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Current Opinion in Biotechnology

Designer protein compartments for microbial metabolic engineering

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Protein compartments are distinct structures assembled in living cells via self-assembly or phase separation of specific proteins. Significant efforts have been made to discover their molecular structures and formation mechanisms, as well as their fundamental roles in spatiotemporal control of cellular metabolism. Here, we review the design and construction of synthetic protein compartments for spatial organization of target metabolic pathways toward increased efficiency and specificity. In particular, we highlight the compartmentalization strategies and recent examples to speed up desirable metabolic reactions, to reduce the accumulation of toxic metabolic intermediates, and to switch competing metabolic pathways. We also identify the most important challenges that need to be addressed for exploitation of these designer compartments as a versatile toolkit in metabolic reprogramming.

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Current Opinion in Biotechnology 2024, **85**:103062

This review comes from a themed issue on **Tissue, Cell & Pathway Engineering**

Edited by **Wilfred Chen** and **Millicent Sullivan**

Available online xxxx

https://doi.org/10.1016/j.copbio.2023.103062

Introduction

Compartmentalization of specialized biological processes is a fundamental feature across all domains of life. Often de□ned as organelles, these compartmentalized structures are classi□ed as phospholipid membrane-bound, protein-shelled, or membraneless (phase- separated) [1]. Historically, the term 'organelle' has been strictly assigned to the cells of eukaryotic organisms; however, we now know that bacteria also possess such subcellular structures (Figure 1), including membrane-bound magnetosomes, protein-shelled carboxysomes, and phase-separated nucleolus-like compartments [2]. Among these organelles, proteinaceous compartments are of particular interest due to their relatively simple constituents, well-studied assembly mechanisms, and signi□cant roles in metabolic regulations, thus making them models for metabolic ef□ciency, speci□city, □exibility, and an inspiration for biomimetic design [3–5].

Engineering microbes for sustainable production of high-value products is a great achievement of metabolic engineering [6]. Target products can be biosynthesized in new hosts by constructing novel metabolic pathways composed of combinations of heterologous enzymes. However, the efforts to generate these products tend to be hindered by bottlenecks that can arise from low enzyme activity and mismatched kinetics due to undesirable microenvironments [6]. Moreover, metabolic interferences can occur between the endogenous and heterologously introduced pathways, potentially resulting in detrimental effects on the pathway yields and cellular physiology. To overcome these challenges, an increasing number of compartmentalization strategies have been developed for organizing cellular metabolism, with the aims of accelerating the rates of intended metabolic reactions, reducing the accumulation of toxic metabolic intermediates (which leads to cellular toxicity), and switching from one metabolic pathway to alternative competing or branching pathways in a controllable manner [3,7,8].

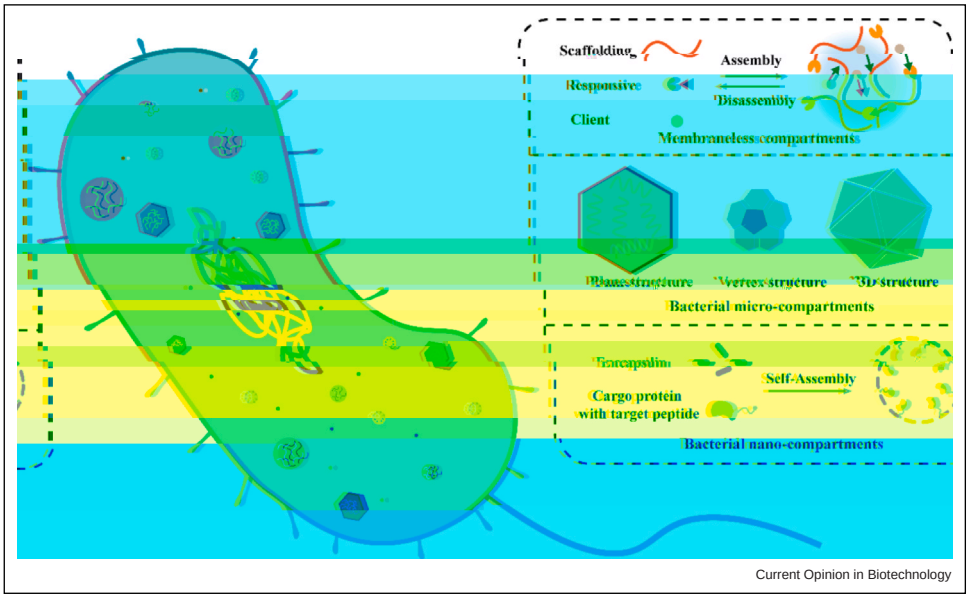
In this review, we □rst introduce the general principles of constructing protein-based compartments, and then discuss how to engineer them for spatial organization of metabolic pathways to speed up desirable metabolic reactions, to reduce the accumulation of toxic metabolic intermediates, and to switch competing metabolic pathways. Finally, we discuss the challenges and perspectives to realize the huge potential of designer protein compartments for metabolic reprogramming in living microbial cells.

