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Theoretical and Practical Aspects of Multienzyme Organization and Encapsulation

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Abstract

The advent of biotechnology has enabled metabolic engineers to assemble heterologous pathways in cells to produce a variety of products of industrial relevance, often in a sustainable way. However, many pathways face challenges of low product yield. These pathways often suffer from issues that are difficult to optimize, such as low pathway flux and off-target pathway consumption of intermediates. These issues are exacerbated by the need to balance pathway flux with the health of the cell, particularly when a toxic intermediate builds up. Nature faces similar challenges and has evolved spatial organization strategies to increase metabolic pathway flux and efficiency. Inspired by these strategies, bioengineers have developed clever strategies to mimic spatial organization in nature. This review explores the use of spatial organization strategies, including protein scaffolding and protein encapsulation inside of proteinaceous shells, toward overcoming bottlenecks in metabolic engineering efforts.

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INTRODUCTION

Metabolic engineering is established as a successful strategy for sustainably synthesizing medically and industrially relevant products using microbes. Using metabolic engineering, desired products can be made in new hosts by expressing novel pathways composed of combinations of heterologous enzymes. Notable successes include the production of artemisinic acid in yeast and 1,3-propanediol (1,3-PD) in *Escherichia coli* (71, 82). As the suite of accessible compounds expands with novel enzyme discovery and enzyme engineering, efforts to generate new products can be hindered by bottlenecks that can arise from cofactor imbalances or mismatched enzyme kinetics (29, 73, 86). These bottlenecks can lead to intermediate accumulation, resulting in toxicity or undesired side products. Moreover, undesirable interactions can occur between native enzymes and engineered pathway intermediates or between engineered pathway enzymes and native metabolites, with detrimental effects on pathway yield and/or cellular health. Nature faces similar challenges for specific metabolic pathways, and various spatial organization strategies have evolved to overcome these challenges.

Spatial organization of metabolism increases control over enzyme stoichiometry, intermediate retention, and pathway enzyme order. For example, polyketide synthesis enzymes are organized in an assembly line that drastically increases pathway efficiency and stereospecificity (50). This assembly line enables rapid intermediate channeling between enzymes. Metabolons present another example of naturally occurring spatial organization. These are multienzyme complexes held together by noncovalent interactions that vary in stability from transient to long-lived (87). Similar to polyketide synthesis, metabolons colocalize pathway enzymes sequentially, increasing pathway flux.

Spatial organization can also be accomplished by enclosing metabolic pathways inside intracellular structures. Eukaryotes achieve this organization via lipid membrane–bound organelles such as mitochondria, lysosomes, and vacuoles (3). These organelles sequester certain cellular processes, providing spatial organization within the cell cytoplasm. Likewise, certain species of

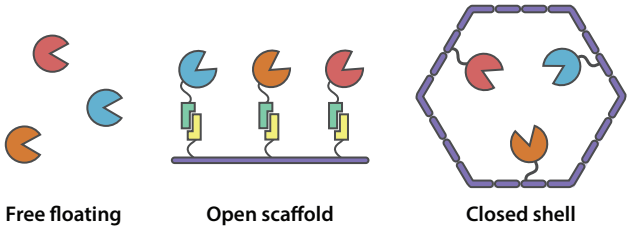

Figure 1

Free-floating, open-scaffold, and closed-shell spatial organization strategies.

prokaryotes organize metabolism using proteinaceous structures called bacterial microcompartments (MCPs) that encapsulate select metabolic pathways. MCPs are composed of a polyhedral protein shell ranging from 100 to 200 nm in size that encapsulates an enzyme core (47). The first MCPs were imaged in 1956 in cyanobacteria (20) and were later revealed to be carboxysomes, a type of MCP that plays a role in carbon fixation (9). MCPs serving various metabolic functions have since been discovered; these include the ethanolamine utilization (Eut) MCP and the 1,2-PD utilization (Pdu) MCP, both of which are used to metabolize niche carbon sources (6, 55).

Inspired by nature, researchers have employed multiple methods to spatially organize heterologous metabolic pathways, including scaffolds and compartmentalization. These methods include repurposing protein complexes from nature for various engineering goals. While some strategies involve engineering complexes that have already evolved for the spatial organization of metabolic pathways, such as cellulosomes and MCPs, others involve adapting viral capsids to impart a new function to serve an engineering goal. Each strategy comes with benefits and drawbacks that lend themselves to different types of metabolic pathways depending on the dominant features of the pathway. In this review, we cover recent strategies and examples of protein-based spatial organization of metabolic pathways toward increased pathway efficiency (**Figure 1**) (**Table 1**). We also discuss future directions and development for more widespread use of these methods for metabolic engineering.

PROTEINS CAN BE COLOCALIZED USING A PROTEIN SCAFFOLD

Early efforts to arrange enzymes spatially used physical tethers to link enzymes together. Inspired by metabolons that induce substrate channeling between pathway enzymes in nature, researchers have directly fused subsequent enzymes together using peptide linkers. This strategy has been successfully employed in *E. coli* to increase production of *n*-alkanes, α-farnesene, and muconic acid by the genetic fusion of two key enzymes in their respective pathways (27, 81, 95). While these examples highlight the advantage of spatial organization for a two-enzyme pathway or a portion of a pathway, direct fusions limit available enzyme stoichiometries and it can be difficult to design fusions to ensure enzyme activity.

