA binding protein	In vitro	Inhibition of translation in protocells	[126]
Supercharged GFPs and RNAs	N/A	N/A	In vitro and in E. coli	N/A	[129]
Tandemly repeating RGG domain of LAF-1	Fluorescent protein	Coiled-coil pair or IDR tag	In vitro, and in Xenopus egg cytoplasmic extract, and in mammalian cells.	N/A	[20]
	Metabolic enzymes	RIAD and RIDD interaction peptides	In vitro and in E. coli	Biosynthesis	[172]
	Native proteins	SZ1 and SZ2 interaction; TsCC (A) and TsCC(B) interaction; FRB and FKBP interaction	In vitro and in living yeast	Sequestration of native protein activities for controlled cell behavior	[173]
Fusion of RGG and photo cleavable site	Fluorescent protein	N/A	In vitro and in S. cerevisiae	Light inducible biomolecular condensate	[131]
FUS	N/A	N/A	In vitro	Modulation of protein phase behavior based on the sequence determinants	[118]
	Fluorescent protein	FUS tag	In vitro	Investigation of the relationship between IDR-IDR interaction and phase behavior	[17]
	Fluorescence complementation probe	FUS tags with varied length	In vitro and in mammalian cells	Investigation of the mechanism underlying client proximity enhancement	[23]
N terminal of FUS (FUS_N) + FTH1	N/A	Optogenetic proteins of iLID and SspB	In vitro and in mammalian cells	Quantitative mapping of intracellular phase diagram	[136]
Fusion of FUS_N and PixD/PixE	N/A	N/A	In mammalian cells	Investigation the role of phase separation in spatial regulation of cells	[140]
	Metabolic enzyme	FUS_N tag	In S. cerevisiae	Biosynthesis	[30]
Fusion of FUS_N and Cry2	Metabolic Enzyme	Cry2 tag	In S. cerevisiae	Biosynthesis	[30]
Fusion of FUS/ DDX4/ HNRNPA1 and Cry2	N/A	N/A	In mammalian cells	Spatiotemporal Control of Intracellular Phase Transitions	[139]
Rationally designed ELPs	N/A	N/A	In vitro	Synthetic condensates across multiple length scale	[143]
Rationally designed protein polymers derived from naturally occurring Pro- and Gly-rich proteins	N/A	N/A	In vitro	Synthetic condensates with tunable lower or upper critical solution temperature	[61].
De novo designed artificial IDRs	Fluorescent protein or enzyme	IDR tag or enzyme complementation	In vitro and in E. coli	Synthetic condensates with predictable phase behavior both in vitro and in vivo	[16]
PSD-95 and SynGAP	N/A	N/A	In vitro and in mammalian cells	Reconstitution of a PSD-like assemblies	[149]
PSD-95, GKAP, Shank3 and Homer	N/A	N/A	In vitro	Reconstitution of PSD for understanding synapse	[154]
	Enzyme	RIAD and RIDD pair	In vitro	Catalyzing cascade reaction	[21]
PB1 and AG	N/A	N/A	In vitro and in living cells	Visualization of protein-protein interactions	[159]
	Proteins related to membrane ruffling and ERK signaling	FRB and FKBP interaction; light dependent LOV2 and Zdk1 interaction	In living cells.	Reversible sequestration and release of protein activity	[158]
$SH3_m$ and PRM_n	N/A	N/A	In vitro and in living cells	Investigation of the relationship between protein valency and phase transition	[33]
	Enzymes	FKBP and FRB interaction	In vitro	Investigation of the mechanism for the accelerated ration rate in biomolecular condensates	[176]
Tandemly repeating human SUMO3 and tandemly repeating SUMO Interaction motif (SIM)	Fluorescent protein	SUMO and SIM interaction	In vitro and in living cells	Investigation of the composition control of biomolecular condensates	[15]
$FKBP_m$ and FRB_n	RNA	Protein-RNA interaction	In vitro and in living cells	Synthetic RNA granule	[29]
Fusion protein of 1AIE-E9-YFP and fusion protein of 4LTB-lm2-RFP	RNA	Protein-RNA interaction	In vitro and in living yeast cells.	Synthetic condensates with tunable phase diagrams.	[162]
ELP-GFP	N/A	N/A	In vitro and in E. coli	Storage of newly synthesized protein	[90]
Amphiphilic scaffold protein derived from ELP	Fluorescent dye	Genetically encoded unnatural amino acid	In E. coli	Synthetic condensates with lipid-like boundary	[91]
Unfolded, structural proteins	Fluorescent protein and enzyme	IDR tag	In vitro and in E. coli	Synthetic membranless organelle in prokaryotes	[72]
CIB1, Cry2, and MP	Proteins involved in diverse functions	Interaction between GFP-client and anti-GFP nanobody	In mammalian cells	Reversible protein inactivation	[115]

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Table 1 (*continued*)

Scaffold-like component	Client-like component	Client recruitment strategy	System	Application	References
	Membrane associated proteins	Phase-forming scaffold tagged client	In mammalian cells	Investigation of Intracellular membrane trafficking	[118]
	mRNA	Protein-RNA interaction	In mammalian cells	Manipulation of the localization and translation of specific mRNA	[119]
FUS/EWSR1 +K2	genetic code expansion system	Protein-RNA interaction (MCP-MS2)	In mammalian cells	An orthogonal translation system with minimum impact on housekeeping translation	[22]
HOTag6/HOTag3 + kinase activity dependent protein-protein interaction	N/A	N/A	In mammalian cells	Visualization of protein kinase activity	[177]
Engineered protein consisting of two repeats of FKBP, mCherry, GFP, and two repeats of FKBP	N/A	N/A	In mammalian cells	Investigation of aggrephagy	[32]
ELP tagged recombinant proteins	N/A	N/A	In vitro	Non-chromatographic protein purification	[178–180]
Affinity ligand-ELP constructs	Recombinant proteins	Protein-ligand interaction	In vitro	LLPS for the tag-free protein purification	[181,182]
ELP tagged therapeutic drugs	N/A	N/A	In mammalian cells and in model animals	Sustained release of therapeutic drugs	[183–185]
ELP tagged anticancer agents	N/A	N/A	In model animals	Targeted delivery of anticancer drugs	[186–189]