Two major types of protein compartments

Protein-shelled compartments

Most prokaryotes rely on protein-shelled compartments to achieve spatial control of cellular metabolism. The protein shells serve not only as protective packaging that concentrates the enclosed enzymes but also as an effective means to mitigate the impact of the external

Figure 1



Protein-based compartments in prokaryotic cells: MLCs and protein-shelled micro- and nanocompartments. The MLCs formed via liquid–liquid phase separation of scaffolding proteins, and the protein-shelled compartments formed by self-assembly of single- or multiple shell proteins.

environment [2,4,9,10]. A growing number of examples of these compartments in nature have been reported, which are diversi□ed by their structures, morphologies, and the metabolic reactions they catalyze [9]. One major class is the bacterial microcompartments (BMCs), which range in size from 40 nm to 600 nm [10]. These compartments have selectively permeable protein shells, which enable diverse organisms to optimize their metabolic processes involved in both anabolic and catabolic metabolism. The only known anabolic BMC is carboxysome [11], which encapsulates the enzymes ribulose-1,5-bisphosphate carboxylase–oxygenase and carbonic anhydrase to enhance CO_2 □xation inside many cyanobacteria and chemoautotrophs. Catabolic BMCs are widely found across bacterial phyla that are usually involved in the metabolism of various organic compounds, such as propanediol utilization BMC and ethanolamine utilization BMC.

Another class is the virus-like nanocompartments called encapsulins, which comprise 60 or 180 copies of a single self-assembling capsid protein giving rise to a diameter of 20–24 nm and 30–32 nm, respectively [5,12]. In general, the nanocompartments possess a less- complex composition and a smaller size than those of BMCs, which are bene□cial for the de novo design and construction. The biological functions of encapsulins are closely related to iron metabolism and defense mechanisms against diverse stresses, which depend on the type of their encapsulated biomolecules. For example, ferritin-like proteins are often encapsulated in the nanocompartments to store iron and protect bacteria from oxidative stress [5].

Phase-separated protein compartments

In addition to the protein-shelled compartments, membraneless compartments (MLCs) have recently been discovered in both eukaryotic and prokaryotic cells [13,14]. Examples include the P-bodies, stress granules, clusters of bacterial RNA polymerase, and cell division proteins, which have been demonstrated to be involved in many cellular processes, such as gene regulation, stress responses, and cellular signaling [15,16]. The formation of these MLCs in cells is generally attributed to biological liquid–liquid-phase separation (LLPS) driven by multivalent interactions of proteins/RNAs [17]. Thus, the MLCs are also called biomolecular condensates. Because of the lack of membrane and shell structures, LLPS-mediated compartments possess higher permeability and faster rearrangement of molecules within the condensates. Moreover, the formation and dissolution of the MLCs can occur rapidly and reversibly in a few seconds [18].

Synthetic construction of protein compartments

Building blocks

The understanding of the principles of compartment assembly provides access to the strategies for constructing synthetic compartments. Usually, the naturally existing

protein compartments are composed of two types of building blocks. One is the scaffolding proteins that are necessary for compartment assembly, while the other is nonessential for compartment formation and termed clients that often localize in the compartments to endow biological functions.

For the formation of BMCs, their shells are usually composed of three distinct scaffolding proteins, including the hexameric BMC shell protein (BMC-H), trimeric BMC shell protein (BMC-T), and pentameric shell protein (BMC-P). BMC-H and BMC-T are somewhat similar according to their amino acid sequences, and form the same basic, hexagonal disk. These two shell proteins assemble into the flat facets of one particular BMC shell that occupy the bulk of the total shell. In addition, they both have large pores that can be up to 14 Å in size in their centers to ensure metabolite flow across the BMC shell. In contrast, BMC-P adopting a flat, five-sided disk shape, is limited to capping the vertices of icosahedrons, and therefore regarded as minor shell components [10]. To help researchers discover and predict new scaffolding proteins capable of self-assembly into BMC shells, a reference database of all currently known examples of BMCs has been developed by surveying their occurrence in all the available genome sequence data [19,20].