More complex pathway organization strategies have relied on tethering multiple enzymes to a single protein scaffold. These scaffolds offer precise control over enzyme stoichiometry and orientation. To accomplish this goal, the enzyme is fused to a tag that colocalizes the enzyme and a given protein scaffold. Tags appending protein interaction domains for this purpose include coiled-coil systems and leucine zipper proteins (26, 36). In this organizational scheme, one of the interacting domain pairs is fused to the enzyme and the other is fused to the scaffold. Interaction tags are genetically fused either N-terminally or C-terminally, depending on which is tolerated best in a particular system. Both termini are often tested when designing protein-based tag fusions, as some proteins may not tolerate fusions on one of the termini. In the event that a fusion

Table 1 Examples of enzyme organization using array, encapsulation, and condensate strategies

Type of enzyme organization strategy employed	Organization system	Number of different enzymes involved	Attachment strategy	Kinetic enhancement factor (fold change)	Reference
Arrayed	NA	2	Genetic fusion of enzymes	3.32	Fujiwara et al. (27)
	NA	2	Genetic fusion of enzymes/zinc finger protein guided assembly	4.8/8.8	Rahman et al. (81)
	NA	2	Genetic fusion of enzymes	317	Wang et al. (95)
	NA	3	Cohesin–dockerin domain interactions	5	Liu et al. (63)
	NA	3	Cohesin–dockerin domain interactions	1.37	Kim & Hahn (52)
	Sequentially arrayed	3	SH3, GBD, and PDZ domain interactions	77	Dueber et al. (21)
	NA	2	Coiled-coil domains attached to PduA nanotubes	2	Lee et al. (59)
	NA	1	SpyCatcher/SpyTag and SnoopTag/SnoopCatcher attached to EutM	NA	Zhang et al. (99)
	NA	2	SpyCatcher/SpyTag and SnoopTag/SnoopCatcher attached to EutM	1.5	Chen et al. (11)
	NA	2	SpyCatcher/SpyTag and SnoopTag/SnoopCatcher attached to EutM	1.24	Liu et al. (64)
	NA	2	*Staphyloccocus aureus* sortase A pentamutant bioconjugation to the T33–21 protein nanocage	2.7	McConnell et al. (66)
	Arrayed onto MDH	3	SH3 domain interaction	97	Price et al. (79)
Encapsulated	VLP	1	Capsid reassembly	NA	Comellas-Aragonès et al. (15)
	VLP	1	Fusion of the scaffolding protein C terminus to protein of interest	Variable depending on P22 VLP shape	Patterson et al. (77)
	Encapsulin	1	Fusion of C terminus of encapsulin targeting peptide	NA	Lau et al. (56)
	Vault protein	1	Fusion of INT to the C terminus of the protein of interest	NA	Kickhoefer et al. (51)
	Vault protein	1	Fusion of INT to the C terminus of the protein of interest	3	Wang et al. (96)
	Vault protein	1	Fusion of INT to the C terminus of the protein of interest	2 to 3	Wang et al. (97)
	HO MCP	3	SpyCatcher/SpyTag and SnoopTag/SnoopCatcher attached to T1 of the HO compartment	NA	Kirst et al. (54)
	Pdu MCP	2	Pdu MCP signal sequences genetically fused to the enzymes of interest	1.63	Lawrence et al. (57)
	Pdu MCP	4	Pdu MCP signal sequences genetically fused to the enzymes of interest	2.45	Lee et al. (59)
	Carboxysome	1	C terminus of CsoS2 fused to enzyme of interest	5	Li et al. (60)

(*Continued*)

Table 1 (*Continued*)

Type of enzyme organization strategy employed	Organization system	Number of different enzymes involved	Attachment strategy	Kinetic enhancement factor (fold change)	Reference
Condensate	NA	2	Genetic fusion of FUS^N to enzyme of interest	6	Zhao et al. (100)
	NA	1	Genetic fusion of RGG to enzyme of interest	1.5	Guan et al. (34)

Abbreviations: Eut, ethanolamine utilization; FUS^N, N terminus of FUS protein; GBD, GPTase-binding domain; HO, *Haliangium ochraceum*; INT, interaction domain; MCP, microcompartment; MDH, methanol dehydrogenase; NA, not available; Pdu, 1,2-propanediol (1,2-PD) utilization; PDZ, PSD95/Discs Large/ZO-1; RGG, N-terminal RGG domain of LAF-1; SH3, Src homology 3; VLP, virus-like particle.

inhibits protein folding or function regardless of the terminus, a flexible linker sequence may be placed between the tag and the protein (4, 12). Flexible linkers often need to be optimized for sequence, length, and composition because the optimal linker will be protein tag dependent. Thus, while protein tagging is necessary for scaffolding enzymes, this procedure often requires multiple design–build–test cycles to find the most optimal tagging scheme.

One popular protein interaction pair used in protein scaffolding is the cohesin–dockerin domain pairing from cellulosomes. Cohesin domains are located on the aptly named scaffoldin protein. This system allows more enzymes to be scaffolded onto the same structure while also offering direct control over the orientation and stoichiometry of the enzymes. The use of cohesin–dockerin pairs from different organisms allows individual pathway enzymes to be ordered in a genetically encoded manner on the scaffold. For example, a three-enzyme methanol oxidation pathway, composed of an alcohol dehydrogenase (ADH), a formaldehyde dehydrogenase, and a formate dehydrogenase, was scaffolded using three distinct cohesin–dockerin pairs (63). In this case, the scaffolded enzymes produced five times more NADH than the unscaffolded case. Cohesin–dockerin pairs were also used to scaffold a 2,3-butanediol production pathway in *Saccharomyces cerevisiae* (52). In this study, three enzymes (acetolactate synthase, acetolactate decarboxylase, and 2,3-butanediol dehydrogenase) were scaffolded using the same cohesin–dockerin pair from *Clostridium thermocellum*. Kim & Hahn (52) observed that increasing the number of cohesin domains led to a modest increase (37%) in 2,3-butanediol production in a fed-batch format. Because the cohesin–dockerin pairs were all the same, the stoichiometry and order of the coexpressed enzymes were not controlled, but an increase in pathway productivity was still observed.

Another scaffolding strategy for pathway organization takes advantage of protein–protein interactions between metazoan peptide motifs and their cognate adaptor domains, natively used for signal processing. Protein domains such as Src homology 3 (SH3), GTPase-binding domain, and PSD95/Discs Large/ZO-1 (PDZ) are used with their associated peptide ligands to either directly bind enzymes together or bind them to a synthetic scaffold. These domains have been widely used for pathways of varying sizes in *E. coli* and yeast (29). Notably, these domains were used to scaffold the three-enzyme mevalonate pathway consisting of acetoacetyl-CoA thiolase, hydroxymethylglutaryl-CoA synthase, and hydroxy-methylglutaryl-CoA reductase in *E. coli* (21). In this study, Dueber et al. (21) achieved a 77-fold increase over the unscaffolded case when enzymes were scaffolded in a 1:2:2 ratio where subsequent steps were spatially adjacent. Additionally, when the domains were rearranged but the same enzyme stoichiometry was maintained, the authors observed less mevalonate production. This finding demonstrates the ability to directly determine stoichiometries and enzyme order using these domains and shows that this precise control over enzyme locale can affect productivity even when maintaining the same enzyme ratios.

