

1 Theoretical and Practical Aspects of Multi-Enzyme Organization and Encapsulation

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Abstract

The advent of biotechnology 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. The 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 evolved spatial organization strategies to increase metabolic pathway flux and efficiency. Inspired by this, bioengineers developed clever strategies to mimic spatial organization in nature. This review specifically explores the use of spatial organization strategies including protein scaffolding and protein encapsulation inside of proteinaceous shells, towards overcoming bottlenecks in metabolic engineering efforts.

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 in *Escherichia coli* (71, 82). As the suite of accessible compounds expands with novel enzyme discovery and enzyme

engineering, efforts to produce 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, there can be undesirable interactions between native enzymes and engineered pathway intermediates, or between engineered pathway enzymes and native metabolites, each 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 multi-enzyme complexes held together by non-covalent interactions varying in stability from transient to long-lived (87). Similar to polyketide synthesis, metabolons co-localize 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 the mitochondria, lysosomes, and vacuoles (3). These organelles sequester certain cellular processes, providing spatial organization within the cell cytoplasm. Likewise, certain species of 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, and were later revealed to be carboxysomes, a type of MCP that plays a role in carbon fixation (9, 20). MCPs serving a variety of metabolic functions have since been discovered, such as the ethanolamine utilization (Eut) MCP and the 1,2-propanediol (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 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 of these strategies come 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 recently developed strategies and examples of protein-based spatial organization of metabolic pathways towards 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 co-localized 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, the enzymes are fused to a tag that co-localizes 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 pair is fused to the enzyme and the other interacting domain 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 inhibits protein folding or function regardless of the termini, 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 as the optimal linker will be protein-tag dependent. Thus, while tagging proteins is a 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 for more enzymes to be scaffolded onto the same structure while also offering direct control over the orientation and stoichiometry of the enzymes. By employing cohesin-dockerin pairs from different organisms, individual pathway enzymes can be ordered in a genetically encoded manner on the scaffold. For example, a three-enzyme methanol oxidation pathway, comprised of an alcohol dehydrogenase, 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 the unscaffolded case. Cohesin-dockerin pairs were also used to scaffold a 2,3-butanediol production pathway in *S. cerevisiae* (52). In this case, three enzymes (acetolactate synthase, acetolactate decarboxylase, and 2,3-butanediol dehydrogenase) were scaffolded using the same cohesin-dockerin pair from *C. thermocellum*. Kim and Hahn observed that increasing the number of cohesin domains lead 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 co-expressed 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 (GBD) 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, hydroxy-methylglutaryl-CoA synthase, and hydroxy-methylglutaryl-CoA reductase in *E. coli* (21). In this case, Dueber et al. 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, they observed less mevalonate production. This highlights the ability to directly determine stoichiometries and enzyme order using these domains and 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 co-localize pathways enzymes in a scaffold-less complex. In Price *et al.*, SH3 domains were used to co-localize the pathway responsible for converting methanol to fructose-6-phosphate (F6P) (79). Methanol dehydrogenase (Mdh) forms a decamer to which 3-hexulose-6-phosphate synthase (Hps) and 6-phospho-3-hexuloseisomerase (Phi) can be attached. Mdh was fused to the SH3 ligand, and a Hps-Phi fusion was fused to the SH3 domain. Using the SH3 domain interactions, Mdh, Hps, and Phi were complexed together. In addition, lactate dehydrogenase was co-expressed 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 co-localize the methanol to F6P pathway in a scaffold-less complex.

The scaffolds described up to this point have all involved specific domains that are on a generic protein scaffold. However, scaffolds have also been developed that take advantage of their self-assembling properties into various structures. For example, PduA

is a self-assembling hexameric shell protein from the 1,2-propanediol utilization bacterial microcompartment (74). PduA has garnered recent 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. utilized a modified PduA (PduA*) with increased stability and overexpressed it producing nanotubes 20 nm in diameter spanning the length of an *E. coli* cell (59). Enzymes of interest were localized to these self-assembled PduA* nanotubes using the coiled-coil system 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, highlighting how certain peptide fusions can interfere with scaffold self-assembly. This technique was used to scaffold the ethanol production pathway enzymes pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (Adh) onto PduA* nanotubes. Scaffolding Pdc and Adh to the nanotube increased ethanol production by 200%. It was also shown 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 scaffold proteins for both enhancing metabolic flux and for controlling 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 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, alcohol dehydrogenase was immobilized on various EutM homologs resulting in increased stability (99). There was variation in the activity enhancement 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). To simultaneously scaffold two proteins, two EutM constructs were used with each containing either the SpyCatcher or SnoopCatcher tags to match the enzymes with the corresponding tags. This allowed for the direct adjustment of enzyme ratios by changing the ratios of the two EutM constructs. In both cases, 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.* bioconjugated cellulase to the T33-21 de novo protein nanocage using *Staphylococcus aureus* Sortase A transpeptidase (66). This sortase links together 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 a ~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-terminus oligoglycine sequence.