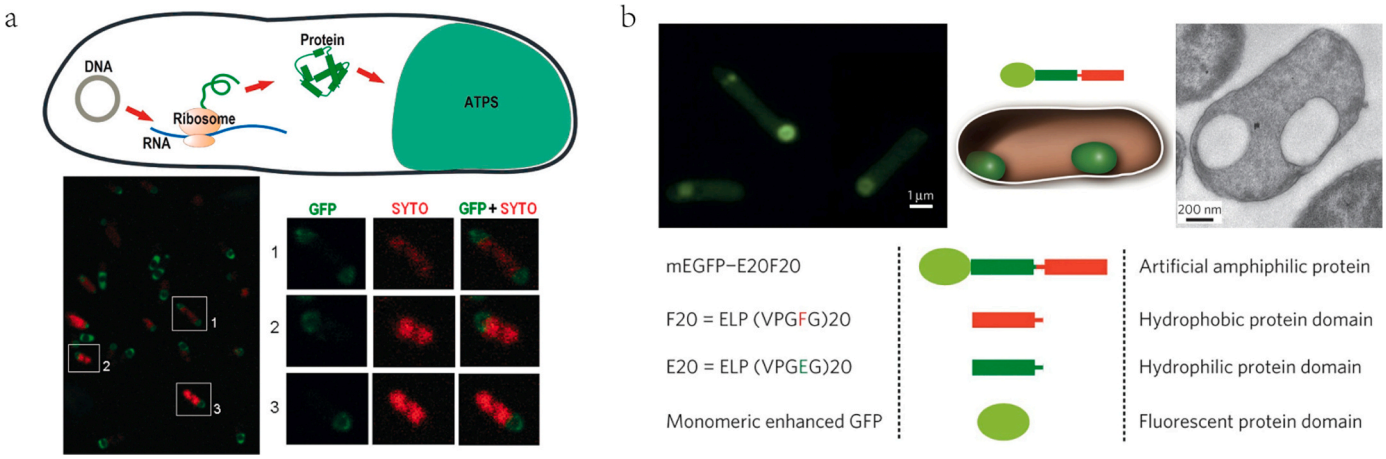

Fig. 9. Synthetic condensates endow prokaryotes with subcellular spatial organization. a, Synthetic biomolecular condensates enable functional partitioning of the prokaryotic cytoplasm into separated spaces. Proteins are synthesized by the protein biosynthesis machinery (red, stained by the fluorescent dye SYTO) and then stored in synthetic condensates (green fluorescence). ATPS, an aqueous two-phase system. b, Construction of synthetic organelles using phospholipid-like amphiphilic proteins. The association of amphiphilic proteins drives the formation of cellular compartments in *E. coli*.
(a) Part a adapted from ref. [178] with permission from American Chemical Society, copyright 2009. (b) Part b adapted from ref. [179] with permission from Nature Publishing Group, copyright 2015.

generate active enzyme (LacZ) which can then convert the substrate FDG to a form of green fluorescein. In engineered *E. coli* expressing LacZΔM15 and αp–A-IDP–mRuby3 condensate, it was shown that the green fluorescence intensity of the green fluorescein was proportional to the molecular weight of A-IDP. Interestingly, the reconstituted enzymes within the condensates showed a significant increase in the catalytic efficiency (K_{cat}). Particularly, the complemented enzymes within condensates comprised of higher molecular weight of A-IDPs showed higher K_{cat}. The authors proposed that protein condensates with increasing molecular weight of A-IDP can sequestrate substrate and enzyme more efficiently, resulting in a higher measured K_{cat}. This study demonstrates that synthetic biomolecular condensate can be used to improve enzyme kinetics, which is consistent with the observation that naturally occurring biomolecular condensates can influence reaction kinetics [19].

Notwithstanding the success of recent studies in employing phase-separated protein scaffolds as reaction vessels to enhance the reaction rate of engineered pathways, two key challenges are faced before realizing the full potential of the phase defined reactors. First, the effects of LLPS on enzymatic reaction is still under debate due to the unique and complex environment within protein condensates. For example, although formation of purinosomes was thought to increase purine de novo biosynthesis, [212] recent studies revealed that the phase-separated purine biosynthetic enzymes did not result in significant rate enhancement in vitro and inside cells [213,214]. To achieve rational design of LLPS mediated reaction vessels, more experimental and theoretical studies are required to elucidate how the unique microenvironment and material properties of biomolecular condensates affect the phase separated enzymes. Second, although researchers have developed several strategies to achieve specific enzyme recruitment into synthetic condensates, it is still difficult to confine desired small molecules (e.g., enzyme substrate) within biomolecular condensates. It is conceivable that reaction rate enhancement will be more pronounced if both enzymes and their substrate were concurrently encapsulated within protein condensates. This is evidenced by a sharp reaction rate acceleration (70-fold) of RNA cleavage by phase separated ribozymes when both of the enzyme and substrate were encapsulated within synthetic condensates [215]. However, compared to the compartmentalization of large biomacromolecules such as RNAs through RNA-protein