The SH3 domain can also be used to colocalize pathways enzymes in a scaffold-less complex. Price et al. (79) used SH3 domains to colocalize the pathway responsible for converting methanol to fructose-6-phosphate (F6P). Methanol dehydrogenase (MDH) forms a decamer to which 3-hexulose-6-phosphate synthase (Hps) and 6-phospho-3-hexulose isomerase (Phi) can be attached. MDH was fused to the SH3 ligand, and a Hps–Phi fusion was fused to the SH3 domain. By use of the SH3 domain interactions, MDH, Hps, and Phi were complexed together. In addition, lactate dehydrogenase was coexpressed to produce NAD^+ for the MDH enzyme. This combination of techniques resulted in a 97-fold increase in F6P production. The MDH decamer acted as a base to colocalize the methanol-to-F6P pathway in a scaffold-less complex.

The scaffolds described above have all involved specific domains that are on a generic protein scaffold. However, scaffolds that take advantage of their self-assembling properties into various structures have also been developed. For example, PduA is a self-assembling hexameric shell protein from the Pdu bacterial MCP (74). PduA has recently attracted interest due to its propensity to self-assemble into nanoscale tubes that can span the cytoplasm of a cell (46, 61, 76). Lee et al. (59) utilized a modified PduA (PduA*) with increased stability and overexpressed it, producing nanotubes 20 nm in diameter that spanned the length of an *E. coli* cell. Enzymes of interest were localized to these self-assembled PduA* nanotubes using the coiled-coil system of de novo–designed heterodimers (26). This system takes advantage of a coiled-coil heterodimer made up of two peptides, acidic (A) and basic (B), that interact tightly when mixed. Lee et al. fused one heterodimer peptide to PduA* and the cognate peptide to enzymes of interest. Interestingly, PduA* assembly into nanotubes was disrupted by fusion of the A peptide, but not the B peptide, showing that certain peptide fusions can interfere with scaffold self-assembly. This technique was used to scaffold the ethanol production pathway enzymes pyruvate decarboxylase (Pdc) and ADH onto PduA* nanotubes. Scaffolding Pdc and ADH to the nanotube increased ethanol production by 200%. The same study also showed that the nanotubes themselves could be localized to the inner cell membrane by scaffolding membrane localizing proteins to the nanotubes. Self-assembling proteins are versatile tools that can be used as scaffold proteins both to enhance metabolic flux and to control cellular localization.

The hexameric protein EutM provides another example of a self-assembling scaffold. This protein from the Eut MCP system forms various structures, including rods and sheets, depending on the EutM homolog used (99). Several different enzymes have been conjugated to EutM in order to create enzyme scaffolds to increase overall activity and stability. Enzymes were directly conjugated to EutM through the SpyCatcher/SpyTag and SnoopTag/SnoopCatcher systems. In one example, ADH was immobilized on various EutM homologs, resulting in increased stability (99). Activity enhancement varied depending on which EutM homolog was used. In addition, two two-enzyme pathways producing either trehalose from soluble starch or tagatose from lactose were scaffolded on EutM (11, 64). Two EutM constructs were used to simultaneously scaffold two proteins, with each containing either the SpyCatcher or SnoopCatcher tag to match the enzyme with the corresponding tag. As a result, enzyme ratios could be adjusted directly by changing the ratios of the two EutM constructs. In both cases, the authors observed increased pathway yield and enzyme stability.

Protein nanocages are another self-assembling scaffold that can be used to organize pathway enzymes. McConnell et al. (66) bioconjugated cellulase to a T33–21 de novo protein nanocage by using *Staphylococcus aureus* sortase A transpeptidase. This sortase links two proteins with a C-terminal LPXTG sequence and an N-terminal oligoglycine sequence, respectively (39). The T33–21 nanocage was selected because the C termini of the subunits are exposed to the exterior of the nanocage (53). The T33–21 subunits were C-terminally tagged with an LPXTG sequence, and two different cellulase enzymes were N-terminally tagged with oligoglycine sequences.

Bioconjugation of two different cellulase enzymes to the surface of T33–21 resulted in an ~2.7-fold increase in enzymatic activity. This technique can be translated to other enzymatic pathways, given that the enzymes are tagged with an N-terminal oligoglycine sequence.

Protein scaffolds enable direct control over enzyme stoichiometry and scaffolded enzyme arrangement. These scaffolds are more modular than direct fusion pairs and, thus, can be applied to many different pathways. Additionally, scaffolds are versatile in that they have been successfully implemented in both in vivo and in vitro settings. Most notably, the use of scaffolds led to increases in yield up to 77-fold over the unscaffolded case. However, their success is still pathway dependent, and a given scaffold may not be the best for every pathway.

PROTEIN SHELLS CAN BE USED TO ENCAPSULATE PROTEINS

The spatial organization methods discussed above have all involved open scaffolds. However, using an organization method that includes a diffusion barrier may be desirable, depending on the enzymes used. Including a barrier can help prevent additional intermediate diffusion away from the enzymes of interest, avoiding toxic intermediate accumulation and undesired side product generation. Several methods to encapsulate enzymes within a self-assembling protein barrier or shell have been developed.

Virus-like particles (VLPs) consist of viral capsids lacking the native infective machinery. They are made of a single or several capsid proteins that self-assemble into a variety of icosahedral geometries and sizes that can be found in nature or designed de novo (94, 98). VLPs can be loaded using a multitude of different methods, including coiled-coil interactions, charge-mediated encapsulation, and covalent fusion of the cargo to the capsid itself (15). Enzymes loaded into VLPs have increased stability in the presence of denaturing conditions like heat or organic solvents (18). In addition, loading enzymes into VLPs introduces a diffusion barrier between the enzyme and their respective substrates that, if manipulated, could regulate enzyme kinetics (31, 83). For example, horseradish peroxidase (HRP) was loaded into the cowpea chlorotic mottle virus (CCMV) VLP (15). To encapsulate HRP, the CCMV VLP was disassembled and subsequently reassembled around HRP in a pH-dependent manner in vitro. The CCMV VLP was permeable to the HRP's substrates and products, and the HRP was functional inside of the CCMV VLP. Because the CCMV VLP partially disassembles as pH changes, the capsid permeability could also be modulated.