In contrast to the shells of BMCs, a typical encapsulin shell comprises a single scaffolding protein that self-assembles into an icosahedral structure [12]. Interestingly, all the encapsulins possess the viral HK97-like fold named after the major capsid protein fold found in the bacteriophage HK97. Despite strong structural similarity between encapsulins and virus capsids, they share little sequence homology. To date, three different encapsulin architectures have been observed and classified by their size and triangulation number describing the complexity of a capsid [5,12].

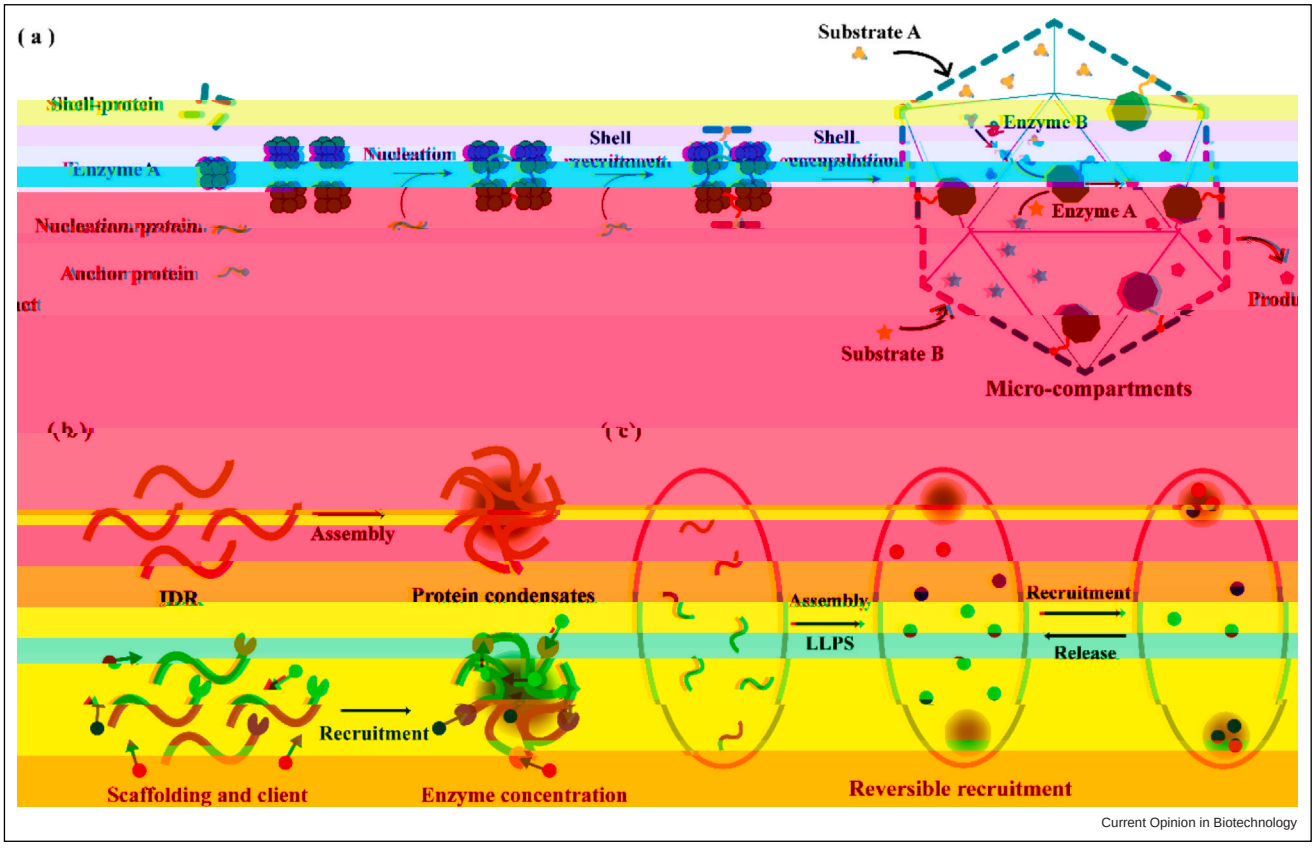
The types of scaffolding proteins with LLPS propensity are more diverse than those of shell proteins. In eukaryotes, well-known examples include the P-granule protein a DDX3 family RNA helicase found in P granules, RNA-binding proteins FUS and TDP-43, and RNA helicase DDX4 [21]. Many candidates have also been identified in prokaryotes, such as the polar-organizing protein Z [22], DNA-binding proteins Dps and Single-stranded DNA-binding protein [14], and bacterial RNA polymerase [23], although some of them need further evidence to prove their LLPS capability intracellularly with endogenous expression levels. The growing evidence also suggests that α-carboxysome CsoS2 protein [24] and β-carboxysome protein CcmM [25] can phase-separate in vitro, which may play a role in carboxysome biogenesis and carbon-fixing function. More intriguingly, artificial disordered proteins such as resilin-like protein, spider dragline silk, and mussel foot proteins are also reported to form liquid condensates through LLPS [26–28]. These proteins share three key features similar to the intrinsically disordered proteins identified to be capable of LLPS within eukaryotic cells: highly repetitive, low complexity, and rich in a few set of disorder-promoting amino acids. To help researchers discover and predict the proteins with LLPS capability, several web-accessible databases have been established [19,29,30].