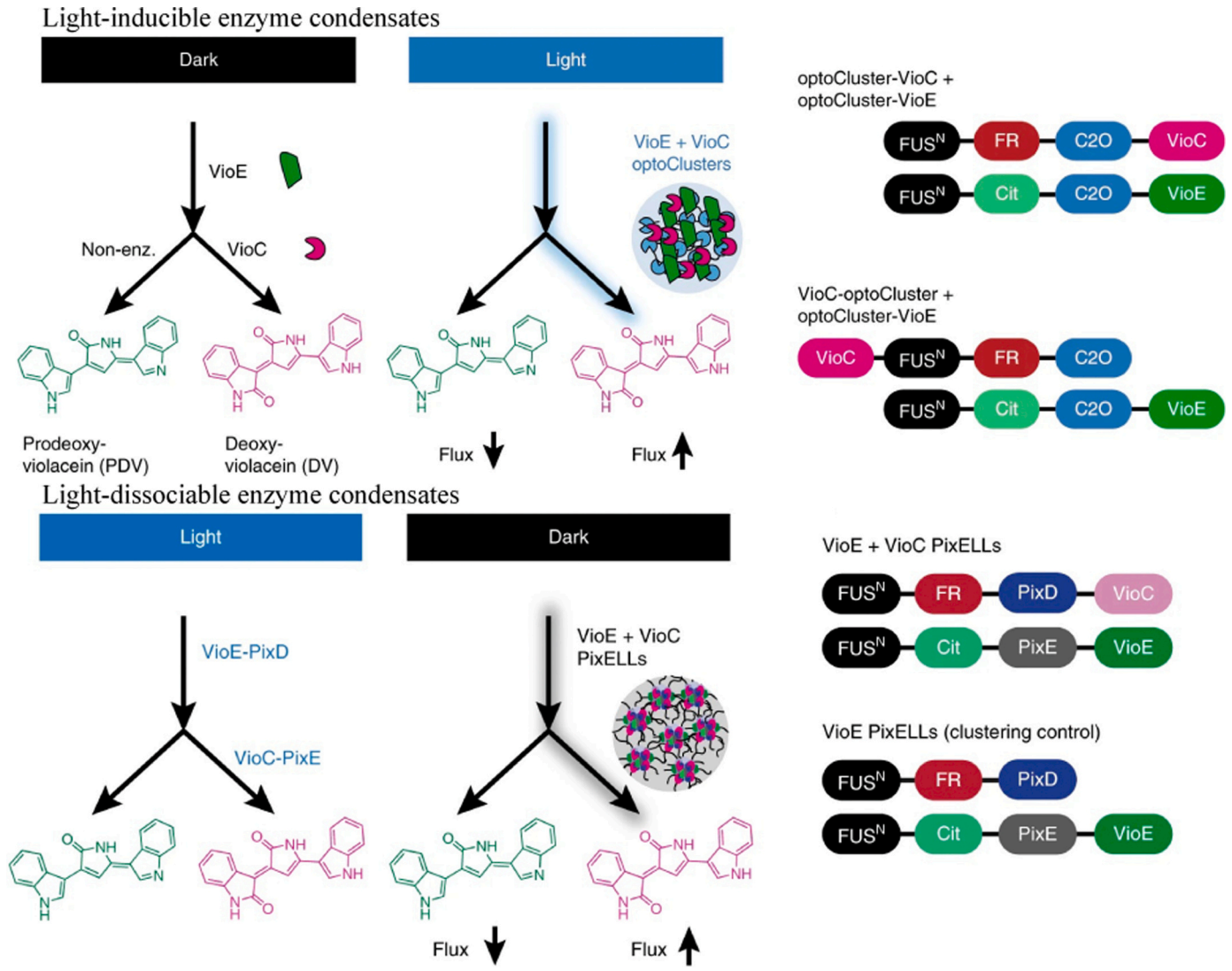

Fig. 10. Diverting metabolic flux at branch point by using light-responsive catalytic condensates. VioE is responsible for the generation of an intermediate, protodeoxyviolaceinate (PTDV), which can be spontaneously oxidized to a green product, prodeoxyviolacein (PDV), or be enzymatically converted to a pink product, deoxyviolacein (DV). Co-localizing enzymes VioE and VioC inside protein condensates can direct more PTDV to enzymatic reaction, leading to an increase in the enzymatic production of DV and a decrease in the production of PDV via non-enzymatic reaction.
Adapted from ref. [30] with permission from Nature Publishing Group, copyright 2019.

interactions, sequestrating substrate of small molecules remains challenge. Thus, more work is needed to develop strategies for targeted small molecular encapsulation.

4.3. *Control of cellular bioactivities*

Precise control of protein activities is attractive in understanding the roles of specific proteins in metabolism and in cellular engineering. However, current strategies such as genetic mutations, small molecular inhibitors and targeted proteolysis are known to have different limitations including off-target effects, poor reversibility, and low spatial resolution [216]. In contrast, naturally occurring biomolecular condensates allow control of specific activities of target biomolecules in a spatiotemporally precise manner by insulating targets away from their active sites to phase-defined compartments. There has been an increasing body of evidence showing that the biomolecular condensates play a vital role in regulation of bioactivities during transcription, translation, and signal transduction [132,217,218]. For example, it has been shown that the yeast translation termination factor Sup35 undergoes phase separation and form protective biomolecular condensates when yeasts are under pH induced stress [219]. The formation of biomolecular condensate makes Sup35 inaccessible to the downstream targets, leading to a protective translation inhibition. Withdrawn of stressful condition leads to the dissolution of the condensates and recovery of protein translation. Inspired by the bioactivities control through the formation of biomolecular condensate in nature, efforts towards reprogramming, mending or creating LLPS in synthetic biosystems holds enormous potential to regulate cellular bioactivities with rapid response and high spatiotemporal resolution.

Using a combination of an optogenetic protein-protein interaction pair and a multimeric protein (MP), Lee et al. created a versatile platform, referred to as light-activated reversible inhibition by assembled trap (LARIAT), to inhibit diverse protein activities in living cells (Fig. 11a, top) [31]. Target protein was fused to Cry2, which can self-associate and then bind to the complementary CIB1 domain upon blue light illumination [220]. CIB1 was fused to CaMKIIα which forms dodecamer under physiological conditions and servers as the multimeric module [221]. Upon illumination, Cry2-tagged target will oligomerize and bind to CIB1-MP, leading to the formation of protein condensates (clusters) which sequesters the targets, leading to an inactivation of the