The bacteriophage P22 VLP has a unique ability to change its size, structure, and permeability in response to temperature changes, making it an attractive enzyme encapsulation candidate (92). The assembled P22 VLP has pores of 2 nm and a diameter of 58 nm. Upon heating, the P22 VLP transforms into an expanded structure at nearly double its original diameter and, with further heating, transforms into a Wiffle ball (WB) structure with ~10 nm pores (67). ADH was encapsulated inside of P22 to investigate how different P22 structures influence encapsulated enzyme kinetics (77). ADH was encapsulated through C-terminal fusion to the C terminus of the P22 scaffolding protein. This fusion was sufficient for encapsulation inside of P22 in the native assembly, the expanded structure, and the WB structure. Loading the ADH AldD in the different P22 VLP structures resulted in different kinetics. This finding demonstrates the tunability of VLP capsid permeability. It also shows that encapsulating enzymes within a VLP is a viable strategy for regulating enzyme kinetics by controlling the assembly and permeability of the VLP capsid.

Encapsulins are another class of self-assembling proteins found in bacteria and archaea that form icosahedral capsid-like structures ranging in size from 24 to 42 nm, similar to VLPs. These capsids contain pores of around 5 Å, creating a selective barrier against larger molecules (1, 90). Encapsulins natively encapsulate enzymes such as peroxidases and other enzymes involved in iron

oxidation, mineralization, and sequestration (10, 17, 30, 68). Enzymes are natively encapsulated into encapsulins using a C-terminal targeting peptide, typically 10–40 residues long, that interacts with a conserved N-terminal helix on the encapsulin protein (1, 17, 30). Genetically, encapsulins and their corresponding encapsulated enzymes tend to be encoded on a single operon, but some have been found further away on the genome (17).

Encapsulins have also been used to encapsulate one or more nonnative enzymes. For example, Lau et al. (56) used an encapsulin derived from *Myxococcus xanthus* to encapsulate Pdc tagged C-terminally with the corresponding targeting peptide. The authors demonstrated that the tagged heterologous Pdc retained activity in the encapsulin. Additionally, they observed that fluorescent proteins were protected from degradation when loaded in the encapsulin capsid.

In another example, Sigmund et al. (85) encapsulated either split mCherry or split luciferase into *M. xanthus*–derived encapsulins and demonstrated recovery of fluorescence or luminescence, respectively, upon assembly of these cargos into encapsulins. This result highlights the ability of encapsulins to simultaneously encapsulate multiple proteins that are active when coencapsulated. Encapsulins are a promising vector to encapsulate enzymes to provide enhanced stability. Additionally, because nearly 1,000 encapsulins have been identified, there are many size, geometry, and pore size options to choose from to suit the pathway of interest (32).

Another strategy for enzyme encapsulation is to use vault proteins. Vault proteins are large ribonucleoproteins found in eukaryotes that form a protein shell that is 420 nm wide and 750 nm long (51). These naturally exist as a complex of proteins and nucleic acids but can be recombinantly assembled using only the major vault protein. These vaults form two major conformations in physiological conditions. The closed conformation is the fully assembled vault and forms under neutral to basic conditions, whereas the open conformation occurs at acidic pHs and comprises two halves of the vault in a separated format (33, 78). These protein vaults have been explored primarily as a vector for drug delivery, but there are some examples of enzyme encapsulation inside of protein vaults (70). To load the vaults, an interaction (INT) domain consisting of the last 162 amino acids of poly(ADP-ribose) polymerase, a component of natural vault protein complexes, is fused to the C terminus of the protein of interest, which then binds to the major vault protein (51). Vault nanoparticles have been used to encapsulate a few different enzymes. Of note are luciferase and manganese peroxidase, both of which were encapsulated via fusions to the INT domain (51, 96, 97). In the case of luciferase, the encapsulated enzyme demonstrated activity similar to that of free luciferase when preincubated with ATP (51). However, when ATP was not preincubated first, the activity was much slower. This result indicates that the vault nanoparticle shell creates a diffusion barrier against ATP, which should be kept in mind for future use.

Additionally, vault protein nanoparticles enhance enzyme stability when encapsulated. When manganese peroxidase was encapsulated, researchers observed increased stability of the enzyme, allowing for enhanced degradation of phenols including bisphenol A, bisphenol F, and bisphenol AP (96, 97). In this case, vaults containing the peroxidase degraded three times as much phenol over the same time period and showed increased degradation against a range of bisphenols.

BACTERIAL MICROCOMPARTMENTS NATIVELY ENCAPSULATE METABOLIC PATHWAYS

Bacterial MCPs represent a separate class of protein containers that natively encapsulate the enzymes, substrates, and cofactors necessary for certain metabolic pathways (5). Because MCPs evolved naturally to encapsulate multistep enzymatic processes, they are particularly relevant candidates for the study of encapsulation as a method for spatially organizing metabolic pathways. Examples of canonical MCPs include the carboxysome, which sequesters RuBisCO for