Encapsulation of clients into compartments

Upon completion of the self-assembly of protein shells, a central cavity is formed, which enables the encapsulation of client molecules. Additionally, the gaps created by the assembly of shell proteins serve as pathways for small molecules to traverse [31]. This inherent structure presents the potential for encapsulating enzymes within the compartments and facilitating efficient biocatalysis. Currently, two primary methods have been employed for encapsulation of intended cargo proteins: fusion protein expression and specific targeting sequence recognition [4,8]. Direct genetic fusion of a desirable cargo to a shell protein is straightforward, but it may lead to undesirable aggregation and loss of client protein activity. To facilitate the encapsulation of enzyme molecules into empty compartments, many researchers prefer the employment of native encapsulation peptides, also called signal sequences that enable precise recognition to the particular shell proteins (Figure 2a). These peptide sequences largely follow a consensus motif, which has guided the design of de novo signal sequences [32,33]. Notably, it is not required to modify shell proteins for the signal sequences to work, however, the detailed interaction mechanism between them is often lacking, which tends to cause unpredictable cargo loading efficiency [33,34]. Conversely, the introduction of known protein–protein interactions such as Post-synaptic density-95, disks-large and zonula occludens-1 (PDZ) domains and their binding partners, and SpyCatcher/SpyTag conjugation, which is highly specific with the formation of an isopeptide bond between the SpyCatcher and SpyTag, can result in stoichiometric loading of clients into the ultimate compartments [35]. However, the placement of cognate binding domains in the shell proteins requires detailed structures of the shell proteins, and the knowledge on how the binding domains are oriented in the assembled compartments.

Similar strategies are employed for localization of client proteins into the MLCs [3]: fusion expression of enzyme molecules and specific recruitment facilitated by engineered peptide modules. In the fusion expression approach, the enzyme molecules are directly fused with the phase-separating scaffold proteins. This method is easy to perform but it tends to cause failure of phase separation and misfolding of the enzymes. Notably, fusion of the client enzymes may impact the phase transition behavior of the scaffolding protein chosen and material properties of the resulting MLCs. On the other hand, a more commonly adopted approach involves interaction polypeptide domains with specific binding capabilities (Figure 2b), such as RIDD–RIAD [36] and

Figure 2



Strategies on encapsulation of clients into compartments. **(a)** The enzymes are encapsulated into BMCs through the native or engineered encapsulation peptides that can specifically interact with the shell proteins. **(b)** The enzymes are recruited into MLCs through the engineered protein–protein interaction domains, which are fused to the phase-separated protein and client protein, respectively. **(c)** Controllable cargo recruitment and release from the MLCs in cells. This is achieved by using the engineered protein–protein interaction domains that act as switches in response to specific stimulating cues.