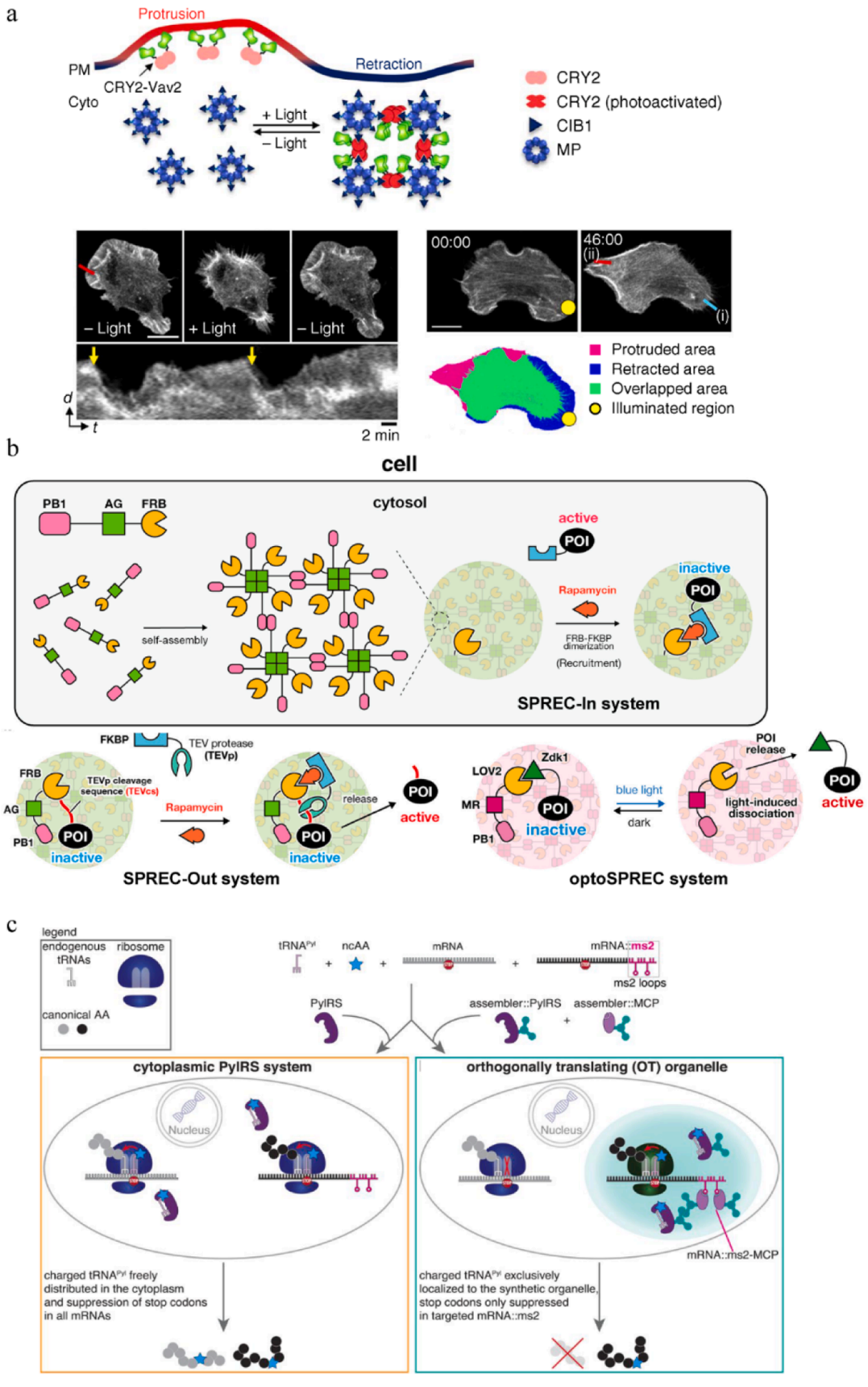

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Fig. 11. Control of protein activity using synthetic condensates. a, Protein inactivation by photo-responsive condensates. Blue light illumination enables the trapping of Vav2 in cytoplasmic condensates, leading to the membrane retraction. b, Stimuli-induced client protein recruitment in synthetic condensate enables control of protein activities. In SPREC-In system, addition of rapamycin leads to the sequestration of proteins from cytoplasm, resulting in the inactivation of the targets; In SPREC-Out system, the initially sequestered client proteins are inactivated in condensates and could be activated following addition of rapamycin which mediates the recruitment of protease into the condensate, releasing the protein target via proximity induced cleavage; In OptoSPREC, reversible client protein sequestration was achieved by applying or withdrawing blue light, enabling repeatable protein activation and inactivation. c, Protein condensates-based orthogonal translation system enables site-specific codon suppression only in the mRNA of POI.
(a) Part a adapted from ref. [31] with permission from Nature Publishing Group, copyright 2014. (b) Part b adapted from ref. [152] with permission from American Chemical Society, copyright 2021. (c) Part c adapted from ref. [22] with permission from American Association for the Advancement of Science, copyright 2019.

targets. It was shown that sequestration of Vav2, which is an activator of Rho small GTPases and is responsible for the membrane protrusion, resulted in a retraction of lamellipodia (Fig. 11a, bottom). Removal of the light restored the Vav2 bioactivity and reversed the morphological effect. The applicability of the LARIAT system was further expanded by incorporation of GFP-binding nanobody into the synthetic condensates, thus allowing inhibition of various GFP-containing proteins in a light dependent manner. Later, the same group refined the LARIAT system, generating mRNA-LARIAT and IM-LARIAT systems, which can insulate mRNAs and membrane-associated GTPases, respectively, resulting in altered cell behaviors [222,223].

More recently, chemogenetic protein condensate namely SPREC-In has been demonstrated to control protein activities by using a combination of PB1-AG fusion-based phase-forming scaffold and a chemically induced dimerization tool (i.e., FKBP and FKBP domains) as shown in Fig. 11b. To control the bioactivities of the target, FKBP and FRB were fused to the PB1-AG scaffold and target protein, respectively (Fig. 11b, top). As a proof-of-principle, Vav2 was selected as the model target. Retraction of lamellipodia was observed following the addition of rapamycin, which are resulted from the chemical-induced recruitment of the FKBP tagged Vav2 from the cytoplasm into FRB-tagged condensates. The author also successfully controlled the ERK signaling pathway by using the adpated system, indicating the utility of the SPREC-In system. Further, by coupling the SPREC-In system with an engineered proximity-dependent protease, a SPREC-Out system has been developed in which the target proteins are initially confined and inactivated in the condensates and could be released from the condensate when the protease was recruited into the condensates via the addition of rapamycin, leading to an activation of the targets (Fig. 11b, left-bottom). Moreover, by substituting the chemically induced dimerization tool with a light-responsive protein dimerization tool (i.e., LOV2 and Zdk1), the authors engineered an optogenetic SPREC (optoSPREC), enabling the reversible protein activation and inactivation through repeatable target protein recruitment and release from the condensates (Fig. 11b, right bottom). It is noteworthy that the optoSPREC inactivates target protein activities in the dark whereas LARIAT system inactivates protein bioactivities upon blue light illumination. This opposite light condition could be useful because having both systems in the same cells could enable bidirectional control. In the same year, Garabedian et al. reported another LLPS system capable of regulating cellular functions such as proliferation, division and cytoskeletal organization through controllable recruitment of native factors into synthetic condensate by applying specific stimuli such as small molecules, altered temperature or light [173]. These studies demonstrate that LLPS is a powerful means to control cellular bioactivities in synthetic biosystems.