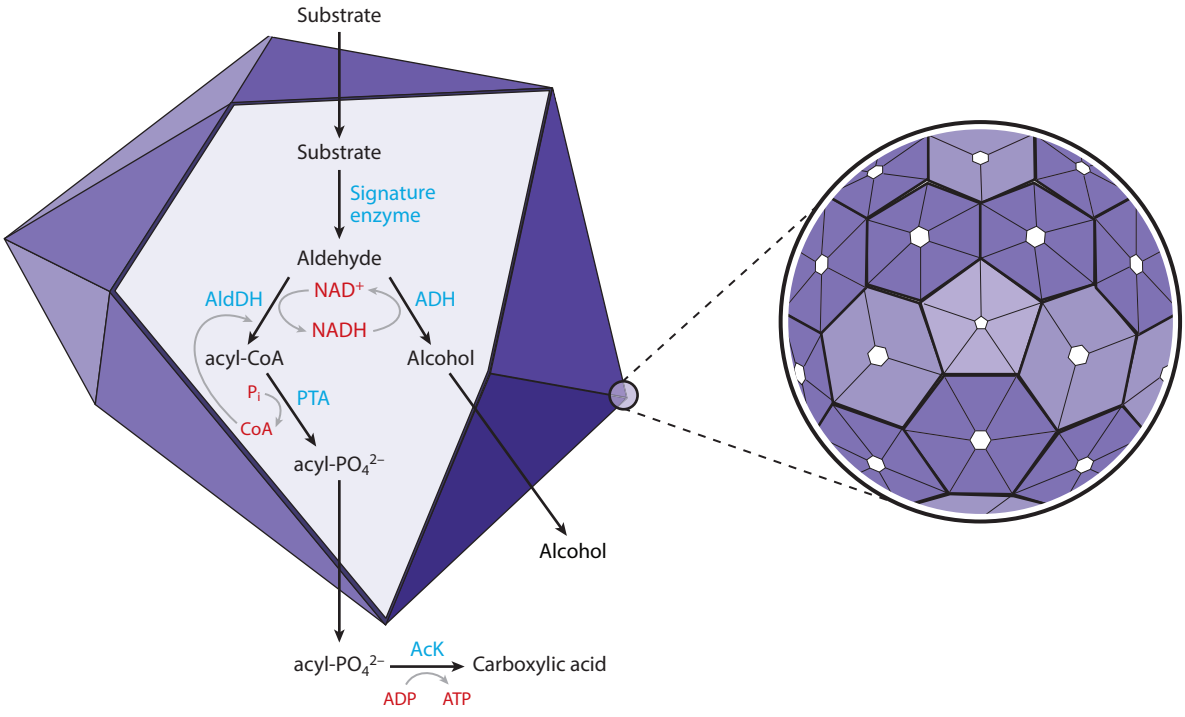

Figure 2

Bacterial MCPs encapsulate metabolic pathways inside a proteinaceous shell. Bacterial MCPs are polyhedral proteinaceous organelles consisting of shell proteins that self-assemble into hexamers, trimers, and pentamers to form the compartment shell. They natively encapsulate pathways that follow a signature form, as shown. Abbreviations: AcK, activated kinase; ADH, alcohol dehydrogenase; AldDH, aldehyde dehydrogenase; MCP, microcompartment; PTA, phosphotransacylase.

carbon fixation, and metabolosomes, such as Eut, which sequesters the ethanolamine degradation pathway, and Pdu, which sequesters the 1,2-PD degradation pathway (84, 88). Metabolosomes natively encapsulate metabolic pathways of a characteristic form, in which the first step produces a toxic intermediate, often at a relatively fast rate compared to a cofactor-dependent second step. The properties of the encapsulated pathways suggest that MCPs are particularly well suited for addressing these types of metabolic engineering bottlenecks (**Figure 2**).

More specifically, the MCP shell is composed of several shell proteins that fall into three subtypes: hexamers, trimers, and pentamers. Unlike other encapsulation vehicles, these shell proteins vary in their pore size, abundance, and ability to self-assemble. Together, the shell proteins act as a diffusive barrier that is thought to be biologically necessary to prevent intermediate escape. These characteristics make MCPs challenging to engineer but also promising, as these structures can accommodate a unique level of complexity while remaining functional.

In addition to reprogramming native MCPs (described in the section titled Native Microcompartment Encapsulation Machinery Can Be Used to Encapsulate Proteins), alternative shell structures based on MCPs have been used to encapsulate pathways to regulate substrate and product exchange between the pathway and the cytosol. The *Haliangium ochraceum* MCP shell (HO-shell) has uncapped vertices resulting in a WB shell structure (91). These uncapped vertices have a gap of 45 Å, which may allow diffusion of larger molecules through the HO-shell in comparison to other MCP shells with pores of less than 10 Å. Kirst et al. (54) used the

HO-shell to encapsulate pyruvate formate lyase (PFL) and a phosphate acyltransferase (EutD) to convert formate and acetylphosphate into pyruvate. PFL and EutD were encapsulated by using SpyCatcher/SpyTag and SnoopCatcher/SnoopTag to attach the cargo directly to tagged shell proteins. Specifically, SpyTag and SnoopTag were simultaneously added to an inward-facing loop within one of the trimers (T1) of the HO compartment, while SpyCatcher was added to the C terminus of PFL and SnoopCatcher was added to the C terminus of EutD. HO-shell-encapsulated PFL and EutD were functional, proving that alternative MCP shells can be used to encapsulate metabolic pathways. Furthermore, use of the SpyCatcher/SpyTag and SnoopCatcher/SnoopTag systems to encapsulate cargo in these shells afforded precise control over loaded enzyme stoichiometry and orientation within the compartment.

NATIVE MICROCOMPARTMENT ENCAPSULATION MACHINERY CAN BE USED TO ENCAPSULATE PROTEINS

Natively, MCPs generally encapsulate enzymes by leveraging interactions between peptide motifs at the N terminus of some of the encapsulated enzymes, also known as signal sequences, and the shell of the compartment (24). Researchers can easily repurpose these signal sequences to encapsulate the desired heterologous enzymes simply by appending a signal sequence peptide tag to the heterologous cargo of interest. Several native signal sequences for canonical metabolosomes have been characterized, including those derived from the Pdu enzymes PduD, PduP, and PduL (23, 24, 65) and the Eut enzymes EutC and EutE (14, 41, 80). These known signal sequences have a common amino acid motif of alternating hydrophobic and hydrophilic residues that forms an amphipathic α-helical structure, which has been hypothesized to interact with MCP shell proteins to facilitate cargo encapsulation (41, 57). Jakobson et al. (42) successfully leveraged the common amino acid motif in signal sequences to develop a suite of de novo signal sequences capable of encapsulating cargo in Pdu MCP shells. Interestingly, evidence suggests that signal sequence cross talk exists for multiple MCP shells due to the common amino acid motif among signal sequences. To prevent the mixing of cargo and MCP shells, *Salmonella enterica* express only one MCP at a time, taking advantage of substrate-regulated suppression of other MCPs (89). This naturally existing means of controlling formation of different MCPs in the cell could enable programming of orthogonal metabolic units in a single organism that can be turned on at different stages of bioproduction (49).

Heterologous cargo tagged with signal sequences can be encapsulated through plasmid supplementation while MCPs are assembling. The amount of heterologous cargo encapsulated in an MCP can be somewhat controlled by how much expression is induced from the plasmid and when expression is induced (40, 41). This plasmid supplementation strategy has been used for a variety of encapsulated pathways, including those for ethanol production and polyphosphate production (57, 62).

