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REVIEW

Phase separation in synthetic biology

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***Background*: The concept of phase separation has been used to describe and interpret physicochemical phenomena in biological systems for decades. Many intracellular macromolecules undergo phase separation, where it plays important roles in gene regulation, cellular signaling, metabolic reactions and so on, due to its unique dynamic properties and biological effects. As the noticeable importance of phase separation, pioneer researchers have explored the possibility to introduce the synthetically engineered phase separation for applicable cell function.**

***Results*: In this article, we illustrated the application value of phase separation in synthetic biology. We described main states of phase separation in detail, summarized some ways to implement synthetic condensates and several methods to regulate phase separation, and provided a substantial amount of identical examples to illuminate the applications and perspectives of phase separation in synthetic biology.**

***Conclusions*: Multivalent interactions implement phase separation in synthetic biology. Small molecules, light control and spontaneous interactions induce and regulate phase separation. The synthetic condensates are widely used in signal amplifications, designer orthogonally non-membrane-bound organelles, metabolic pathways, gene regulations, signaling transductions and controllable platforms. Studies on quantitative analysis, more standardized modules and precise spatiotemporal control of synthetic phase separation may promote the further development of this field.**

Keywords: phase separation; synthetic biology; multivalent interaction; non-membrane-bound organelle; signaling transduction and amplification

Author summary: A substantial amount of physical theories and biological experiments have been developed to uncover the underlying principles of phase separation in biology. The synthetic condensates through phase separation have been used to implement many specific functions in biological systems. In the future, more biomedical functions related to phase separation can be explored, more diseases triggered or accelerated by phase separation may be treated better, more artificial applications can be realized by synthetic condensates.

INTRODUCTION

Since the beginning of life, compartmentalization has been an important criterion to ensure the functioning of biological systems [1]. A cellular compartment has two important properties [2]: (1) it is a boundary that separates the biochemical reaction milieu from its surroundings, so that chemical reactions can be completed in a relatively independent space, without interference from the surroundings, (2) the components within must diffuse quickly and freely, ensuring that biochemical reactions take place inside. Some organelles require membranes to form a physically enclosed environment, called membrane-bound organelles, such as lysosomes [3] and mitochondria [4]. Although membrane-bound organelles are indeed stable and common, they are

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difficult to synthesize in biological systems. Sometimes the membrane itself is not necessary to implement functions, and the formation of a compartment without a membrane is guided by simple physical principles which are easier to regulate and perform better under some conditions. These compartments such as nucleoli [5], stress granules (SGs) [6,7], processing bodies (P-bodies), RNA and protein-containing bodies in embryos of *Caenorhabditis elegans* [8], promyelocytic leukemia (PML) bodies, germ granules, nuclear speckles or Cajal bodies [9], are called non-membrane-bound organelles [1].

In the field of physical chemistry, the transformation of a single-phase system into a multiple-phase system is defined as phase separation. In thermodynamics, chemical materials flow to regions with lower chemical potential rather than lower concentrations [10,11], leading to uniform distribution of components. Edmund Beecher Wilson anticipated that the cytoplasm might include "a mixture of liquids" with "suspended drops of different chemical nature", which gave researchers a new concept of the cellular physical environment [12]. In later years, researchers speculated that phase separation might be associated with certain diseases and proposed novel theories. Until 2009, phase separation was observed in the rapid fusion and diffusion P-bodies [8], indicating that phase separation in living cells provides a way to concentrate certain molecules and exclude others to create order in the crowded chaos of living cells (Fig. 1).

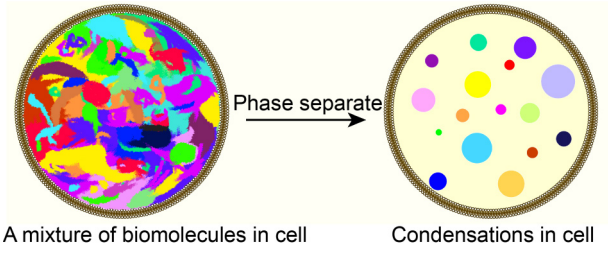

Figure 1. Phase separation in biological systems. Cell is the basic unit of organism structure and function. How to assemble various components in the right time and space to carry out their corresponding functions is a problem that needs to be solved. Cells have evolved a series of organelles, including membrane-bound organelles, such as nuclei, lysosomes, etc., and non-membrane-bound organelles, such as nucleoli, stress granules. The conversion of a single-phase system into a multiple-phase system is defined as phase separation in thermodynamics.

FROM NATURAL BIOLOGY TO SYNTHETIC BIOLOGY

In recent years, phase separation was frequently found in both physiological and pathological conditions [13]. Phase behaviors play an important role in gene regulation, cellular signaling, the formation and maintenance of synapses, as well as the pathophysiology of neurodegenerative diseases [14]. Comprehensive understanding of phase separation in biological systems can help synthetic biologists understand the mechanisms and design principles of phase separation.

In transcriptional elongation and mRNA processing, numerous factors compartmentalize the transcription apparatus through phase separation [15–19]. Phase separation can selectively recruit different components and regulate the phosphorylation of domains in different cellular positions [20–23]. This property can be used to trigger and control synthetic biological processes. In chromatin structure regulation, phase separation provides an explanation for the regulation of chromatin structure [24–26] and compartmentalization [27–29]. Phase separation of heterochromatin protein 1 (HP1) [30], histone H3K9 methyltransferase (SUV39H1) [31–33] and tripartite motif containing 28 (TRIM28) [34] integrates modification and recognition, which leads to a continuous positive feedback loop [35], resulting in H3K9 methylation and spread of HP1 along the nucleosome DNA [36,37]. This in turn leads to the compartmentalization of chromatin and the formation of regions that inhibit recombination and transcription [33]. The H3K9 trimethylation (KDM4A) inhibition promotes heterochromatin compaction and slows DNA replication, and finally activates tumor-cell-intrinsic immunity in squamous cell carcinoma (SCC) [38]. Moreover, linker histone H1 also contributes to phase separation of heterochromatin domains in cells. However, the interaction between H1 and other proteins such as HP1 is more important in phase separation of heterochromatin organization [39]. Therefore, different cooperative functional components can form condensates through phase separation.

Phase separation is widely utilized in the initiation of cellular signaling [40,41]. The localization and recruitment of membrane receptors, adapter proteins, ligands of adaptor proteins is a process of phase separation through multivalent interactions, which eventually leads to the formation of a complex compartment on the cytomembrane [42–45]. This process is rate-limiting and called gamma-distribution which is referred to in stoichiometry as "kinetic proofreading" and inhibits the spontaneous membrane localization of ligands and prevents spontaneous signal activation [42,44]. Phase separation due to cellular signaling can also occur in the cytoplasm. In the innate immune system, DNA arising in the cytoplasm binding to cyclic GMP-AMP synthase (cGAS) induces phase separation to form robust liquid-like droplets where cGAS is activated. This DNA-

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induced phase separation driven by the multivalent interactions between DNA and the DNA binding domains of cGAS [46,47] enhances the production of cyclin GMP-AMP (cGAMP) and activates stimulator of interferon genes (STING), which is essential for controlling the expression of host defence genes [48].

Phase separation is also closely related to the formation and maintenance of synapses in the nervous system. Rab3-interacting molecule (RIM) and RIM-binding protein (RIM-BP), which are considered to be among the main organizers of active zones, form condensed and dynamic assemblies through liquid-liquid phase separation (LLPS) via specific multivalent bindings [49–51]. In addition, LLPS provides a possible mechanism for the construction of presynaptic active zones and postsynaptic densities [52,53]. This highly enriched and dynamic structure can further guide researchers to understand synaptic plasticity by modulating phase separation and the physiology leading to diseases when it is disorganized.