Apart from control of endogenous biological activities, insulation of exogenous bioactivities has also been achieved using LLPS. One challenge in genetic code expansion (GCE) system is the unwanted modification of nontargeted proteins [224]. This is particularly true for eukaryotes, in which the abundance of the most widely used amber stop codon (UGA) is about 20%, leading to an unavoidable stop codon suppression in nontargeted proteins. To minimize the background decoding of stop codons of nontargeted proteins, Reinkemeier et al. have developed a LLPS-based orthogonal translation system, which allows incorporation of unnatural amino acids (ncAAs) only into POI site-specifically in living cells [22]. This synthetic organelle has three parts: an mRNA targeting part using major capsid protein (MCP) and its binding partner ms2 RNA stem loops, [225] an orthogonal tRNA/tRNA synthetase (RS) pair, [226] and an assembler system using a combination of phase-forming domain FUS/EWSR1 and spatial targeting kinesin truncations (K2) [152,227]. In addition to colocalization of GCE within protein condensates, kinesin truncations allow another level of spatial control of GCE system by targeting the synthetic organelles to the microtubule-plus ends. As a result, the components of the GCE system and the mRNA of a POI are specifically concentrated inside the condensate, inhibiting the cross-reactivity of GCE with the host's protein biosynthesis machinery. Highly specific introduction of noncanonical amino acids to the POI was observed using synthetic condensates, whereas other mRNAs outside the protein droplet were not translated efficiently.

The LLPS mediated bioactivity control works on the premise that most client proteins can be recruited into the phase-defined space instead of residing at their normal locations where they exert influence on their substrates. It was shown that cells expressing low to moderate expression of scaffold-like proteins along with relative higher expression of client-like proteins only exhibited partial sequestration of the target proteins, which may lead to the leaky activities of the targets [158,173]. To achieve high partitioning efficiency of the targets into biomolecular condensates, the scaffold proteins should be expressed at relatively higher level than that of client proteins, providing sufficient binding sites for client recruitment. In this regard, orthogonal induction systems may prove useful in balancing the expression levels of client proteins and scaffold proteins. In addition, promoter engineering and adjustment of gene copy number can also be used to enhance scaffold expression. Besides balanced expression level of scaffold proteins and client proteins, there is compelling need to develop efficient client recruitment strategies.

4.4. *Probing intracellular processes*

Probing intracellular events in real time and in a non-invasive manner is attractive not only for understanding physiological and pathological processes but also for monitoring disease progression and for evaluating response to therapeutic treatments. However, monitoring the intracellular events with high spatial and temporal resolution remains challenging due to the narrow dynamic range, slow response, and/or large signal-to-noise ratio of current reporters and biosensors. In contrast, nature has evolved to use phase separation to sense and response to intracellular and extracellular changes. Living organisms can sense even subtle changes in temperature, pH, and salt concentrations, leading to a system-wide adaptation and promotion of cellular fitness through formation and dissolution of biomolecular condensates. For example, cyclic GMP-AMP synthase (cGAS) is a cytosolic DNA sensor that induces STING-dependent interferons by producing the second messenger cGAMP [228]. Previous study has shown that the DNA sensing is dependent on the phase behavior of cGAS [207]. It has been shown that multivalent binding between cGAS and DNA drive the formation of cGAS-DNA condensates depending on the length of DNA and zinc concentration. Similarly to sensing the intracellular changes, living cells have been shown to sense environmental changes using phase separation [229,230]. Biomolecular condensates have several properties which make them as ideal candidate for the construction of

phase-separation based probes. First, reporters such as fluorescent proteins can be recruited and concentrated within protein droplets, enabling signal amplification. Second, phase separation is expected to have a fast kinetics, allowing fast response to cellular changes. Third, phase separation is known to have high cooperativity (i.e., individual phase-forming biomolecules cooperate to change their phase behaviors in response to subtle changes in intracellular conditions), allowing design of probes with high sensitivity.

There is increasing interest in the development of LLPS-based probes to investigate diverse intracellular events such as protein-protein interactions, autophagy and kinase signaling [32,159,177]. Zhang et al. reported a phase separation-based kinase probe capable of detecting dynamics of kinase signaling in cells, in animal tissue and in living animals, referred to as SPARK (separation of phase-based activity reporter of kinase) [231]. Although genetically encoded fluorescent biosensors (e.g. FRET biosensors) have been widely used to probe kinase activities in the cellular context, live imaging of kinase signaling in living animals remains challenging due to the small fluorescence ratio change [232,233]. In view of this limitation, instead of reporting the changes of intensities of donor and/or acceptor fluorescent proteins by FRET, the SPARK system can phase-separates and concentrates fluorescent proteins inside droplets upon kinase activation, resulting signal changes of the number of imaged fluorophores per pixel. SPARK system contains three parts: (1) fluorescent protein EGFP that provides fluorescent signal; (2) homo-oligomeric coiled coils (HOTag6 and HOTag3) that provide multivalent interactions; (3) kinase-specific substrate peptide and corresponding phosphopeptide-binding domain that enable kinase activity dependent protein-protein interaction (Fig. 12). The activation of kinase enabled phosphorylation of corresponding peptide substrates and the resultant phosphorylated peptides associated with phosphopeptide-binding domains, leading to the formation of highly concentrated EGFP puncta. Besides probing the dynamics of protein kinase A, the author further designed and constructed an ERK reporter by swapping the kinase dependent interaction module (kinase-specific substrate peptide and corresponding phosphopeptide-binding domain), enabling investigation of ERK signaling which is related to the animal development, in cells, in animal tissue and in vivo. Notably, SPARK-based probes feature intensive brightness and simple fluorescent pattern, enabling examination of kinase activity in a quantitative way based on raw images without time-consuming data processing.