Signal sequence–tagged heterologous cargo can also be encapsulated when expressed from the genome. A genomic platform to encapsulate enzymes at tunable levels was recently developed using combinations of different native Pdu signal sequences at different loci on the Pdu operon to achieve a range of encapsulation levels (72). An advantage of integrating the genes of heterologous cargo in the Pdu operon is that induction of the operon results in simultaneous expression of the Pdu MCP shell and the heterologous cargo. Additionally, genomic incorporation avoids issues with plasmid retention and the requirement for an inducer for bioproduction applications. Nichols et al. (72) utilized fluorescent reporters integrated into the *S. enterica* genome to investigate the influence of expression level and signal sequence choice on encapsulation levels in the Pdu MCP. The fluorescent reporters were integrated at the location of natively encapsulated enzymes on the Pdu operon and resulted in varied levels of reporter expression. Additionally,

the encapsulation efficiency of the reporters at a given locus varied depending on which signal sequence was fused to the fluorescent reporter. By combining locus-dependent reporter expression and the choice of signal sequence fused to the fluorescent reporters, the authors achieved a wide range of encapsulation levels, which was expanded to coencapsulate multiple fluorescent reporters in the Pdu MCP. The type of cargo also influenced encapsulation levels. Each of these factors should be accounted for when encapsulating heterologous pathways in an MCP.

MULTIPLE HETEROLOGOUS PATHWAYS HAVE BEEN SUCCESSFULLY ENCAPSULATED IN MICROCOMPARTMENTS

Multienzyme pathways have been encapsulated in MCPs using both native encapsulation mechanisms and alternative encapsulation methods. For example, Lawrence et al. (57) constructed an ethanol nanobioreactor by encapsulating Pdc and ADH in a minimal Pdu MCP shell, resulting in a 63% increase in ethanol production compared with an unencapsulated control. The authors produced a minimal Pdu MCP shell by using a plasmid-based expression of select *pdu* genes from *Citrobacter freundii* in *E. coli* (75), and they used a combination of native signal Pdu signal sequence tags to target Pdc and ADH for encapsulation. This study was one of the first to show heterologous pathway encapsulation in an MCP.

In addition to increasing pathway flux, pathway encapsulation in MCPs is hypothesized to sequester toxic or volatile intermediates. Lee et al. (58) took advantage of this property to encapsulate the pathway that produces 1,2-PD from glycerol and contains the toxic intermediate methylglyoxal. This four-enzyme pathway was encapsulated in the same minimal Pdu MCP shell as in the studies by Lawrence et al. (57). Tagging the pathway enzymes with native signal sequences resulted in variable (15–90%) reductions in specific enzymatic activity. Despite these reductions in activity, encapsulation of the 1,2-PD-producing pathway resulted in an ~100% increase in 1,2-PD production compared with an unencapsulated control. Surprisingly, signal sequence–tagged enzymes without the Pdu MCP shell proteins resulted in an ~240% increase in 1,2-PD production, likely due to a signal sequence–mediated aggregation of enzymes that increased pathway flux via enzyme colocalization. This example highlights the importance of considering or even comparing both colocalization and encapsulation options when optimizing a given heterologous pathway.

The tunable permeability of the MCP shell can be used to create specific microenvironments within the MCP. Carboxysomes, the carbon-fixing MCPs mentioned above, natively provide an anaerobic microenvironment inside of their oxygen-impermeable carboxysome shell (48). The carboxysome's anaerobic microenvironment has been repurposed to encapsulate oxygen-sensitive enzymes in aerobically grown *E. coli* cultures (60). For example, Li et al. (60) developed a hydrogen nanobioreactor by encapsulating the oxygen-sensitive [Fe-Fe] hydrogenase HydA in α-carboxysomes. HydA was fused to the C terminus of carboxysome shell protein CsoS2 to target it for encapsulation and plasmid-based expression to generate MCPs in *E. coli*. Encapsulation of HydA in α-carboxysomes increased hydrogen production by ~500%, suggesting that the carboxysome shell protected HydA from oxygen.

Another property that can be tuned when using MCPs for encapsulation is their geometry. Aside from the use of self-assembling MCP proteins to generate scaffolds (discussed above), there are other methods that retain the more closed environment characteristic of encapsulation in MCPs. For example, Mills et al. (69) knocked out the vertex protein PduN from the Pdu MCP system to create elongated MCP structures. These structures, called microtubes (MTs), still contain the rest of the expected MCP shell proteins and MCP-associated enzymes, albeit in a slightly different geometry—cylindrical rather than polyhedral. When these MTs were evaluated for function in comparison to a regular MCP geometry, the cells with the MTs functioned similarly to typical MCPs but had a slight increase in intermediate buildup.

To explore how this geometry shift might alter pathway kinetics, Mills et al. (69) developed a kinetic model to explore the apparent change in the surface area–to–volume ratio between MTs and MCPs. This model demonstrated that keeping the surface area of the MTs the same as that of the spherical MCPs while increasing the enzyme concentration led to nearly identical behavior between the two. In contrast, keeping the volume of the MTs the same as that of the spherical MCPs while increasing the surface area was predicted to lead to increased diffusion of the initial substrate across the MCP barrier, resulting in an increase in the apparent rate of the first reaction. In the case of the Pdu pathway, this increase would lead to an increased accumulation of the toxic intermediate propionaldehyde. While this study was limited to analysis of the native pathway, similar calculations could elucidate how heterologous metabolic pathways of interest with different pathway kinetics may benefit from the availability of the substrate to the enzymatic core by using MTs instead of spherical MCPs.

Just as there are different-sized VLPs, MCPs also vary in size, which can affect pathway performance. An example is the engineering of the glycyl radical–associated MCP group 2 (GRM2)-type MCP found in *Klebsiella pneumoniae* (45). In this study, the shell components of the GRM2 MCP were expressed heterologously in *E. coli* in different combinations. These MCPs natively lack any trimers and instead have four types of hexamers and one type of pentameric shell protein. By leaving out certain shell proteins, Kalnins et al. (45) generated small MCPs of around 20–40 nm as well as larger MCPs of around 200 nm. Notably, in order to observe particles, the pentameric vertex protein cmcD must be present. The authors also encapsulated native GRM2 cargo proteins to these simplified MCP structures. This study created alternate-sized MCP-like structures that are somewhat simpler than the full systems. A simpler system permits rapid engineering that can tailor it to a pathway of interest, since there are fewer shell proteins to consider when making changes.