Protein scaffolds allow for direct control over enzyme stoichiometry and scaffolded enzyme arrangement. These scaffolds are more modular than designing direct fusion pairs and thus can be applied to many different pathways. Additionally, scaffolds are

versatile in their uses as they have been successfully implemented in both in vivo and in vitro settings. Most notably, scaffolds have resulted in 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

Thus far, the spatial organization methods discussed 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 avoid additional intermediate diffusion away from the enzymes of interest, avoiding toxic intermediate accumulation and undesired side product generation. There have been several methods developed to encapsulate enzymes within a self-assembling protein barrier or shell.

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 covalently fusing the cargo to the capsid itself (15). Enzymes loaded into VLPs have been observed to 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 2 nm pores and a diameter of 58 nm. Upon heating, the P22 VLP will transform into an expanded structure (EX) at nearly double its original diameter and with further heating, will transform into a "wiffleball" (WB) structure with ~10 nm pores (67). Alcohol dehydrogenase (AdhD) was encapsulated inside of P22 to investigate how the different P22 structures influenced encapsulated enzyme kinetics (77). AdhD was encapsulated by C-terminally fusing it to the C-terminus of the P22 scaffolding protein (SP). C-terminus fusion to the SP was sufficient for encapsulation inside of P22 in the native assembly, the EX structure, and the WB structure. Loading AldD in the different P22 VLP structures resulted in different kinetics, showcasing the tunability of VLP capsid permeability. It also demonstrates 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, which interacts with a conserved N-terminal helix on the encapsulin protein (1, 17, 30). Genetically, encapsulins and their corresponding encapsulated enzyme 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 non-native enzymes. For example, Lau et al. used an encapsulin derived from *Myxococcus xanthus* to encapsulate a pyruvate decarboxylase tagged C-terminally with the corresponding targeting peptide (56). In this case, they demonstrated that the tagged heterologous pyruvate decarboxylase 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. 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 (85). This highlights the ability of encapsulins to encapsulate multiple proteins simultaneously that are active when coencapsulated. Encapsulins are a promising vector to encapsulate enzymes to provide enhanced stability. Additionally, because there nearly 1000 encapsulins have been identified, there are many options to choose from with the varying size, geometry, or pore size 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 (MVP). 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 primarily been explored 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 domain (INT) 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 MVP (51). Vault nanoparticles have been used to encapsulate a few different enzymes. Of note are luciferase and manganese peroxidase, which were both encapsulated via fusions to INT (51, 96, 97). In the case of luciferase, the encapsulated enzyme demonstrated similar activity to free luciferase when preincubated with ATP (51). However, in the case where ATP was not preincubated first, the activity was much slower. This indicates that the vault nanoparticle shell creates a diffusion barrier against ATP and should be kept in mind for future use.

Additionally, vault protein nanoparticles provide enhanced stability of enzymes 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 were able to degrade three times more 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 multi-step 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 carbon fixation, and metabolosomes, such as the ethanolamine utilization compartment (Eut), which sequesters the ethanolamine degradation pathway, and the 1,2-propanediol utilization compartment (Pdu), which sequesters the 1,2-propanediol degradation pathway (84, 88). Metabolosomes natively encapsulate metabolic pathways of a characteristic form, where the encapsulated pathways contain a toxic intermediate, a cofactor dependent second step, and typically contain a kinetically fast first step followed by a slower second step in the pathway, suggesting that MCPs are particularly well-suited to generally address these types of metabolic engineering bottlenecks (Figure 2).

More specifically, the microcompartment 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 both

make MCPs a challenge 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 below), 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 “wiffle ball” shell structure (91). These uncapped vertices have a gap of 45 Å which may allow the diffusion of larger molecules through the HO-shell when compared to other MCP shells with pores of less than 10 Å. Kirst et al. used the HO-shell to encapsulate pyruvate formate lyase (PFL) and phosphate acyltransferase (EutD) to convert formate and acetylphosphate into pyruvate (54). In this case, 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 encapsulated metabolic pathways. Further, 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 MCP 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 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 alpha-helical structure which has been hypothesized to interact with MCP shell proteins to facilitate cargo encapsulation (41, 57). Jakobson et al. 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 (42). Interestingly, evidence suggests that there is signal sequence crosstalk for multiple MCP shells due to the common amino acid motif among signal sequences. To prevent the mixing of cargo and MCP shells, bacteria have been observed to express only one MCP at a time, taking advantage of substrate-regulated suppression of other MCPs (89). This naturally existing route to controlling formation of different MCPs in the cell has the potential to 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 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 inducer for bioproduction applications. Nichols et al., 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 (72). 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. This was expanded to co-encapsulate multiple fluorescent reporters in the Pdu MCP. In addition to locus, expression level and selected signal sequence, 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 MCPs