Watanabe et al. reported an LLPS-based reporter to analyze protein-protein interactions (PPIs) in living cells, referred to as Fluoppi. PPIs are fundamental bioprocess for many cellular functions. However, the measurement of PPIs is hampered by the limited dynamic range and quantitative reproducibility. To overcome these limitations, the authors utilized PPI-dependent LLPS to visualize protein interactions. To this end, the potential interaction domains (X and Y) were fused to the AG and PB1, respectively, generating fusions of PB1-X and AG-Y. As mentioned above, both PB1 and AG are known to exist as oligomers and AG itself is a fluorescent protein. They found that the formation of fluorescent puncta is dependent on the interactions between X and Y. Particularly, PB1-X and AG-Y mediated fluorescent puncta were observable only if there are interactions between X and Y domains. By using Fluoppi, a variety of PPIs and drug-induced PPI blockage were successfully analyzed. This study also showed that the fluorescent oligomer, AG, can be replaced with other oligomeric fluorescent proteins, indicating the flexibility of the system. For example, the author successfully used a reporting containing orange-emitting FP, Kusabira-Orange (KO), to analyze the histamine-induced oscillatory binding between CaM and M13 in cancer cells. It is noteworthy that the Fluoppi has been commercially available on the market. Taken together, these studies open the door to designing and developing LLPS-based probes for the quantitative and precise analysis of cellular processes related to diverse functions and provide an emerging area in the development of bimolecular condensate-based smart materials with custom designed functions for cell biology, diagnosis and biomedicine.

4.5. Purification of recombinant proteins

In order to obtain homogenous recombinant proteins, a common approach is to express the proteins with interaction tags and subsequently purify them using affinity chromatography. Although the chromatography-based methods have been extensively utilized and are ideal for producing recombinant proteins on a laboratory scale, it does have limitations when it comes to large-scale production. This is primarily due to the high cost of resins and large volumes of buffers, and time-consuming process involved in the chromatographic purification. LLPS provides a potential solution to these limitations. Previous study has shown that phase-forming IDRs can be purified to homogeneity with extremely high yield without chromatography by cycling between one- and two-phase regime of their phase diagram [16]. Particularly, phase-forming proteins will separate into condensed phase from the

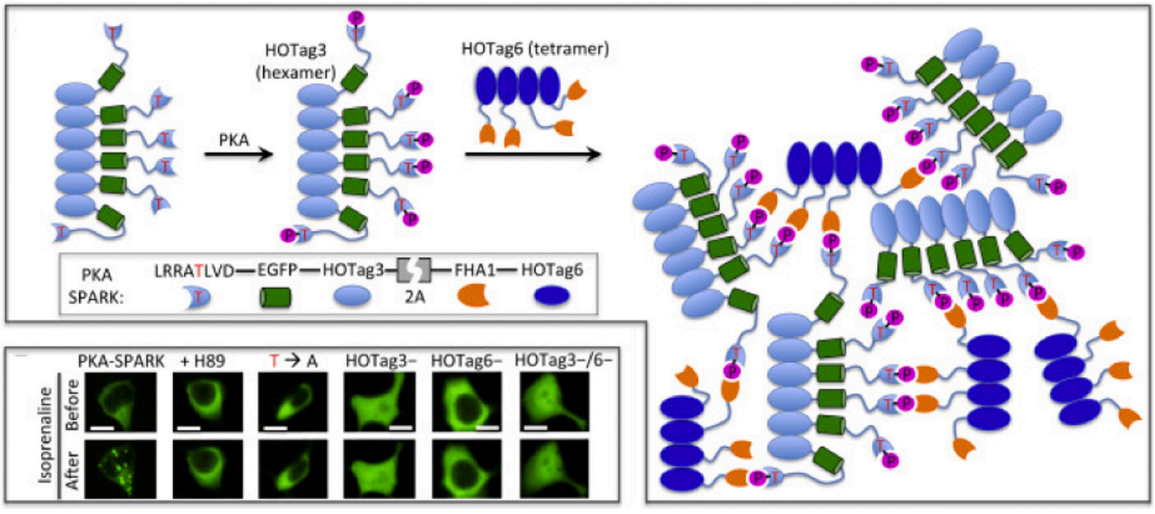

Fig. 12. Probing intracellular kinase activity using phase-separated reporter SPARK. Protein multivalent interaction drive the formation of intensively fluorescent droplet in a kinase dependent manner. Upon kinase activation, the phosphorylated peptides bind to the corresponding phosphopeptide-binding domain, leading to the formation of fluorescent puncta in cells. Isoprenaline is used to activate protein kinase A (PKA). P, phosphate group. H89, PKA inhibitor; T→A, mutation of threonine to alanine; HOTag3-, HOTag3 was removed; HOTag6-, HOTag6 was removed; HOTag3-/6-, HOTag3 and HOTag6 were removed.
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soluble fraction by applying certain solution conditions which promote phase separation, enabling isolation of target proteins simply by centrifugation. After removing the supernatant containing cellular contaminants, the target proteins can be recovered by dissolving the condensed phase through altering the solution conditions to promote the dissolution of the condensed phase. It is noteworthy that the proteins in the condensed phase are distinct from irreversible aggregation or inclusion bodies, in which the proteins are often inactive or lose most of their activities [129,166]. Taking advantages of the reversibility of a soluble-insoluble phase transition, LLPS provides a facil, economical, and scalable alternative to traditional methods for protein purification.