FUTURE PROSPECTS

Researchers Have Begun to Leverage Liquid–Liquid Phase Separation as a Scaffold-Less Enzyme Organization Strategy

Liquid–liquid phase separation (LLPS) refers to the formation of condensates through liquid demixing. Examples of these condensates in biology include membrane-less organelles such as the nucleolus, Cajal bodies, and stress granules (7, 8, 28). Researchers have successfully created similar condensates by fusing peptides of intrinsically disordered regions (IDRs) to proteins of interest (19). These condensates resulted in the localization of proteins of interest without the use of a protein scaffold. A study by Zhao et al. (100) used the N terminus of the FUS protein (FUS^N) as the IDR to localize enzymes of the branched violacein pathway and divert flux down specific branches. Fluorescent reporters attached to FUS^N formed fluorescent puncta, suggesting that FUS^N is sufficient for condensate formation. Two of the violacein pathway enzymes were tagged with FUS^N, resulting in a 6-fold enhancement in product formation and an 18-fold enhancement in product specificity.

Guan et al. (34) employed LLPS to organize enzymes by using the N-terminal RGG domain of LAF-1 (RGG) as the IDR (22). They fused the light-emitting enzyme NanoLuc to RGG and used it to visualize condensate formation (35). The formation of condensates containing RGG domain–fused NanoLuc was controlled by temperature, ionic strength, and protein concentration (34). The overall concentration of NanoLuc increased by more than 10-fold, and reaction rates increased by ~1.5-fold. Colocalization of enzymes using LLPS is a promising new strategy to not only enhance enzyme kinetics but also increase enzyme concentrations and product specificity.

Computational Modeling Can Be a Valuable Tool for Spatially Organizing Heterologous Pathways

Successful encapsulation of heterologous metabolic pathway MCPs has shown that encapsulation is a viable strategy to increase pathway flux in specific cases. However, each success has required optimization of pathway enzyme encapsulation due to the different encapsulation efficiencies of individual signal sequences as well as the variable effects of signal sequence fusions on specific enzymatic activity. Furthermore, comparisons of encapsulation and colocalization strategies (43, 58) suggest that encapsulation is not always the most optimal spatial organization strategy. To overcome these challenges, researchers can take advantage of computational models to guide selection of a spatial organization strategy. Modeling can allow for faster identification of both an optimal organization strategy and ideal enzyme ratios than trial-and-error experimental approaches.

Computational modeling of enzyme pathway kinetics is a promising way to predict which spatial organization strategy would most enhance metabolic pathway flux and how that strategy can best be applied to a metabolic pathway. For example, a computational model has been developed to compare the effects of scaffolding a metabolic pathway versus encapsulating a metabolic pathway inside of an MCP (**Figure 3**) (43). The model also analyzed how the Pdu MCP enhances the flux of the native Pdu pathway. When enhancing pathway flux, it is pertinent to consider possible intermediate leakage from the pathway, the pathway kinetics, and substrate accessibility. To that end, the model was used to analyze parameters including cell membrane permeability to intermediates, Pdu enzyme kinetics, and external substrate concentrations. Another parameter was the permeability of the MCP shell, since shell permeability influences both pathway flux and intermediate retention. The interplay of these parameters determined whether an open scaffold or full MCP encapsulation was predicted to be the optimal spatial organization strategy. Interestingly, in some cases no spatial organization was predicted to be most beneficial to pathway flux, indicating that spatial organization is not always necessary to enhance pathway flux. The optimal organizational strategy can also differ depending on whether the goal of enzyme organization is to maximize pathway flux or reduce intermediate leakage. This type of model has also been applied to a more general and simplified compartment system using Michaelis–Menten kinetics at steady state (93). In this study, the authors explored the relationship between permeability and enzyme rates to determine

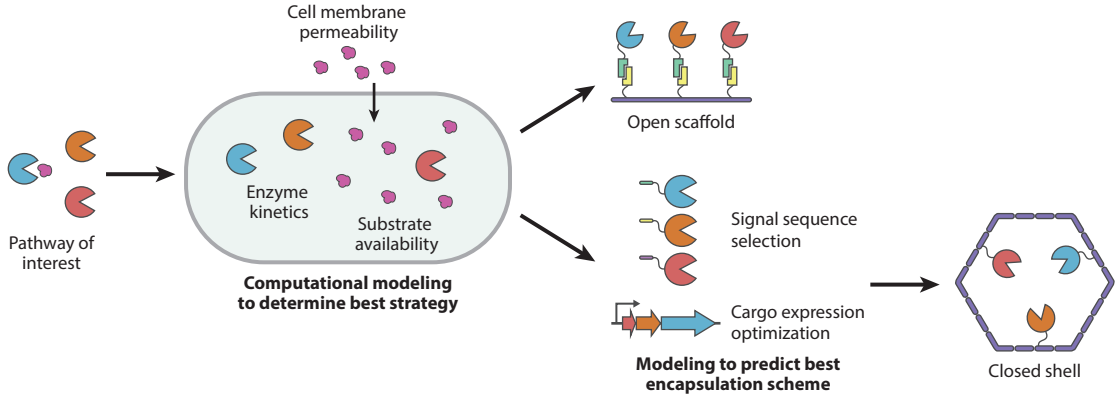

Figure 3

Spatial organization pipeline. To spatially organize heterologous pathways, modeling should be employed to determine the best spatial organization strategy. If the best strategy is pathway encapsulation inside a microcompartment, modeling may be used to predict the most optimal encapsulation scheme for the pathway of interest.

when a particular pathway would benefit from encapsulation. For additional details regarding how compartment models inform the field, we recommend the review by Huffine et al. (38).

Another way to model spatially organized systems is to use stochastic simulation such as the Gillespie method. Conrado et al. (16) constructed a stochastic model of the biosynthesis of 1,2-PD that performed similarly to mass action kinetics–based models when compared using a spatially homogeneous system. By subdividing the reaction space, the authors utilized the spatial capabilities of stochastic modeling to model the increase in catalytic efficiency when enzymes were confined to particular subdivisions, or compartments. Other computational toolboxes, such as Smoldyn, can model stochastic and spatial details to predict behavior about compartmentalization in a number of forms (2).