PDZ–PDZlig pairs [37]. These peptides are fused with the phase-separating proteins and the target enzyme molecules, respectively. Through the speci□c interactions between the interacting peptides, the enzymes are recruited into the MLCs. It is worth to note that many interaction domains such as the light-responsive Cryptochrome 2–Cryptochrome-interacting basic-helix-loop-helix pair [38] and the temperature-responsive TsCC(A)–TsCC(B) [39] can act as switches in response to various stimuli and, thus, have great potential to endow the synthetic MLCs with controllable client recruitment and release properties (Figure 2c). This feature is unique to the MLCs because of their higher permeability and dynamic properties when compared with their protein-shelled counterparts.

Synthetic compartments for metabolic engineering

Repurposing protein-shelled compartments

With the increasing understanding of the structure and biological functions of protein-shelled compartments, there have been several remarkable reports of the heterologous expression and engineering of these compartments for the development of metabolic nanoreactors (Table 1) [4,8,10,40]. By recruiting enzymes and metabolites into the compartments, it is possible to favorably control the kinetics and stability of metabolic processes. Moreover, spatial restriction of these processes can reduce metabolic crosstalks and the accumulation of toxic metabolic metabolites.

As one of the most well-known BMCs, carboxysome has not only been successfully reproduced but also repurposed for hydrogen production in heterologous host *Escherichia coli* [33,34]. Taking advantage of the anaerobic microenvironment inside the BMC shell, an oxygen-sensitive [Fe–Fe] hydrogenase has been targeted to the rumen of synthetic BMCs by N-terminal fusion with an encapsulation peptide, which resulted in increased hydrogen production by ~500% in aerobic culture of the engineered *E. coli* cells (Figure 3a) [34]. In a more recent study, two enzymes, including an oxygen-

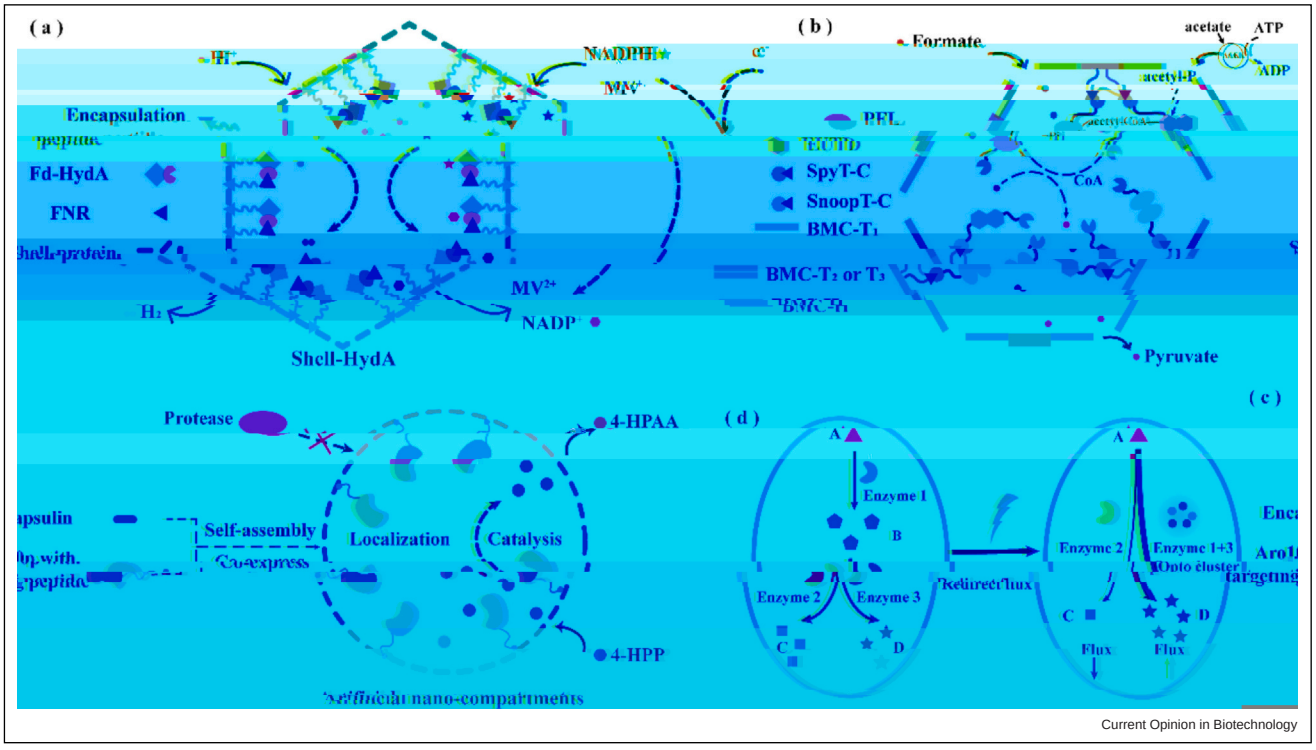
Table 1

Typical synthetic protein compartments.