The ELPs which exhibit thermally triggered soluble-insoluble phase transition are the most widely used phase-forming IDRs for recombinant protein purification. One advantage of ELPs is that ELPs phase separate and dissolve under mild conditions. In addition, the phase transition temperature (*T*t) of ELPs can be tuned by altering the amino acids composition, the chain length, and/or the type and concentration of salt in solution [234]. This tunability makes ELP an excellent choice for the purification of diverse proteins. Moreover, the ability of ELP to phase separate reversibly is retained when fused to target proteins [235,236]. In a pioneering work, Chilkoti and Myer reported a nonchromatographic purification strategy for the purification of recombinant proteins by fusing ELP to target proteins [178]. In this study, the soluble fraction of the target proteins is first isolated from the insoluble cellular debris by centrifugation at temperature below the *T*t of ELP fusion. Then, salt addition and/or raising the temperate above *T*t lead to the phase separation of the target proteins into the condensed phase (insoluble). The phase separated target proteins then were separated from the soluble fraction by centrifugation again. The target proteins in the condensed phase are then recovered by reversing the phase transition through lowering the temperature of the solution below *T*t. While this process, namely inverse transition cycling, has been successful in purifying proteins with varying physicochemical properties and molecular sizes, it does have a drawback. Additional protease treatment is required for the removal of the ELP tag, which compromises the simplicity of the method. In view of this limitation, Wood and coworkers developed a strategy in which a self-cleaving intein domain is incorporated into the ELP-based protein purification system, enabling the self-cleavage and removal of the ELP tag from target proteins under mild conditions [179]. Although the intein containing ELP system is convenient and efficient, the limitation is that intein often suffers from premature cleavage during protein expression in vivo, resulting in the decrease of the final yields of target proteins. To solve this problem, the same group refined the self-cleaving ELP system by splitting the intein domain into two fragments: the N-terminal fragment of intein is fused to an ELP tag, while the C-terminal fragment is flanked by an ELP tag and target protein [180]. The cleaving activity of intein is active only if the two intein fusions were assembled in vitro by incubation. By controlled activation of intein, premature cleavage was avoided, and the final yields of the target protein were improved. In addition to intein, affinity ligand-ELP constructs have also been used to purify ELP tag-free proteins by affinity capture followed by phase separation [181,182]. For example, protein A was fused to ELP to capture and purify monoclonal antibodies [182]. Compared to direct fusion of ELP to target proteins, ligand-ELP-based affinity purification obviates the need for ELP cleavage.

4.6. Drug delivery

Phase-separated compartments have been used as a potential platform for drug delivery. The advantages of LLPS based drug delivery system including protection of drugs from degradation and denaturation, improvement of the pharmacokinetic properties, and enchantment of their biodistribution [183–185,237]. Drugs of various sizes and types, including small molecules, peptides, proteins, and nucleic acids, have been delivered by using LLPS systems made of different polymers [238]. However, for drug delivery systems based on chemically synthesized polymers, concerns have been raised about the potential adverse effects on human health, which raises questions regarding the biocompatibility and biosafety [239,240]. In comparison, protein and peptide-based LLPS systems, such like ELPs, are composed of naturally occurring amino acids and thus can be degraded into nontoxic byproducts by natural metabolisms [241.] Another advantage is that, in case of protein-based drugs, they can be simply attached to the protein-based delivery system by genetic modification. Furthermore, the fully genetically encoded carrier system offers unparalleled molecular precision without polydispersity, which represents an obstacle to the chemically synthesized polymers, enabling the fine tune of the physiochemical properties of the delivery system.

Phase separated proteins have been utilized as drug depots for sustained delivery of drugs. The advanatges of prolonged formulation include reduction of dosing frequency, improvement of patient compliance, and matainance of steady state drug levels for optimal therapeutic outcomes. This is particulary true for the treatment of chronic diseaes such as diabetess which requieres a routine administration of drugs. In a previous study, Amiriam and cowaorkers develoopped an LLPS-based injectable depot for sustained delivery of GLP-1 (Glucagon-like peptide-1), which is an anti-type 2 diabetic peptide [183]. Although GLP-1 posseses many attractive features, the short half-life hampers its application in the treatment of type 2 diabetes due to rapid renal clearance and proteolytic degradation [242]. The short half-life necessitates frequent injections, causing undesired side effects and poor patient compliance [243]. To achieve extended GLP-1 circulataion time, the anti-diabetic petide is fused to ELP. The author found that the GLP-1-ELP fusion exbited improved resistance to proteolysis compared to GLP-1 monomer owning to the higher molecular weight, providing a means for increasing the plasma half-life of the circulating peptide. Moreover, the ability of ELP domain to phase seprate was retained in GLP-1-ELP fusion, leading to the formation of a drug depot through a phase transition triggered solely by body heat post a single subcutaneous injection. The drug depot further enhanced the half-life of the GLP-1 fusion by providing sustained release of bioactive GLP-1-ELP fusion, consequetnly resulting in pronlonged glucose control in mouse model of type 2 diabetes for up to 5 days, 120 times longer relative to that of native peptide. Similarly, an ELP based drug delivery system for another antidiabetic drug (FGF21, Fibroblast growth factor 21) has been successfully designed, allowing for a significantly extended half-life [184]. The appllication of LLPS in sustained releae formulations is not limited to anti-diabetes drugs. For example, Shamji and coworkers reported a drug depot for sustained release of Interleukin-1 receptor antagonist (IL-1Ra) towards treatment of osteoarthritis by using ELP as drug carrier [185]. These studies demenstrate the utility and fexibility of LLPS based-drug delivery systems for sustained release of drugs.

LLPS have also been engineered for the targeted delivery of drugs for the treatment of cancer [186–189]. Liu and coworkers have developed a unique method for the brachytherapy of solid tumor based on LLPS, by which the therapetic phase-separating IDR-tagged radionuclides undergo a solube-insolube phase transition upon intratumoral injection and thus form a radioactive depots within the tumor [186]. Although brachytherapy has demonstrated potential advantages over systemic administration of anticancer agents, such as mild side effects and high local concentration of therapeutics, there are several disadvantages that need to be considered. These include the complex nature of placement procedures and, if necessary, the removal of the depot [244]. To address these issues, a biocompatible and biodegradble redionuclide-ELP conjugate with a *T*t of ~28 ℃ was designed and constructed, enabling the formation of an injectable depots. Particularly, redionuclide-ELP conjugates are solube when temperature is lower than *T*t and form a drug depot upon intratumoral injection due to body heat, eliminating the complex and unfavorable surgical procedures for implantation. In addition, it was shown that conjugation of ELP to therapeutic radionuclide and formation of viscous depot protected the radionuclide from dehalogenation, leading to a longer residence time (one week) in the