Dynamic reorganization of subcellular space may explain pathological protein aggregation in neurodegenerative diseases [54,55]. Fused in sarcoma (FUS), a prion-like protein containing intrinsically disordered domains (IDR) is associated with the neurodegenerative disease Amyotrophic Lateral Sclerosis (ALS). During pathogenesis, IDR can undergo phase separation and the transition from liquid droplets to aggregation is accelerated by pathogenic mutations [56,57]. LLPS, which is driven by the positively charged microtubule-binding domain of tau, is based on coacervation with negatively charged molecules. This process promotes amyloid formation, which can lead to the occurrence of Alzheimer's disease (AD) [58–60]. In Huntington's disease, a poly-glutamine (polyQ) region and a proline-rich region in huntingtin protein responsible for the disease [61], also cause phase separation regulated by the protein concentration and the length of the polyQ region [62]. In addition to AD and ALS, protein aggregates are also pathognomonic for Parkinson's disease (PD) and frontotemporal dementia (FTD) [63]. Thus, the process from health to disease could be recreated by synthetic biologists through phase separation and the transition from liquid to aggregates should be more thoroughly studied. Disturbing this transition by synthetic biology is a potential therapeutic principle for patients.

Synthetic biology aims to recombine and even *de-novo* design new artificial life forms with specific functions [64]. It provides an ideal tool to optimize functions that are already available in natural systems, as well as implement artificial functions which are not found in natural systems [65]. At the same time, an artificial system is a potential "to build to learn" platform for understanding biological processes like biochemical reactions, signal transduction, biological evolution, and even the origin of life [66]. Synthetic biology is concerned with two main aspects: on the one hand searching for interchangeable modules from natural living system that can be assembled into artificial system [67], while on the other hand using artificial modules to produce emergent behaviors, creating artificial life and functions.

Phase separation is a phenomenon that should capture the interest of synthetic biologists, since: (1) The components can undergo phase separation from the surrounding environment or even form non-membrane-bound organelles which can implement certain functions without disturbing other components outside the organelles, or more precisely, it can limit the design of artificial functions to one compartment to reduce the impact on native biological systems. (2) Phase separation has a large dynamic range, fast kinetics and it can be triggered by certain inducers, which makes it an ideal potential platform for the detection of target signals in biological systems. (3) A liquid-like state induced by phase separation in living cells is a good model for certain biological behaviors as the material in the liquid-like state can diffuse freely. These processes are fast enough within the compartment that biochemical reactions can take place [2], which means that the formation of a liquid-like phase provides a potential reaction hub for artificial synthesis. Thus, we focus on LLPS in synthetic biology. (4) Phase separation is a critical process in the formation of living systems. It has been postulated that coacervates formed by phase separation were vital in the origin of life [68], which is the process of forming a multi-molecule system from biomolecular components. Phase separation-based synthesis of RNA oligonucleotides, selection of long RNA molecules by ribozyme catalysis and protection from the external environment are all plausible roles for LLPS in the primordial earth [69]. This implies that introducing phase separation into artificial living systems is a rational way to mimic their natural genesis and study the origin of life. Therefore, phase separation is a suitable mechanism for regulating biological processes, and increasing numbers of studies confirmed its existence and properties in natural biological systems [70]. Consequently, designing and regulating phase separation artificially can help researchers better understand and control natural biological systems.

THE PHYSICAL PRINCIPLE OF PHASE SEPARATION

In order to have a thorough understanding of phase separation, we consider the free energy and the chemical potential first. In thermodynamics, the basic definition

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of free energy is the energy available in a system to work. A system tend to approach a state that has the minimum free energy. The basic definition of chemical potential is the energy per molecule. The chemical potential identifies the necessary work that must be done to add one certain molecule to a system, and describes the tendency to change the number of the component molecules in a system. Thus, there is a tendency to reduce the number of molecules in a certain type when the chemical potential is higher. The function of free energy in a system of non-interacting solute molecules is unimodal. Therefore, when different components are attached to each other, phase separation does not occur (unimodal). However, in the presence of interactions disfavoring the close proximity of different components, phase separation occurs (multimodal) (Fig. 2A). Consequently, components can sometimes undergo phase separation but sometimes not. Materials flow to regions with lower chemical potential rather than lower concentration [10,11], which is why the components form organelles in the cell instead of distributing uniformly. In the case of mixing, the chemical potential curve becomes monotonic, and in the case of phase separation, the chemical potential can be equal for two different compositions, so that a de-mixed state is thermodynamically stable (Fig. 2B).

Whether a solution is able to undergo phase separation in the cell, and whether phase separation is nucleation-limited or diffusion-limited depends strongly on the concentration, the type of solution, and environmental factors including temperature, pH and salt type, etc. [71]. Phase diagrams are used to define a series of conditions that result in 1-phase regime or 2-phase regime, and nucleation or spinodal decomposition. The binodal line shows the boundary whether molecules are or not able to undergo phase separation and it describes the region where phase separation is thermodynamically favorable. When the component concentration is lower than the saturation concentration, the component is diffuse in solution. Beyond binodal line, the system is in the one-phase region. Within the binodal line is a spinodal line. The spinodal line shows the boundary between two different states: (1) nucleation-limited phase separation, a metastable region where phase separation can only occur under sufficiently large global perturbations; (2) diffusion-limited phase separation, a stable region where phase separation can occur under any local perturbation (Fig. 2C). This thermodynamic concept that a single fixed saturation concentration is a necessary feature for phase separation through homotypic interactions, such as protein-protein interactions via IDRs. However, phase separation through heterotypic multicomponent interactions, such as protein-RNA interactions are very common in biology. Phase separation through heterotypic multicomponent interactions does not exhibit a fixed saturation concentration. It is governed by high-dimensional phase behavior whose saturation concentration values vary with component concentrations [72]. Moreover, the saturation concentration can also be controlled by changing the valency of molecular assembly and/or the affinity between molecules. A framework for multivalent interactions influencing the process of phase separation has been developed and it demonstrated that increasing valency or affinity of components is more likely to enable phase separation at lower concentrations [10].

The properties of phase separation, including liquid, solid, gel, and aggregates, are determined by the mode of interaction and concentration of interacting molecules. A Liquid-like droplet rearranges its components within the droplet and exchanges its components with the surroundings at short time [2]. LLPS is reversible. Liquid-like droplets can exhibit fusion or diffusion, and it can exhibit fluorescence recovery after photobleaching (FRAP). The shape of a liquid is defined by surface tension and it can be modified easily [73]. A liquid has no memory of its shape. We use the concept of viscosity to describe a liquid. Therefore, it is very common for liquid-like droplets to exhibit spherical shape under 3D imaging. A solid has an arbitrary shape and it has a memory of its shape for very long times. This property is called shear elasticity, which does not occur in a liquid-like droplet. The rearrangements of components in a solid are extremely rare [2]. A solid is irreversible. Gels can be divided into two classes: chemical gels and physical gels. The bonds linking the subunits of chemical gels are covalent chemical bonds. While, the bonds linking the subunits of physical gels are physical interactions. Biological gels are typical examples of physical gels. In physics, a gel is formed by reversible cross-links, which are held together by weaker interactions than a solid [74]. A gel can exhibit both solid-like behavior and liquid-like behavior. It has the properties of viscous and elastic. Gel-like phase separation is reversible, although the molecules are arrested. Liquid-like phase separation and gel-like phase separation can further transition to protein aggregates, which often occur in pathological amyloid-like fibers in neurodegenerative diseases [63,73,75,76]. Components in the aggregates stick together with a strong interaction that does not allow rearrangement. The large aggregates are made up by many units and each unit consists of many protein molecules [74,77]. These protein molecules are denatured clusters which contain a number of proteins with β-sheets [74,77]. Aggregates are irreversible, hence the molecules are arrested (Fig. 2D).