Name	Size in diameter	Heterologous host	Scaffolding block	Client encapsulation strategy	Function
[illegible]					
BMC ([illegible])	n/a	[illegible] MB001 (DE3)	Self-assembly of PduABJknt shell proteins	C-terminal fusions with synthetic interaction peptides (PDZ, SH3, and GBD) or with encapsulation peptides (D18, P18)	Redesigning and engineering BMC for use in [illegible] [58]
BMC ([illegible])	~100 nm	[illegible] BL21 (DE3)	Self-assembly of CsoS2, CsoS4AB, CsoS1CAB, and CsoS1D shell proteins	N-terminal fusions with encapsulation peptide (C-terminus of CsoS2)	Hydrogenase encapsulation to improve hydrogen production [34]
BMC ([illegible])	~100 nm	[illegible] BL21 (DE3)	Self-assembly of CsoS1/1D, CsoS2, and CsoS4A/B shell proteins	Specific interaction with shell protein	Provides a modular platform for efficient assimilation of CO2 [59]
BMC ([illegible])	~40 nm	[illegible] BL21 (DE3)	Self-assembly of BMC-H, along with BMC-T1T2T3 protein	SpyTag/Catcher, SnoopTag/Catcher pair with Tag fusion to T1 shell protein	Build a prototype synthetic formate-utilizing BMC [35]
Encapsulin ([illegible])	~26 nm	[illegible] PK2-1D	Self-assembly of shell protein (EncA)	N-terminal fusions with targeting peptide (C-terminus of the EncC protein)	Provides a modular platform for programming synthetic compartmentalization in eukaryotes [41]
Virus capsid (bacteriophage P22)	~56 nm	[illegible] BL21 (DE3)	Self-assembly of P22 coat protein	N-terminal fusions with P22 scaffold protein	Provides stability and protection to the [NiFe]-hydrogenase for improved hydrogen production [40]
Virus particle ([illegible])	~41–46 nm	[illegible] CEN.PK2-1 C	Self-assembly of VP1 shell protein	C-terminal fusions with VP2C- targeting peptide	Increase D-glucaric acid production by encapsulating an unstable and rate-limiting enzyme MIOX [42]
[illegible]					
-	n/a	[illegible] BY4741	LLPs of FUS^N domain	Genetic fusion with FUS^N and light-associable Cry2olig or light-dissociable PixD/E	Enhance product formation and specificity [47]
	0.7–1 μm	[illegible] BY4741	LLPs of Sup35, Med1, FUS, RGG, or A-IDP domains	Genetic fusion with phase-separated proteins	Enhance methanol assimilation and increase n-butanol yield [48]
	n/a	[illegible] BL21 (DE3)	LLPs of synthetic spider silk protein MaSp1, MaSp2, or resilin-like proteins	Genetic fusion with phase-separated proteins	Develop artificial organelles with on-demand functions in prokaryotes [26]
	~200 nm	[illegible] BL21 (DE3)	LLPs of RGGRGG domain derived from LAF-1	Genetic fusion with RIDD for pairing with RIAD-tagged scaffold	Enhancing α-farnesene production [45]
	n/a	[illegible] BL21 (DE3)	LLPs of RGG domain derived from LAF-1	Genetic fusion with RIDD for pairing with RIAD-tagged scaffold	Increasing the production of 2′-fucosyllactose [46]
	n/a	[illegible] BL21 (DE3)	LLPs of FUS domain or tandem SIM–SUMO repeats	Genetic fusion with Cry2 for pairing with CIB1-tagged scaffold	Regulation of metabolic reactions [44]

a DDX3 family RNA helicase found in P granules (LAF-1); PDZ, Post-synaptic density-95, disks-large and zonula occludens-1 (PDZ); Regulated IRE1α-dependent decay (RIDD); Regulatory subunit I anchoring disruptor (RIAD); Cryptochrome 2 (Cry2); GTPase binding domain (GBD); arginine–glycine–glycine repeat (RGG); Artificial intrinsically disordered proteins (A-IDP); interacting motifs (SIMs)-Small Ubiquitin-like Modifier (SUMO)