Additional aspects of encapsulation strategies that should be considered include the geometry of the compartment as well as diffusion limitations from the encapsulation barrier itself, if it exists. For example, as discussed above, Mills et al. (69) used kinetic modeling to compare the predicted performance of MTs with that of spherical compartments. While diffusion across the compartment barrier is limited by the permeability of the shell, the MTs have two ends that may or may not have the same permeability. When diffusion through the ends was assumed to be the same as free diffusion, accumulation of the toxic intermediate increased, leading to a result similar to the case of no compartmentalization. However, when diffusion through the ends was more similar to the Pdu shell or blocked entirely, the performance of the MTs was closer to that of the spherical compartments. Therefore, depending on the system used, diffusion through uncapped tube ends could limit the utility of the compartmentalization strategy. In this case, modeling helped explore the effect of this feature of MTs.

Hinzpeter et al. (37) explored how both the size of a compartment and enzyme density affect the compartment's productivity. They used carboxysomes as a guide for parameter choices. They discovered that there is a critical compartment size that allows for maximum productivity, as well as a switch between maximal enzyme packing and partial enzyme packing as the optimal strategy. The critical compartment size also depends on the permeability of the compartment shell to the substrates and intermediates, highlighting the role of modeling in directing the engineering of compartment systems for a given pathway.

Additionally, different types of diffusive barriers may be desirable, depending on the cell environment in which that the metabolic pathway will exist. For example, in pyrenoids, which are organelles found in many photosynthetic organisms that encapsulate RuBisCO, active transport of substrate across the pyrenoid barrier may not be required for ideal function, depending on the environmental concentration of CO_2 (25). Using a reaction–diffusion model, Fei et al. (25) determined that the optimal function of the pyrenoid-based CO_2-concentrating mechanism requires a sufficient physical barrier against CO_2 leakage and proper enzyme localization. Additionally, at higher concentrations of CO_2, active transport was not required to have optimal function. This finding demonstrates that the external environment may not require maximum rates of substrate transport across a diffusion barrier for proper function. The application of such models is a key first step in the use of computational modeling to inform metabolic engineers about the influence of spatial organization strategies on flux through their target pathway.

In addition to predicting the most optimal spatial organization method, computational models can be used to guide selection of ideal enzyme ratios for a given pathway. To date, many enzyme encapsulation and scaffolding studies have focused largely on demonstrating that a given metabolic pathway is functional upon encapsulation or scaffolding. However, precise control and consideration of enzyme stoichiometries are essential for maximizing pathway flux in any pathway of interest. Computational models can address this gap by combining existing data on encapsulation and scaffolding efficiency with knowledge of enzyme kinetics to predict not only the most

optimal encapsulation or scaffolding scheme for a metabolic pathway but also the enzyme stoichiometries that should be targeted. Depending on the results of these calculations, researchers could determine, a priori, a set of optimal experimental parameters such as enzyme expression levels and cargo-loading tags that control enzyme stoichiometry. Doing so could enable more efficient use of design–build–test cycles for pathway organization.

Pathway Encapsulation in Microcompartments Should Be Further Studied to Realize Their Use as a Spatial Organization Method

MCPs represent a promising pathway-encapsulating platform because they naturally encapsulate multiple enzymes in nature. However, while there are many hypothesized advantages of encapsulation in MCPs, such as increased intermediate retention, diverted flux away from competing pathways, and private renewing cofactor pools for encapsulated enzymes (13, 44, 65), the degree to which these hypothesized benefits transfer to nonnative pathways is poorly understood. To this end, encapsulation of heterologous pathways with features that enable testing of these specific hypotheses would be highly valuable to the field. For example, encapsulation of a branched pathway would provide insight into how well MCPs promote intermediate retention and flux diversion for nonnative pathways. Alternatively, the ability of MCPs to retain a private cofactor pool for an encapsulated heterologous pathway could be investigated by encapsulating heterologous cofactor-dependent enzymes with and without a cofactor recycling enzyme in the MCP (13, 65). Pathway encapsulation in MCPs is a promising tool for metabolic engineering but requires further study to understand how pathways of interest stand to benefit from encapsulation.

CONCLUSION

Metabolic engineering has helped expand the number of molecules that we can produce using cells. However, introducing new pathways to cells can result in undesirable outcomes. If there are mismatches in expression or kinetic properties of relevant pathway enzymes, intermediates may accumulate over time, resulting in cell toxicity if the intermediate is toxic, or generation of undesired side products due to native metabolism in the cell. To help alleviate these issues, nature has evolved multiple methods to spatially organize metabolic pathways. Spatial organization of pathways can increase local concentrations of required enzymes and substrates that then help increase flux through the desired pathway. Additionally, some natural systems contain a diffusion barrier that can sequester toxic or reactive intermediates, preventing said intermediates from diffusing freely in the cell.

Protein scaffolds have been used in a variety of settings and constructions to increase the yield and stability of enzymes in metabolic pathways. While this approach has been fairly successful for a number of pathways, there are many choices for protein scaffold systems that come with their own complexities and limitations. Notably, most protein scaffolds are still open to the environment, so if the pathway of interest produces a toxic intermediate, an encapsulation strategy may be more desirable.

Many spatial organization strategies provide a diffusive barrier between the external environment and pathway enzymes. They often take the form of a capsid, especially in the case of VLPs and encapsulins. These strategies have mostly been demonstrated to improve the stability and longevity of single enzymes, though multienzyme pathways could potentially be incorporated depending on the encapsulation strategy. To this end, MCPs are emerging as another system for spatial organization. Because they natively encapsulate pathways that generate a toxic intermediate and have a kinetic mismatch between pathway enzymes, MCPs are a promising candidate for use with other metabolic pathways that suffer from the same limitations. Several heterologous

pathways have been encapsulated in MCPs; however, their full utility has not been extensively explored to date.

Further studies should be carried out to help elucidate which types of pathways will benefit the most from spatial organization. Kinetic modeling has helped answer this question but needs to be refined to properly account for the complexity that comes with implementing spatial organization methods. In this way, a combination of modeling and experimental studies will help expand the toolkit for spatially organizing metabolic pathways, increasing their utility across many desired applications.

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