The separation of a solution phase depends on the concentration, identities of the molecules, and also the

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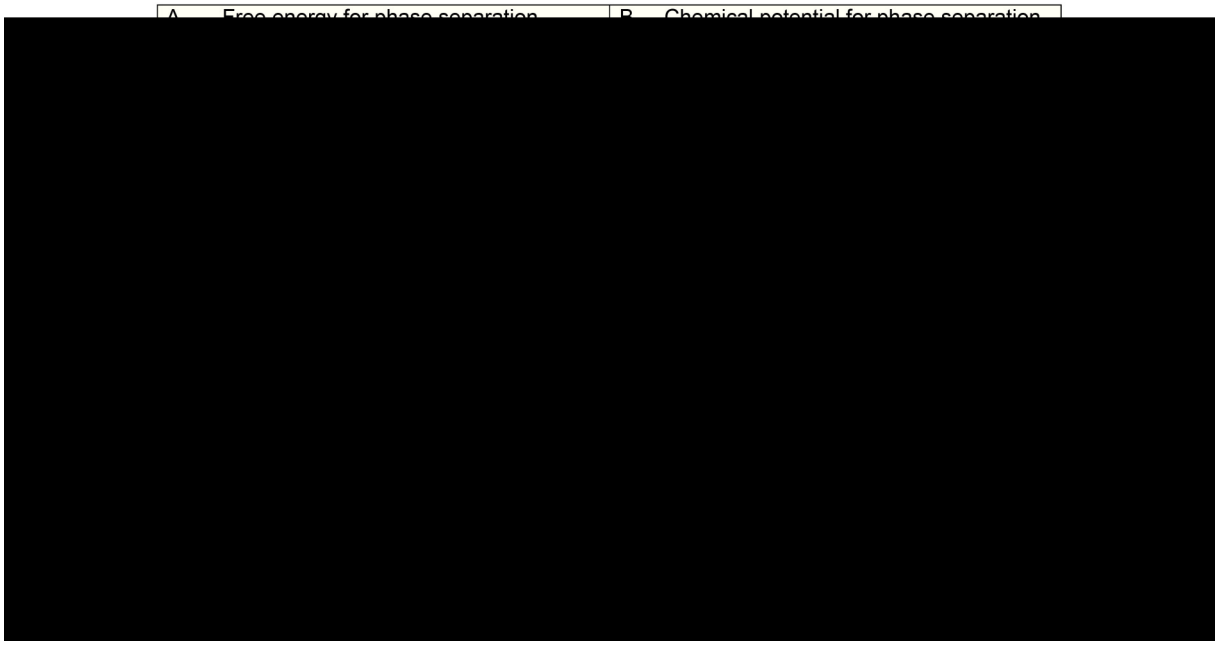

Figure 2. Thermodynamics of phase separation in biology. (A) Phase separation does not occur on the unimodal line (blue curve). However, when solute molecules interact (dashed curve and arrow), phase separation occurs on the multimodal line (green curve). (B) The chemical potential curve becomes monotonic (blue curve) in the case of mixing. However, when solute molecules interact, the chemical potential curve becomes non-monotonic (dashed curve and arrow), the demixed state is thermodynamically stable (green curve) when phase separation takes place. (C) The binodal line shows the boundary between 1-phase regime and 2-phase regime. The spinodal line shows the boundary between nucleation-limited phase separation (the region between the spinodal line and the binodal line) and diffusion-limited phase separation (the region under the spinodal line) (D) The properties of phase separation, including of liquid, gel and aggregation is determined by interactions strength, the binding affinity, and concentration.

environmental conditions. These conditions include salt type, temperature, co-solutes, pH, and the volume excluded by other molecules [58,78–82]. Notably, this is true in both natural and synthetic biological systems, whereby all factors in the surrounding medium influence the interaction strength among molecules [73]. For example, ubiquilins (UBQLNs) undergo LLPS under physiological conditions, but the change occurs more easily with NaCl and increasing protein concentrations [83]. In order to learn more about the physical properties of LLPS in biological systems, it is useful to recapitulate physical phase diagrams. Phase diagrams can show concentration-dependent thresholds for assembly [84], which are easy to regulate by tuning promoter intensity. In the future, regulating the physical environment may be used as an effective approach to regulate the occurrence of phase separation.

IMPLEMENTING AND REGULATING PHASE SEPARATION IN SYNTHETIC BIOLOGY

Phase separation is an essential process for the formation of condensates. Thus a pivotal step in the synthesis of organelles is to induce and regulate phase separation both *in vivo* and *in vitro*. In recent years, researchers have systematically studied methods for achieving phase separation and established many ways to regulate phase separation.

Implementing phase separation in synthetic biology

Based on the formation of condensates via phase separation in natural biological systems, the specific domains or proteins undergoing phase separation can be purified from natural systems. Researchers systematically propose methods to design artificial non-membrane-bound organelles through LLPS in synthetic biology. These are mainly based on multivalent interactions, which can occur in numerous different types of molecules, including proteins with multiple domains interacting with other partners, and through IDRs that have multiple sites interacting with other components [85,86]. There are two main ways to implement multivalent interactions, either by introducing multivalent interacting structures,

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such as intrinsically disordered regions (IDRs) from natural proteins, or designing multivalency and interacting structures artificially using repeating motifs.

Intrinsically disordered regions

Intrinsically disordered regions (IDRs) are key drivers of LLPS [87,88], especially in natural biological systems [89] (Fig. 2A). IDRs are special protein domains with multiple motifs [80,90–92], interacting with each other through van der Waals forces, electrostatic attraction, and hydrophobic effects [93] (Fig. 3A). In natural biological systems, many RNA-binding proteins have modular architectures in which different IDRs are appended to various RNA-binding domains, including RNA recognition motifs (RRMs) and motifs rich in arginines and glycines (RGG repeats) [94,95], and enzymatic domains [73,96]. These motifs drive phase separation, whereby their multivalent binding to RNA can further promote this process.

In synthetic biology, introducing IDRs is a common method to induce LLPS. Phase separation of a protein of interest can be achieved by fusion to FUS [96–98], an RNA-binding protein involved in RNA transcription, splicing, transportation and translation [99,100]. FUS undergoes physiologically reversible and rapid phase separation between hydrogel, liquid-like and dispersed states [57,101–104], which are stabilized by hydrogen bonding formed by the core residues 39–95 in the low-complexity domain [105]. In the light-based control of synthetic organelles, fusing the N-terminal IDR of FUS to Cry2 leads to rapid phase separation [96]. Similarly, fusing the N-terminal IDR of FUS to PixD or PixE leads to reversible phase separation [106]. In designed membrane-less organelles enabling codon reassignment of selected mRNAs, proteins that are essential for translation fused with FUS can undergo phase separation [98].

In addition to fusing FUS to proteins of interest, there are several other strategies to form condensates via phase separation. Folded modular binding domains and β-structures are also critical to phase separation [86,105,107]. For example, low-complexity aromatic-rich kinked segments (LARKS) [108,109], can undergo phase separation through multivalent interactions of β strands [108,110]. Prion-like domains (prLDs) in non-prion RNA-binding proteins or prion proteins [105], containing amyloid cross-β structures, can also undergo phase separation [111]. However, β character is seldom observed in liquid droplets formed through LLPS [112,113]. These structures can potentially be used to induce condensates via phase separation by fusing these specific domains to proteins of interest in synthetic biology.

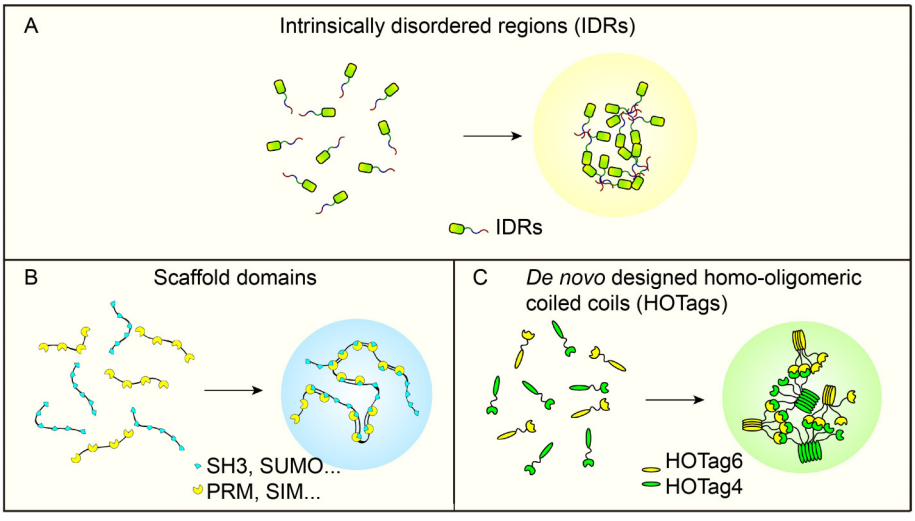

Figure 3. Three designs to achieve phase separation in biological systems. Generally, phase separation can be undergone by interactions and multivalency. (A) IDRs are multiple interacting motifs, called intrinsically disordered regions, which is also a main way to drive LLPS both *in vivo* and *in vitro*. (B) Multivalent interactions are the main methods to achieve phase separation both *in vitro* and *in vivo*. Many proteins with oligomeric interactions (such as SH3-PRM, SUMO-SIM) can be constructed into multiple repeats. Oligomeric interactions induce the aggregation of specific proteins and expand the chemical or biological components involved in the process of phase separation, while multivalency enables formation of oligomeric assemblies. (C) *De novo* designed homo-oligomeric coiled coils, called HOTags, can also drive multivalent interactions, not limited by the difficulties to DNA combination, and not making the protein extremely large. HOTag4 can self assemble into hexamer after expression. HOTag6 can self assemble into tetramer after expression.