tumor. Moreover, the phase separated redionuclide conjugates inhibitited cancer cell growth by irradiating the tumor from the inside out with low systemic toxicity even at high does, resulting in the improvenent of survival in mice with implanted tumor xenografts relative to that of soluble, control radionuclides. To further improve the in vivo retention of the ELP-based therapeutic depot for solid tumor, a photosensitizer was introduced into the system, yielding a photoradiation-controlled intratumoral depot capable of photo-cross linking of the ELP- radionuclide conjuagtes into stable hydrogel when light is applied [189]. As an alternative to the body heat triggered formation of depot within tumor, targetted delivery of cancer drugs can also been achieved by locally heated the tumor region to temperature higher than body temperature [187,188]. In a proof-of-principle study, a thermally responsive ELP with a *T*t sighltly higher (41 ℃) than body temperature was designed [188]. It was shown that hyperthermia treatment of the tumors implanted in mice led to a ~2-fold increase accumulation of the thermally responsive ELP in tumors compared to the same ELP under normothermia. The combinantion of LLPS-based targetted delivery of cancer drugs with hyperthermia treatment is attactive since hyperthermia can synergistically enhance tumor cytotoxicity of the drugs [245]. These studies demonstrate that LLPS holds great promise for the development of effective drug delivery systems towards cancer treatment. This is especially significant considering the complex physiology and morphology of tumors, which pose significant obstacles to successful treatment approaches.

5. Conclusion and outlook

Compared to the biomolecular condensates in living systems and the complex coacervation of polymers in non-living systems, the design and construction of LLPS in synthetic biosystems is still in its infancy and several important challenges must be overcome before fulfilling the potential. One important consideration of this emerging technology is to develop fully biorthogonal phase-forming scaffolds. Although naturally occurring IDRs as well as folded proteins have been used for the synthetic LLPS, these phase-forming scaffolds may encapsulate unwanted biomolecules other than the targets through non-specific interactions, [129] which is particularly true in the context of complex intracellular environment, leading to the interference with the native metabolism and even lethality damage. Moreover, there is mounting evidence that LLPS are closely related to the pathological processes such as neurodegenerative diseases, cancer, and infectious diseases which raises the concern of the cytotoxicity of the natural IDRs-based phase forming scaffolds. In a recent study, the cytotoxic effects of the RGG scaffolds were analyzed by monitoring the OD600 of the engineered *E. coli* and no noticeable cytotoxicity was observed [24]. Similarly, one of the previous studies demonstrated that the PB1-AG fusion protein-based scaffolds had no observable cytotoxic effects on mammalian cells upon transient expression [158]. Nevertheless, further study is required to investigate the cytotoxic effects of LLPS on biological systems across long time scale. In addition, to avoid unintended client recruitment, it will be necessary to deepen our understanding on how natural biomolecular condensates recruit client and how to control the biomolecular components, which will inform the design of recruitment strategies with high efficiency and specificity in LLPS of synthetic biological systems. Besides, de novo designed phase-forming scaffolds will prove useful to prevent cross-reactivities [16].

Even though liquid-liquid phase separation (LLPS) leads to different applications such as catalyzing reactions, control of bioactivities and probing cellular signaling, the effects of hardening or aging on the functions of the phase-separated bioactivities in synthetic biological systems should be carefully examined. As we have discussed, previous studies have established that many phase-separated proteins undergo a process known as hardening or aging both in vitro and in vivo, through which the liquid-like materials become more solid-like which in turn alters the bioactivities within the condensed phase [246]. In other words, unlike the coacervation of polymers, most of the biomolecular condensates in living systems are far from equilibrium. However, whether the aging is also applied to LLPS in synthetic biological systems remains elusive and the effects of the time-dependent material properties on the encapsulated functions have yet to be systematically investigated in most of the previous works. For example, most of the LLPS-based reactors showed enhancements of reaction rates for the encapsulated enzymes, [21,24,30] but little is known whether the phase-defined reactors are also subjected to a phase transition process and how the aging process influences the enzymatic reactions. It is conceivable that the more solid-like reactors will counteract the enhanced reaction rates by lowering the diffusion of reactants and/or altering the conformation of the encapsulated enzymes akin to the enzymes immobilized on a solid matrix/support. Further study is needed to investigate the relationship between the functions and the time-dependent material properties of the LLPS in synthetic biosystems. In addition, it will be important to further our basic understanding on how nature maintains the LLPS away from hardening, which will prove useful for the development of a robust LLPS system for biological applications.

The ultimate goal of LLPS in synthetic biosystems is to spatiotemporally orchestrate chemical reactions in complex solution environments, like nature does. Living systems have offered tremendous inspiring examples on how to balance a wide range of thermodynamic interactions to form/dissolve LLPS upon request. Even though we are still far from understanding how living systems control LLPS, recent progress has demonstrated several factors that modulate the LLPS formation. Thanks to the progress, various LLPS have been designed and constructed in synthetic biosystems using proteins containing IDRs and folded proteins as phase-forming scaffolds. In parallel, LLPS in polymer solutions is a well-established research area. In contrast to living systems, LLPS in polymer solution is often at equilibrium state and the thermodynamic interactions are relatively simple and can be conveniently tuned. As a result, many mathematical models have been developed to describe the thermodynamic process. Despite the distinct difference between LLPS in living systems and polymer solutions, they both share the same thermodynamic fundamentals. Therefore, the available models for LLPS in polymer solutions could not only be applied to living systems but also synthetic biosystems.

The synthetic biosystem lies in between the living cell and the polymer solution. Not only can the progress in both systems provide key insights into the design and construction of LLPS in synthetic biosystems, but also the progress in synthetic biosystems can help to elucidate LLPS in living systems. More importantly, the uniqueness of synthetic biosystems may lead to the formation of LLPS under certain conditions that may not be achievable in either living systems or in polymer solutions, thereby making it a powerful platform for spatiotemporally manipulating chemical reactions to enable novel synthetic strategies and advanced functionalities.

Declaration of Competing Interest

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

Data Availability

No data was used for the research described in the article.

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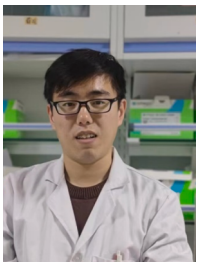

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