Figure 3



Synthetic protein compartments for metabolic engineering. **(a)** Development of a synthetic carboxysome by sequestering a catalytically active hydrogenase within the protein shell to boost production of hydrogen. **(b)** Construction of a synthetic formate-utilizing BMC by encapasulating both PFL and phosphate acyltransferase within the protein shell for the bioconversion of formate and acetate into pyruvate. **(c)** Construction of a synthetic encapsulin system by encapsulating a tetrameric pyruvate decarboxylase enzyme Aro10p into the capsid to produce 4-hydroxyphenylacetaldehyde (4-HPAA), an important precursor of benzylisoquinoline alkaloids. **(d)** Development of light-switchable MLCs enabling selective co-compartmentalization of certain enzymes in a complex metabolic pathway to divert metabolites toward one branch pathway.

sensitive pyruvate formate lyase (PFL) and a phosphotransacetylase (EutD) of *E. coli*, have been compartmentalized into a synthetic BMC toward the production of pyruvate from formate and acetate (Figure 3b) [35]. Of particular interest, SpyTag/SpyCatcher and SnoopTag/SnoopCatcher adaptor systems that are orthogonal in isopeptide bond formation, have been used for enzyme conjugation to the shell proteins, which therefore colocalized the catalytic activities of the above two enzymes within the compartments [35]. Although active BMC can be successfully assembled within the recombinant *E. coli* cells expressing all the necessary protein components, whole-cell biotransformation of formate and acetate into pyruvate remains to be demonstrated in future studies.

As a more engineerable and modular tool, encapsulins require single scaffolding proteins for their assembly, and thus offer the potential for precise installation and functioning of the enclosed enzymes. This has been recently demonstrated via the construction of a synthetic encapsulin platform in the model eukaryote *Saccharomyces cerevisiae*. The presented nanocompartments were able to selectively encapsulate heterologous proteins of interest, thereby protecting the clients from degradation as well as serving as a nanoreactor for enzymatic catalysis without undesired metabolic crosstalk. Typically, this encapsulin system paired the scaffolding protein (termed capsid) with a tetrameric pyruvate decarboxylase enzyme Aro10p that was C-terminally fused to the targeting peptide, thereby encapsulating Aro10p to create the synthetic encapsulin as a benzylisoquinoline alkaloid nanoreactor (Figure 3c) [41]. In order to increase the loading capacity and assembly efficiency, a synthetic compartment based on murine polyomavirus virus-like particles (VLPs) has been developed recently in the baker's yeast *S. cerevisiae*. The assembled compartment is ~50 nm in diameter and has a theoretical maximum loading of 72 cargo proteins per particle, providing a larger loading capacity than the above encapsulin system. In addition, this VLP-based compartment has been shown to increase the production of D-glucaric acid by encapsulating the key enzyme *myo*-inositol oxygenase (MIOX), which is unstable and rate-limiting in D-glucaric acid biosynthesis [42]. Finally, it should be noted that encapsulins have rarely been employed for metabolic engineering in the microbial workhorse *E. coli*. This might be due to the following

reasons: 1) natural encapsulins are primarily identified from GC-rich organisms such as *Actinobacteria*, and their encoding genes are rather difficult to be heterologously expressed in *E. coli*, and 2) homogeneous loading of multiple enzyme cargoes into the engineered encapsulins can be rather difficult due to the macromolecularly crowded intracellular environments of *E. coli*.

Engineering phase-separated protein compartments

It is widely recognized that phase separation results in a compartmentalized enrichment of substrate and/or enzyme molecules, which is beneficial for accelerating the enzymatic reaction rates and product formation (Table 1). As a demonstration, an enzyme cascade has been recruited into a protein condensate in vitro by rapamycin-induced protein–protein interaction pairs for SUMOylation, and the reaction rates can be increased up to 36-fold in the phase-separated droplets compared with the surrounding bulk [43]. In an in vivo example, the reaction rates of luciferin and catechol oxidation were demonstrated to increase by 2.3- and 1.6-fold, respectively, via light-responsive enrichment of the enzymes into the phase-separated compartments [44].