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Designing artificial multivalency and interacting structures

The phase-separation behavior of IDRs is critically reliant on multivalency and interactions [85,86]. A classical example of multivalency implemented by protein architectures encoding multivalent domain interactions is lipid bilayers of protein clusters, which contains the membrane-bound adhesion receptor nephrin, as well as two cytoplasmic partners, the cytoplasmic protein N-WASP and adaptor protein Nck [114]. Nck has three SH3 domains that bind multiple proline-rich segments in N-WASP, as well as one SH2 domain that binds to multiple phosphotyrosine sites in nephrin, leading to the formation of a condensate under the membrane [115]. This separation is governed by the phosphorylation degree of nephrin, indicating that phase separation in living cells can be controlled by kinases [116].

Several independent multivalent pairs were demonstrated to undergo phase separation *in vitro*, including (1) proteins with repeats of human SUMO3 (polySUMO) and proteins with repeats of the SUMO interaction motif (polySIM) [85,117] (Fig. 3B); (2) proteins with repeats of the second SH3 domain from Nck (polySH3) and proteins containing repeats of a proline-rich motif (PRM) from Abl1 (polyPRM) [85,116] (Fig. 3B); as well as (3) the PTB proteins (containing four RNA recognition motifs [RRMs]) and RNAs with five repeats of the RRM recognition element UCUCU (polyUCUCU) [116,118].

However, these independent pairs are limited by the underlying design. Overly strong binding affinity between the components may make the complex extremely large and poorly regulated. This may disturb the structure and impact its functions due to steric hindrance. In recent studies, *de novo* designed homo-oligomeric coiled coils, called HOTags, were demonstrated to also be able to drive multivalent interactions (Fig. 3C). Most *de novo* designed coiled coils are short peptides of about 30 amino acids [119] that can self-assemble after transcription. Therefore, they are ideal tags for implementing multivalency. Previously, several coiled coils were characterized in protein *de novo* design studies [119–121]. In order to implement high multivalency, coiled coils with high stoichiometry are preferred. These coiled coils can form tetramers, pentamers, hexamers, and heptamers, which have been fully characterized in recent works [122].

Changes in the binding valency will shift the landscape and material properties of condensates. Increasing and decreasing valency through genetic manipulations can impact some vital quantifiable aspects of phase separation including concentration thresholds, size, location, or material state (liquid-like or gel-like) of the droplets [71]. For example, in an *in vitro* system of scaffold stoichiometry dictating client recruitment [85], clients are poor competitors of the interaction between scaffolds. Therefore, only the scaffold that is in stoichiometric excess will have free sites which are accessible to its cognate client in either phase. The biological behavior of intrinsically disordered arginine/glycine-rich RGG regions is valency-dependent. Its transition temperatures differ significantly depending on the number of RGG repeats. Compared to single and tandem RGG, triple RGG phase separate more readily and form condensates below higher temperatures [123].

Phase separation on the basis of interaction can occur spontaneously or after stimulation. In artificial systems, it can be triggered by stimuli such as small molecules, light and phosphorylation. Interactions between different proteins without multivalency are insufficient to induce and maintain phase separation and then form condensates, but they can induce phase separation and expand the chemical or biological components involved in the process of phase separation.

In conclusion, proteins undergo phase separation through multivalent interaction, which can be achieved through various methods, including fusing IDRs to proteins of interest, designing artificial multivalency and interacting structures, or fusing folded modular binding domains and β-structures to proteins of interest. These approaches offer a number of potential strategies to regulate phase separation in synthetic biology.

Regulating phase separation in synthetic biology

Phase separation can be induced exogenously or occur spontaneously. There is an increasing toolbox of dimerization modules that can regulate phase separation. Small molecules and phosphorylation of proteins in cellular signaling are commonly used to induce phase separation. Light control is widely used to trigger phase separation due to its spatial and temporal regulation properties [96,106,124,125]. The physical environment, including protein concentrations, temperature, pH, and the surrounding medium, also plays an important role in the regulation of phase separation.

Spontaneous phase separation

Phase separation can occur spontaneously. Small ubiquitin-like modifier (SUMO), acting in post-translational modifications is such an example. Previous studies have shown that SUMO mediates protein-protein interactions by binding to a SUMO-interaction motif (SIM) on receptor proteins [126]. This interaction is primarily mediated by a stretch of four residues

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containing 3−4 hydrophobic amino acids (I, V or L), which is a conserved property of SIMs [127]. Therefore, multivalent SUMO and SIM have been used as the core system for spontaneously induced phase separation *in vitro* [85]. However, inducible phase separation is also needed, especially in synthetic biological systems.

Small molecule regulation in phase separation

The interactions between certain components that can undergo phase separation must to be triggered by small molecules. For instance, the interaction between FKBP (12-kDa FK506 binding protein) and Frb (FKBP-rapamycin binding domain) can be robustly induced by rapamycin. Multivalent FKBP and Frb, assembled using HOTags, can undergo phase separation when incorporating rapamycin [122]. Another commonly used group of small molecules, phytohormones, have also shown potential for regulating phase separation. Abscisic acid (ABA) is a major phytohormone that regulates plant stress responses, and its receptors are proteins from the PYR-PYL-PCAR family [128]. Previous structural and biochemical studies have provided a working model for PYL-mediated ABA signaling [129]. In the absence of ABA, type 2C protein phosphatases (PP2Cs), including ABI1 and ABI2, are fully activated and pyrabactin resistance like (PYL) proteins are present as inactive homodimers, which are unable to bind and inhibit PP2Cs, resulting in an incompatible conformation of the CL2loop. Following ABA induction, the CL2loop undergoes a structural rearrangement that brings it closer to the ABA-bound pocket, so that the interaction between PYLs and PP2Cs can be implemented [130].

Moreover, recent pharmaceutical research has shown that lipoamide and its analogues, such as lipoic acid, can regulate phase separation of FUS protein, and their efficacy was verified at different expression levels in HeLa and iPS cells, as well as *in vivo* in *C. elegans* and *Drosophila* [131]. The treatment of neurodegenerative diseases remains challenging, and there is no doubt that this research brings new ideas for the treatment of neurodegenerative diseases.

Light control in phase separation

Light is a powerful tool for synthetic biology, due to its special properties as an inducer, including the fact that: (1) light control can be triggered by specific light, which is an orthogonal and easily implemented system; (2) light control can be made completely reversible in living cells even after multiple cycles of activation [96]; (3) light control enables precise temporal and spatial control of intracellular processes, which highly desirable for many applications in life sciences [124]; (4) light control has a fast response, which can take effects even in seconds [96]. Due to these benefits, light control was applied to induce local condensations in cells. These local condensations, which arise via controlled changes in multivalency and interaction triggered by light, are called optodroplets. Some of these optodroplets are reversible and represent a powerful potential way to achieve a better understanding of biological systems [132]. Several light control systems are available [133–135], which use engineered multivalency, like IDR or scaffold proteins, and light-triggered protein-protein interaction, like PhyB-PIF [132], Cry2 [96,124,136], and PixE-PixD [106,134] to drive LLPS in specific regions in living cells. In addition, fusing IDRs to Cry2 leads to rapid light-induced localized clustering through phase separation, which exhibits a threshold effect in terms of light intensity and concentration [73,96]. The *Arabidopsis* red-light-inducible phytochrome (PhyB-PIF) system is a common optogenetic tool, which comprises the phytochrome B (PhyB) protein and the basic-helix-loop-helix (bHLH) transcription factor phytochrome interaction factor (PIF; PIF3 or PIF6) [137]. This pair of proteins are induced to bind under far-red light, and the binding is reversed within seconds of exposure to far infrared light. However, it is otherwise stable for hours in the dark. A generalizable light-inducible organelle targeting system, triggered by PhyB-PIF, which is fast, reversible and titratable [132]. Therefore, the interacting pair PhyB-PIF is a good tool to trigger reversible condensates via phase separation [136]. Light control induces condensates not only through light-triggered protein-protein interaction, but also through light-triggered protein cleavage. A composite construct containing 2 RGG domains, a photocleavable protein (PhoCl), and a solubilization domain form condensates containing 2 RGG domains upon 405 nm illumination [138]. Moreover, light control can not only induce protein condensates but also disperse coacervated proteins. PixELLs (Pix Evaporates from Liquid-like droplets in Light), an light control system for the disassembly of proteins, exhibited spatial memory and was able to convert shallow gradients into sharp boundaries [134].

APPLICATIONS OF PHASE SEPARATION IN SYNTHETIC BIOLOGICAL SYSTEMS

Phase separation has been used widely in synthetic biology in recent years. Optodroplets have been developed as a potential tool for studying biological systems. In the future, researchers may be able to identify drug targets using optodroplets to recreate the pathology of diseases that are associated with phase separation [139].

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This technique allows researchers to observe neurodegeneration in real time. In research tools for biological functions, phase separation can be used as a signal amplifier because of its specific dynamic properties, and it can also be visually detected by coupling to fluorescent proteins. For biological functions, phase separation can regulate branching metabolic reaction and increase product of interest [106]. Inspired by natural biological systems, phase separation is likely to promote positive feedback and kinetic proofreading in cellular signaling, enabling the development of rapid and precise signal transduction. Even more encouragingly, researchers recently designed artificial non-membrane-bound organelles via phase separation to use natural and synthetic amino acids to produce proteins with new functions [98], providing a potential route toward customized organelles that can be used to implement orthogonal translation and protein engineering in living cells.

Signal amplification and visualization

Condensate formation is controlled by scaffold components, providing a mechanism for signal amplification. Owing to the amplification effect of phase separation, weak signals and low concentrations of inducing chemicals can potentially be visually detected, especially with the assistance of fluorescence imaging technology (Fig. 4A). As mentioned above, oligomeric interactions can trigger phase separation. Phosphorylation is an important modification of proteins, especially those related to cellular signaling. This natural mechanism attracted the attention of scientists' studying artificial systems. A phosphorylation-based reporter system was built based on the formation of a liquid-like phase, which is called separation of phases-based activity reporter of kinase (SPARK). Scientists described GFP-based kinase reporters whose phase separation is induced by kinase activation via multivalent interactions, forming intensely fluorescent droplets [122]. These receptors are reversible while offering high brightness, large dynamic range, and fast kinetics. They have kinase substrate peptides that are phosphorylated upon kinase activation and then bind to a phosphopeptide-binding domain, with the resulting multivalency leading to highly concentrated (10×) EGFP droplets. Researchers fused the protein kinase A (PKA) substrate sequence to EGFP followed by HOTag3, and fused forkhead-associated domain 1 (FHA1) to HOTag6. FHA1 and the substrate undergo a PKA activity-dependent protein-protein interaction, and the two HOTags, HOTag3 and HOTag6, introduce multivalency. After the activation of PKA, multivalent protein-protein interactions are induced, leading to the formation of EGFP droplets via phase separation.

Designer non-membrane-bound orthogonally translating organelles

A major goal in the engineering of artificial biochemical reactions inside cells is to develop techniques to design and manufacture organelles and proteins that do not negatively affect the native cellular systems. Synthetic organelles are a simple appendage that allows the organism to customize new pathways and behaviors in a controlled way. Recently scientists developed a non-membrane-bound organelle through phase separation, where only one type of mRNA, stop codon suppression machinery, and ribosomes, realize spatial targeting to only synthesize specific proteins [98]. Protein translation is a complex multiple-step process in which different aminoacylated tRNAs, cognate tRNA synthetases, ribosomes and numerous other factors must collaborate to synthesize polypeptide chains. Thus, modifying protein translation or other cellular processes, such as transcription and post-translational modification, is a great challenge in synthetic biology and also a goal of many scientists. Inspired by the concept of phase separation, a non-membrane-bound organelle was generated and shown to be able to synthesize proteins containing non-canonical amino acids [98] (Fig. 4B). It includes the following components: (1) An mRNA-targeting system, whose mRNA is fused to two ms2 RNA stem loops, creating an mRNA::ms2 fusion encoding the protein of interest. Ms2 loops bind specifically to major capsid protein (MCP) to form a specific and stable mRNA::ms2-MCP complex in cells; (2) A tRNA/RS suppressor pair, which enables the coding of noncanonical amino acids; (3) The assembler, which is the key component required to create a dense phase or condensate, in which all of the parts mentioned above can form an orthogonally translating organelle. A major innovation of this synthesis system is the ability to use special materials to implement synthetic biological functions in a limited space, called synthetic organelles, with minimal impact on the host, which means that these synthetic organelles are more like natural organelles and adaptable to the cellular environment. This standardized design approach for constructing artificial organelles can be used in biological research, allowing the concentration of customized tasks into a specifically designed non-membrane-bound organelle. Since these synthetic organelles restrict biochemical reactions to a compartment, they can be used as a nanoscale bio-reactor, isolating toxic intermediates and allowing the artificial degradation of certain proteins.

Membrane-bound organelles and non-membrane-bound organelles can be accommodated in a cell at the same time, and designed to allow them to interact. The interaction between non-membrane-bound organelles

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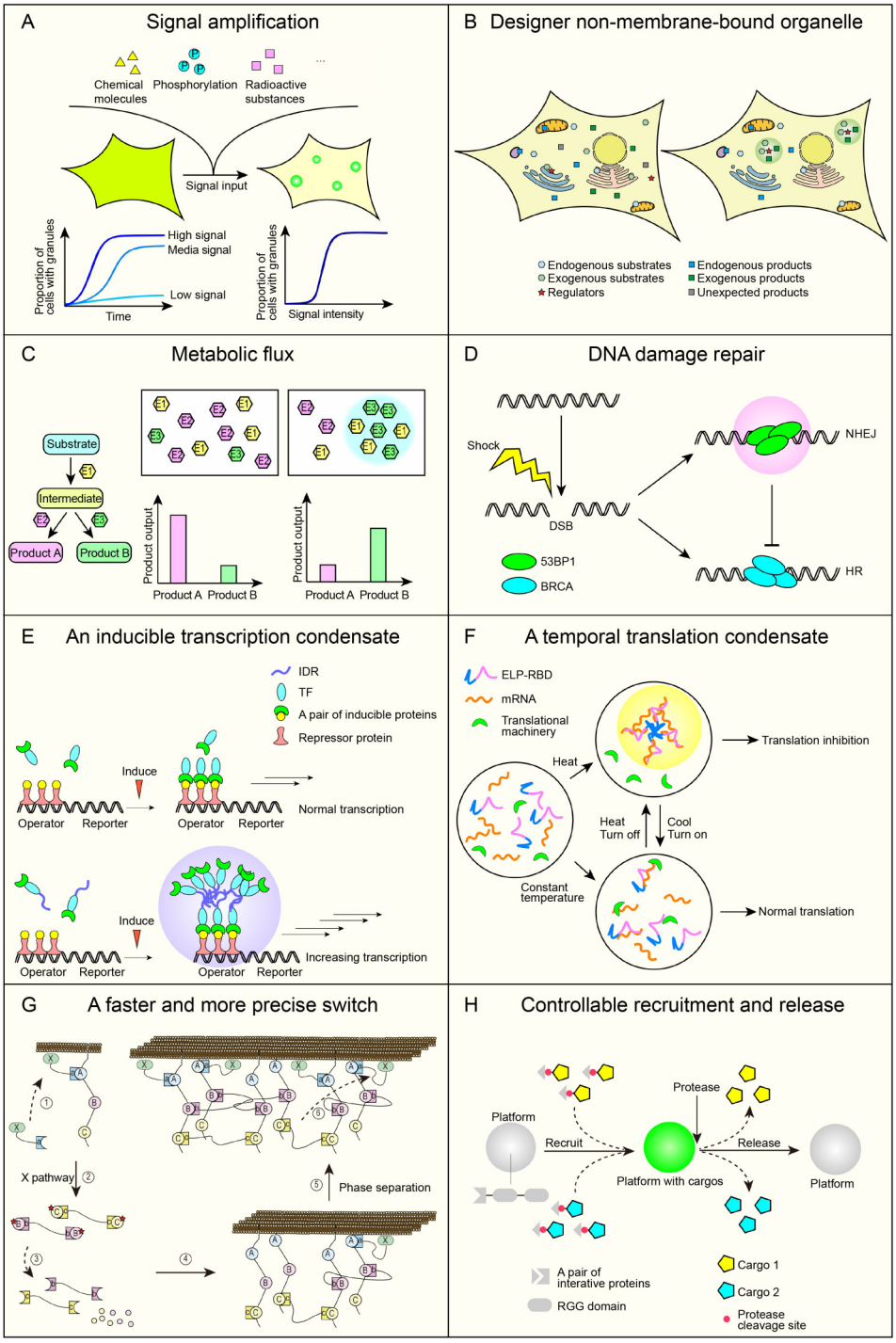