Compartmentalization of the pathway enzymes involved in the synthesis and utilization of toxic intermediates is promising to improve the production of target products by overcoming the toxicity issue of the metabolic intermediates. Recently, a phase-separated compartmentalization strategy has been developed to improve the production of a sesquiterpene, α-farnesene in engineered *E. coli*. The two overexpressed enzymes Idi and IspA were recruited into synthetic RGG protein condensates through the peptide–peptide interaction pair RIAD–RIDD, which alleviates the cytotoxicity of Idi-catalyzed reaction product leading to an appreciable enhancement in production of α-farnesene [45]. Using a similar condensation strategy, four enzymes involved in de novo synthesis of 2′-fucosyllactose were simultaneously recruited into the RGG condensates and the production titer and yield of the target product were increased significantly, in comparison to the strains expressing free-floating pathway enzymes [46].

Metabolic pathways may have multiple branches, which makes it difficult to increase the production of a desired compound through a specific pathway. Unlike the traditional knockout or knockdown of the competing pathway genes, synthetic MLCs provide a new approach for metabolic regulation by compartmentalizing specific pathway enzymes. As a pioneering example, light-responsive MLCs were constructed and employed to trigger formation and dissolution of metabolically active enzyme clusters in yeast cells (Figure 3d). This switchable clustering strategy enhanced target product formation by sixfold and product specificity by eighteen-fold by decreasing metabolic flux through the competing pathway [47]. More recently, two intriguing switch tools were similarly developed to control the size and rigidity of the synthetic MLCs in yeast. By using these tools, methanol assimilation was significantly enhanced via alleviating toxicity of formaldehyde, and n-butanol yield was increased through switching from oxidative to non-oxidative glycolysis [48]. This synthetic MLC provides a novel strategy to channel C1 feedstock into value-added chemicals.

Conclusion and future perspectives

Metabolic compartmentalization by protein compartments fulfils three important functions within the cells: establishing unique chemical environments, providing protection of reactive metabolites, and enabling switchable regulation of metabolic pathways [1,3,49]. In contrast with the self-assembled BMCs and encapsulins with protein shells, MLCs are biomolecular condensates without the restriction of any enclosing membranes. In addition, due to unique formation mechanism involving LLPS, MLCs can be liquid droplet-like, and are more dynamic in exchanging molecules with their surrounding environments, thus enabling faster material exchange and offering greater ease of artificial design and construction.

In the near future, metabolic compartmentalization in the interdisciplinary fields of biophysics, biochemistry, and synthetic biology will receive a rapidly increasing attention [50,51]. Through protein self-assembly or phase separation, synthetic protein compartments can be tailor-designed with on-demand dynamics and biological functions, which not only offers new versatile tools to engineer biology but also provides insights into the fundamental mechanisms by which the cells spatially organize their cellular metabolism. Although the remarkable advances in engineering protein compartments have been achieved in the past few years, substantial challenges remain to fully realize their potential in metabolic engineering. First, the design principles underlying protein self-assembly and phase transitions are still limited. It may be tackled by developing high-throughput screening and characterization platforms of scaffolding proteins to generate big data for deep learning, which will support the rational and predictive design [52–55]. Second, the unique structures and physical states of the synthetic protein compartments, and their dynamic transitions in living cells remain underexplored. To this end, it is vital to develop in situ characterization methods to detect and probe the protein compartments for real-time monitoring and quantitative evaluation of their physicochemical properties and functional outcomes [15,17,56]. Third, limited strategies are available for efficient encapsulation of pathway enzymes within the compartments for metabolic engineering. This challenge may be alleviated by the

deeper understanding of the assembly mechanisms of protein compartments [10,57], and modular optimization of the building blocks by synthetic biology [3,54].

CRediT authorship contribution statement

ZGQ and XXX formulated the idea of compressively summarizing the current updates on synthetic protein compartments. FZ drafted the paper with contributions from YJZ. FZ drew the figures. The final paper was written through contributions from all authors. All authors have given approval to the final version of the paper.

Data Availability

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

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

This work is supported by the National Key Research and Development Program of China (Grant No. 2022YFC3401700 to Z.-G.Q.) and the National Natural Science Foundation of China (Grant 32071414 to X.-X.X., and grants 32270107 and 22075179 to Z.-G.Q.).

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