Figure 4. Applications of phase separation in synthetic biology. Phase separation has unique and excellent properties in biological function realization. (A) A schematic of signal amplification. Phase separation can be used as a signal amplification because of its unique dynamic properties of high sensitivity and fast response. (B) A schematic of designer non-membrane-bound orthogonally translating organelles in cell. Endogenous metabolites and exogenous metabolites are phase separated which provide each biochemical reaction a relatively isolated but interconnected compartmentalization. (C) A schematic of metabolic flux in a branched pathway and the regulation of phase separation in the branched pathway. (D) A schematic of regulation DNA damage repair pathway. (E) A schematic of an inducible TFs condensate for increasing transcription activation and gene expression. (F) A schematic of a synthetic condensate for temporally "turn on" or "turn off" translation. (G) A schematic of faster and more precise synthetic switch in cellular signaling systems. (H) A schematic of controllable recruitment and release of cargo. Cargos with interaction domains can be recruited into a platform and released under the induction of proteases.

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and membrane-bound organelles may be divided into several categories: (1) membrane-bound organelles can be partitioned into condensates via phase separation for storage and transport; (2) membranes can provide a platform for condensates assembly and even regulate their dynamics; (3) condensates can transport on membrane-bound organelles via phase separation and the translocation of cargos across membrane-bound organelles can be modulated by phase separation [140]. For biological functions, they may exchange substances, or even complete a biochemical reaction together. In natural biological systems, the broadly expressed RNA-binding protein TIS11B forms a non-membrane-bound organelle, called TIS granule, enriching membrane protein-encoding mRNAs with multiple AU-rich elements. TIS granules enable the translation of mRNAs with AU-rich elements at an ER subdomain, and specific protein-protein interactions can only occur in TIS granules [141], representing the first known non-membrane-bound organelle interwoven with a classical membrane-bound organelle. In the future, researchers will continue to explore the correlation between membrane-bound and non-membrane-bound organelles, and more examples in natural biological system may be found. Although no artificial non-membrane-bound organelles interwoven with classical membrane-bound organelles have been designed to date, it is not only a new way to study the nature of phase separation, but also a novel way to expand the application prospects of phase separation in synthetic biology.

Determining the direction of biological pathways

Tight regulation of cellular metabolism is essential for all life on earth [142]. The main purpose of metabolic engineering is to divert natural cellular metabolites to synthetic pathways in order to maximize the synthesis of target products. Traditional approaches rely on overexpressing the enzymes that are required for the formation of the product, and reducing the expression of competing enzymes However, the overexpression of engineered pathways often leads to the accumulation of toxic intermediates, and strong protein overexpression itself can be deleterious for cell growth [106]. Thus, to achieve high production requires each enzymatic reaction in the pathway to be balanced, which can limit the accumulation of toxic intermediates and prevent loss of intermediates to branching pathways. Mathematical models and experimental studies demonstrated that enzyme clustering can induce more targeted metabolic fluxes to solve these problems. The intermediate from the first step can have a higher probability of reaction through the enzyme in the second step before diffusing away from the cluster when sequential enzymes in the pathway are co-localized [143]. Designing protein scaffolds that recruit metabolic enzymes can increase the effective concentrations of intermediates and thus increase the metabolic flux, while limiting the amounts of intermediates below toxic levels [97]. This scaffolding strategy builds up a substrate channel, which can (1) prevent the loss of intermediates to competing pathways or diffusion; (2) protect unstable intermediates from solvent and decrease the transit times of intermediates; (3) circumvent unfavorable equilibria and kinetics imposed by bulk-phase metabolic concentrations, and finally, increase the synthesis of the target products.

LLPS has the property of clustering and condensing many substances. This property results in a different reaction environment and different concentrations between the resulting non-membrane-bound organelles and the bulk intracellular medium, which can increase the reaction rate. In a recent study, light-controlled systems were used to trigger the assembly and disassembly of active enzymes [106]. Light-switchable clustering, which is induced by the interactions of Cry2 photolyase homology domain under irradiation at 450 nm, enhanced product specificity 18-fold and the production efficiency 6-fold by decreasing the accumulating of intermediates and reducing competing metabolic fluxes (Fig. 4C). In addition to branching pathways, synthetic condensates of sequential enzymes in one single pathways can also significantly accelerate the biocatalysis efficiency and increase the final product [144].

Phase separation can also be used to regulate the DNA damage response pathway and may help reduce CRISPR off-target effects. Phase separation determines liquid-like behavior of DNA repair compartments in DNA damage response (DDR). Numerous DDR proteins undergo phase separation and form a complex compartment to repair damaged DNA [145]. The DDR is triggered by DNA damage sensor proteins, which are then recruited to DNA break sites [146]. The adaptor protein MDC1 binds γH2AX to assemble the ubiquitin ligases and recruits p53-binding protein 1 (53BP1) [147–149]. 53BP1, whose C-terminus is sufficient for the formation of liquid-like droplets and includes an oligomerization domain, undergoes phase separation at DNA break sites and forms a liquid-like droplet together with MDC1 and γH2AX [150]. The phase separation of 53BP1 not only promotes the recognition and repair of DNA damage sites, but also controls the expression of p53 targeted genes, integrating DNA damage recognition with cell fate decision [150,151].

CRISPR is a highly efficient and widely used gene editing technology, which is based on the induction of DSB at a specific target sequence, which is then repaired using the endogenous DNA repair mechanisms. As Cas proteins only play a role in inducing the DSB,

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controlling the repair process is important for regulating the direction and success rate of gene editing. Phase separation may help control this procedure. There are two main repair pathways dealing with DSB in eukaryotic cells: non-homologous end-joining (NHEJ), which can re-ligate the broken ends of a severed DNA molecule [152]; and homologous recombination (HR), which requires large stretches of very similar or even identical DNA sequences elsewhere in the genome to serve as templates for DNA repair [153]. The 53BP1 protein, which antagonizes the resection of DSBs, preserves DSB ends and thereby promotes NHEJ [147]. In natural biological systems, regulating 53BP1 retention or recruitment at DSBs causes the cell to push the DSB repair pathway toward either HR in S-G2 or NHEJ in G1, by controlling the extent to which a DSB is resected [154]. Based on these studies, artificially regulating phase separation of 53BP1 may convert HR to NHEJ, and thereby direct the cell fate. It is possible that when 53BP1 undergoes an artificial phase separation at the broken DNA end, the cell preferentially engages NHEJ, which increases the productive rate of random mutations and indels, whereas disrupting phase separation of 53BP1 can force cells to induce HR, which is more precise and promotes genetic stability (Fig. 4D).

Synthetic condensates for gene regulation

Controlling gene regulation *in vivo* and *in vitro* revolutionizes our understanding of cell behavior and accelerates invention of precise gene therapies. Inspired by natural phase separation in gene regulation that the condensate of C-terminal domain of RNA polymerase II, coactivators and transcription factors (TFs) at super-enhancers (SEs) ensure robust transcription of essential genes [20,22], a synthetic TFs condensate has been designed in mammalian cells and mice. The synthetic TFs condensate is based on TetOff system, which consists a tetracycline operator (TetO), a tetracycline repressor (TetR) that constitutes the DNA binding domain, a transactivating domain (TAD), and a nuclear localization signal (NLS) [155]. Both dynamical model and experiment result show that a higher local concentration of TAD increases reporter expression [136]. The synthetic TF- consists TetR, TAD and NLS, while the synthetic TF+FUS is connected with the N-terminal IDR of FUS. Consequently, TF+FUS forms condensates at the TetO sites, leading to the locally increased TAD density, and then increasing promoter activity and gene expression [136]. Moreover, fusing light-inducible proteins with TF+FUS can design a light-inducible TFs condensate to increase transcription activation (Fig. 4E).

Another way to control gene expression is to regulate the translation process. In order to provide extrinsic control of translation, a synthetic condensate temporally inhibiting translation has been designed in protocells [156]. The RNA-binding domains (RBD) fused with elastin-like polypeptides (ELPs) forms condensates in response to heat. ELPs are *de novo* designed stimuli-responsive peptide polymers composed of repeat domains [157–159]. Proteins fused with ELPs have the property of thermoresponsiveness that is the lower critical solution temperature (LCST) phase behavior [156,160,161]. This approach is a good way to implement phase separation of target proteins. After heating to a temperature above the cloud point temperature (T_{cp}), RBD-ELP co-phase-separate with mRNA chains into liquid-like condensates. These protein-mRNA-rich condensates are spatially separated from translational machinery components, and finally inhibit the migration of mRNA chains (Fig. 4F). Changing the temperature to $T>T_{cp}$ or $T<T_{cp}$ can "Turn-On" or "Turn-Off" the translation, which temporally regulates translation in a programmable manner [156].

Developing a faster and more precise switch for synthetic cellular signaling systems

In natural cellular signaling through membrane receptors, LLPS can suppress accidental activation and trigger high-frequency activation. Consequently, it is an ideal switch to turn on cellular signaling at the level of membrane receptors. In synthetic biology, scientists hope to achieve precise and quantitative regulation of cellular signaling. An ideal potential switch is not only conducive to the study of dynamic biological properties, but also conducive to the construction of precise artificial receptors and corresponding cellular signaling. Moreover, LLPS enables the formation of positive feedback loops, which introduce a self-stabilizing function and rhythmic properties into cellular signaling. In the future, such synthetic signal switches may be used in biological and clinical medicine applications such as *in vivo* drug delivery and targeted-therapy. Unlike normal cells, cancer cells have mutations that lead to the constitutive activation of intracellular signaling pathways [162,163]. Thus, phase separation of membrane receptors, adaptor proteins and the ligand of the adaptor protein based on the kinetic proofreading system may be able to specifically sense cancer cells and release drugs to kill them.

In addition, researchers developed a tunable light-inducible system based on the synthetic interaction between the LOV2 domain and an engineered PDZ domain [164], which can recruit Ste5 to the cell membrane and trigger cellular signaling. Inspired by this trigger system, protein-protein interactions can be combined with Ste5, and multivalent interactions can be

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introduced between transmembrane proteins and adapter proteins, which can gradually undergo phase separation under the membrane, leading to a positive feedback loop. This in turn can speed up cellular signaling in synthetic biological systems. Here, we provide a potential design. ① Protein X is recruited to the membrane by protein-protein interactions (for example, interaction between the LOV2 domain and PDZ domain induced by light). ② Process ① activates downstream signal transduction (for example, the MAPK pathway), here we call it X pathway activity to identify the downstream signaling activity induced by the recruitment of protein X to the membrane. ③ Proteins B and C in the cytoplasm are linked to a label (for example, degron), which degrades when X pathway is activated. Then, adapter proteins b and c are released. ④ Released proteins b and c bind to proteins B and C attached to the membrane. ⑤ Process ④ leads to clustering and gradually realizes phase separation in three-dimensional space under the membrane. ⑥ Phase separation of transmembrane proteins A-B-C can help recruit more proteins X. Consequently, the recruitment of proteins X by protein-protein interaction is faster than Process ① and it activates downstream signaling, which comes back to ② (Fig. 4G). This process constitutes a positive feedback loop, producing a faster and more precise switch for cellular signaling systems with a higher response rate to upstream signaling.

A platform for controllable recruitment and release of cargo

A major goal of synthetic biology is programmable regulation and control of biological behavior through sequential logic. Condensates formed by phase separation can be used as a platform for the precise recruitments and release of target proteins. Liquid-like droplets can be logically controlled by proteases. Respectively placing tobacco etch virus (TEV) protease recognition sequence (termed x) and human rhinovirus 3C protease (HRV3C) recognition sequence (termed y) between the two intrinsically disordered arginine/glycine-rich RGG domains in RGG-RGG (resulting in RGG-x-RGG-y-RGG) produces an AND gate. The liquid-like phase behavior of single, tandem, and triplet RGG in turn dampens the response. Therefore, droplets treated with TEV (or HRV3C) protease alone remain condensates at room temperature, while the addition of the other protease in the next step leads to the disassembly of droplets [123].

This logically controlled condensate can be used as a platform to recruit and release cargo through phase separation. In this case, the cargo is placed between the two RGG domains. These three parts are linked by two different protease cleavage sites (resulting in RGG-x-cargo-y-RGG). Consequently, the cargos can be recruited through phase separation and released by proteases. It should be noted that there are also other efficient designs to implement a platform for controllable recruitment and release of cargo through phase separation. By fusing a pair of interacting proteins, one with multivalent RGG and one with the cargo, and then placing protease cleavage sites between the cargo and interacting proteins can also be used to recruit and release cargo through phase separation [123] (Fig. 4H). These versatile systems have applications in the compartmentalization of proteins, which enables the construction of synthetic dynamic platforms with programmable and controllable phase behavior in living cells.

PERSPECTIVES OF PHASE SEPARATION IN SYNTHETIC BIOLOGICAL SYSTEMS

Many issues must be resolved before phase separation finds wider applications. More efforts are needed to clarify the underlying physicochemical and biological mechanisms and propel further utilization of phase separation.

Quantitative analysis in living cells and technical improvement

As an organizing principle in living cells, phase separation not only allows us to examine known phenomena from a new perspective, but also brings new hope to the field of synthetic biology. However, the physiological environment in the cell is highly complex, it is often still unclear whether there is a real causal relationship between the regulation of phase separation and biological functions, and how the phase separation itself is precisely regulated is also incompletely understood [165,166]. Some problems impede the development of applications based on phase separation: (1) Many experiments are based on the results of *in vitro* protein studies [10]. Phase separation may be possible in tube, and proteins at very high concentrations may also be able to undergo phase separation in a cell, but the physiological relevance of such phenomena in natural systems is unclear. Compared with phase separation *in vitro*, it is hard to control phase separation *in vivo* via different parameters because nontrivial controls and parameters like the concentration of proteins of interest are required to ensure that the results are not due to secondary effects. (2) Many *in vivo* experimental results are induced by over-expression. Researchers evaluated the data from the most recent 33 studies, collectively making claims for 50 examples of *in vivo* LLPS for a range of cellular systems and organisms. It was found

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that 34 examples were the result of over-expression, which can itself cause significant phase separation, in which molecules tend to thermodynamically separate to the high-concentration phase from the low-concentration phase. Moreover, slight overexpression can cause significant changes in molecular phenotypes [165]. (3) It is difficult to regulate changes in key parameters *in vivo* and conduct quantitative research. Consequently, many experiments are overly reliant on descriptive feature evidence, such as spherical droplets, or fusion and diffusion between droplets. Most studies are indirect and observational. Not only are there no statistics, but most studies are based on protein overexpression data [165]. (4) FRAP is practically the "gold standard" in the field of LLPS, but technical limitations in terms of the required instrumentation restrict its wider application. Furthermore, measurement and analysis of key parameters in phase separation such as molecular diffusion coefficient and molecular concentration is still not convenient enough.

In the future, phase separation should be further explored in the following aspects: (1) Techniques for verifying phase separation need to be further developed and improved. (2) More *in vivo* experimental evidence should be gathered to collect more parameters *in vivo*. (3) More quantitative data rather than descriptive results should be provided. Recently, a novel method for studying salt-dependent rheology and surface tension of protein condensates has been developed. Optical traps can be used to characterize the rheology and the surface tension of a major component of P granules, and the results demonstrated that protein condensates display viscoelastic liquid-like material properties influenced by salt concentration. Thus, this novel method may enable more quantitative studies leading to a more comprehensive and deeper understanding of the physics of protein condensates and their behavior in cells [167]. This is expected to be a major trend of phase separation research in synthetic biology.

Developing more standardized modules for phase separation

The methods for inducing phase separation in synthetic biological systems are based on two main strategies: proteins with multiple domains, or proteins with IDRs that have multiple sites. However, in some cases, phase separation cannot be achieved when functional or targeted proteins are connected with these modules. This may be due to structural differences such as steric hindrance of the protein that prevents the phase separation. Therefore, more standardized modules that can induce and better regulate phase separation should be developed. More polymers similar to HOTags found in nature or *de novo* designed tags that can be used to achieve multivalency should be developed. For oligomeric interactions, more methods of induction, such as ion-based control should be implemented to expand the applications of phase separation, and develop more modules with higher orthogonality, higher sensitivity and faster response. For example, the super uranyl-binding protein (SUP) interacts with uranyl with extremely high affinity and selectivity. A protein network with multivalent SUP was designed to gather and harvest uranyl [168]. Ion-based control is a conditional trigger that can be used to sense and report ions. Phase separation-based ion reporters or enrichment devices would be more precise and sensitive. Thus, ion-based control would be a valuable tool for implementing phase separation in synthetic biology.

The physical and chemical properties of IDRs that can undergo phase separation need to be further studied and more proteins containing IDRs or *de novo* designed IDRs that can undergo phase separation are needed. Prediction of phase separation properties and sequence analysis for phase separation can direct the exploration of proteins that are likely to undergo phase separation *in vivo* or *in vitro* [117,169,170]. Some analysis tools and phase separation predictors were developed by biophysicists, such as DrLLPS [171], LLPSDB [172], PhaSepDB [173], PhaSePro [174], PSPredictor [71,170,175]. In the future, prediction of phase separation properties and sequence analysis for phase separation may play a crucial role in phase separation in synthetic biology.

Precise spatiotemporal control of phase separation

In order to further understand the molecular mechanisms that drive phase separation in biological systems, researchers need techniques to find, control, and regulate phase separation in living cells. In previous studies, researchers regulated the aggregation degree of different liquid components in living cells to induce the emergence and dissolution of liquid-like droplets [176,177]. For example, light control is suitable for clarifying how signaling dynamics are converted into cellular responses. Researchers can change the dynamics of pathway activation while monitoring the influence on downstream processes in living cells in real time. Recently, it was shown that repeated, optimally-spaced pulses of Erk activity driven by light could be used to maximize target gene expression [178]. Moreover, the optimal frequency for gene expression coincides with that observed in living cells cultured under highly proliferative conditions [178,179]. Electrogenetic control is a new generation of tools allowing precise spatiotemporal control. Electrogenetically controlled

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cellular insulin release for real-time control of glycemic has been developed [180]. Electrical pulse stimulation leads to membrane depolarization and calcium influx, which activates the calmodulin/calcineurin pathway, which induces the expression of the gene of interest. A promising strategy would be to express a protease gene under electrical pulse stimulation. Which would then leads to the disassembly of droplet composed of RGG-x-RGG-y-RGG, as discussed above. Therefore, small molecules and electrogenetic controls also allow precise control. Thus, due to their properties and phase separation characteristics, these special controls can be regulated temporally, leading to fast or slow, intermittent or constant, temporary or chronic activation, which can be used as a more precise signal or a more quantitative reporter in biological systems.

CONCLUSION

Phase separation has brought great changes to synthetic biology, both in research methods in biotechnology and in the implementation of biological functions. Here, we summarize several developed and still to be developed applications of phase separation in synthetic biology (Fig. 5). Thanks to its special dynamic properties, phase separation can be used as a faster and more precise switch in cellular signaling systems, both in natural organisms and in synthetic biology. Many modules can be used to form synthetic non-membrane-bound organelles through phase separation, which can separate endogenous and exogenous reactions to make them harmless to cells. Moreover, because of the physical properties of the liquid state, LLPS can be used as a nanoscale bioreactor that focuses metabolic fluxes to increase the synthesis of target products. Based on its high sensitivity, fast response and visual detection, phase separation can be used as a sensor or a reporter. Moreover, light-based phase separation was used as a valuable tool for the basic study of biological systems. Phase separation in gene regulation has been studied by many scientists. The regulation of gene expression, post-transcriptional modification and protein synthesis by

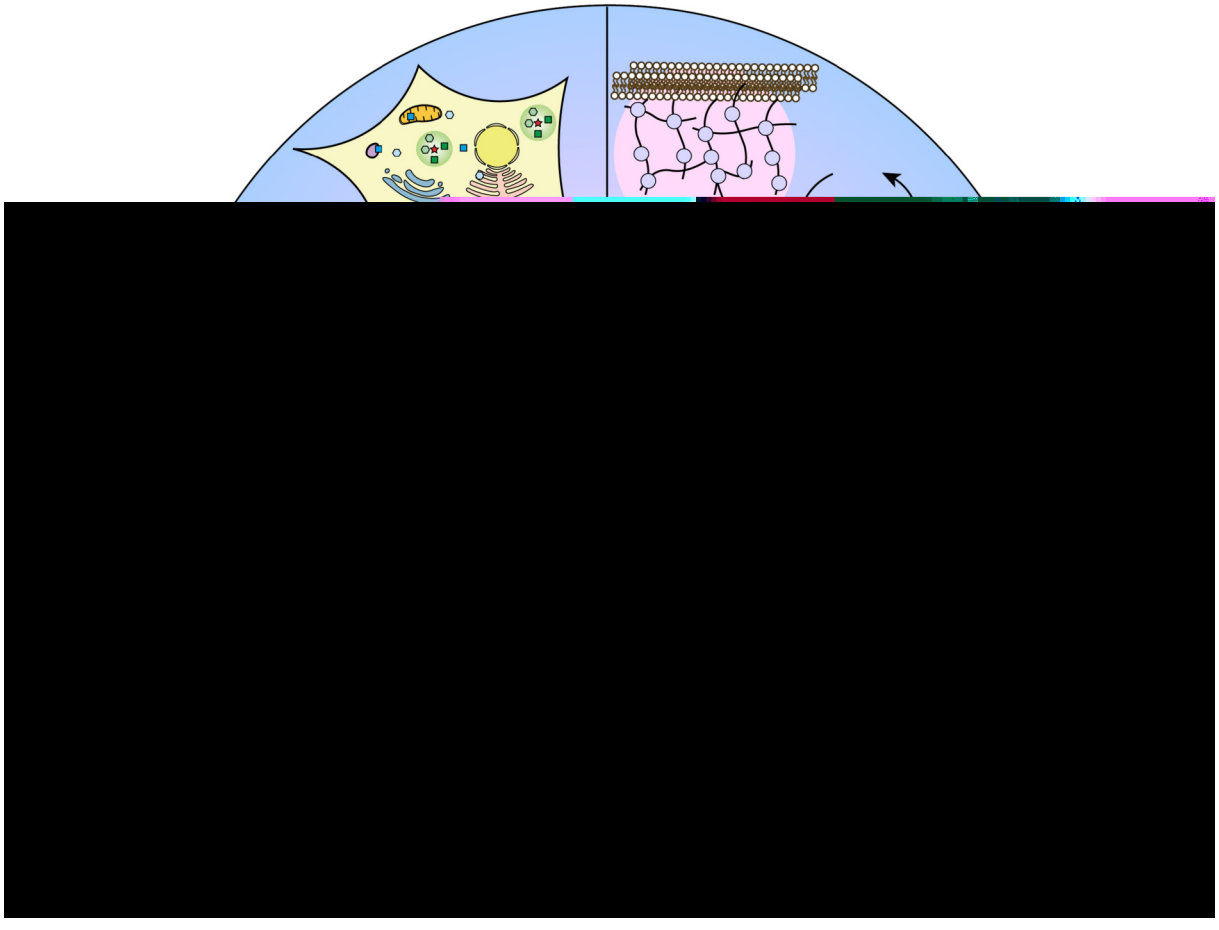

Figure 5. Phase separation in synthetic biology. Phase separation in synthetic biology has many potential functions in signal transduction, metabolic reaction, spatiotemporal control, gene regulation, designer organelle, sensor and detector.

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controlling the process of phase separation, which may provide new ideas for the treatment of diseases, is just around the corner.

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COMPLIANCE WITH ETHICS GUIDELINES

The authors Shuyu Shi, Wen Si, Xiaoyi Ouyang and Ping Wei declare that they have no conflict of interests.

This article is a review article and does not contain any studies with human or animal subjects performed by any of the authors.

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