Multi-enzyme pathways have successfully been encapsulated in MCPs using both native encapsulation mechanisms and alternative encapsulation methods. For example, Lawrence et al. constructed an ethanol nanobioreactor by encapsulating pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (Adh) in a minimal Pdu MCP shell (57), resulting in a 63% increase in ethanol production compared to an unencapsulated control. In this work, a minimal Pdu MCP shell was produced using a plasmid-based expression of select *pdu* genes from *Citrobacter freundii* in *Escherichia coli* (75) and a combination of native signal Pdu signal sequence tags were used 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. took advantage of this property and encapsulated the pathway that produces 1,2-PD from glycerol and contains the toxic intermediate methylglyoxal (58). This four enzyme pathway was encapsulated in the same minimal Pdu MCP shell as in Lawrence et al. (57, 75). Tagging the pathway enzymes with native signal sequences resulted in variable reductions in specific enzymatic activity ranging from a 15% - 90% reduction in activity. Despite these reductions in activity, encapsulation of the 1,2-PD producing pathway resulted in a ~100% increase in 1,2-PD production compared to an unencapsulated control. Surprisingly, signal sequence-tagged enzymes without the Pdu MCP shell proteins resulted in a ~240% increase in 1,2-PD production, likely due to signal-sequence mediated

aggregation of enzymes that increased pathway flux via enzyme co-localization. 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 subsequently been repurposed to encapsulate oxygen sensitive enzymes in aerobically-grown *E. coli* cultures (60). For example, Li et al. 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*. In this work, encapsulation of HydA in α -carboxysomes resulted in ~500% increased hydrogen production, suggesting that the carboxysome shell protected HydA from oxygen.

Another property that can be tuned when using microcompartments for encapsulation is their geometry. We discussed above how self-assembling MCP proteins can be used to generate scaffolds, but there are other methods that also still retain the more closed environment characteristic of encapsulation in MCPs. For example, *Mills and Kennedy* et al. knocked out the vertex protein PduN from the Pdu MCP system to create elongated MCP structures (69). These structures, also 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 as compared to a regular MCP geometry, the cells with the MTs were still able to function similarly to typical MCPs but with a slight increase in intermediate buildup.

To explore how this geometry shift might alter pathway kinetics, a kinetic model was developed to explore the apparent change in the surface area to volume ratio between MTs and MCPs. This model elucidated that keeping the surface area of the MTs the same as 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 spherical MCPs while increasing the surface area was predicted to lead to increased diffusion of the initial substrate across the MCP barrier, leading to an increase in the apparent rate of the first reaction. In the case of the Pdu pathway, this would lead to an increased accumulation of the toxic intermediate propionaldehyde. While this initial 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 impact pathway performance. One example of this is the engineered of the glycyl-radical associated microcompartment group 2 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. were able to generate small MCPs around 20-40 nm as well

as larger MCPs around 200 nm. Notably, in order to observe particles, the pentameric vertex protein cmcD must be present. They also were able to encapsulate native GRM2 cargo proteins to these simplified MCP structures. This work provides alternate sized MCP-like structures that are somewhat simpler than the full systems. A simpler system permits rapid engineering in order to tailor it to a pathway of interest, since there are less 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 successfully created similar condensates by fusing peptides of intrinsically disordered regions (IDRs) to proteins of interest (19). These condensates result in the localization of proteins of interest without the use of a protein scaffold. In Zhao *et al.* the N terminus of the FUS protein (FUS^N) was used as the IDR to localize enzymes of the branched violacein pathway and divert flux down specific branches (100). Fluorescent reporters attached to FUS^N formed fluorescent puncta, suggesting FUS^N was sufficient for condensate formation. Two of the violacein pathway enzymes were tagged with FUS^N resulting in a 6-fold enhancement to product formation and an 18-fold enhancement to product specificity. Guan *et al.* also used LLPS to organize enzymes by using the N-terminal RGG domain of LAF-1 (RGG) as the IDR (22, 34). The light-emitting enzyme

NanoLuc was fused to RGG domain and used to visualize condensate formation (35). Formation of condensates containing RGG domain fused NanoLuc could be controlled by temperature, ionic strength, and protein concentration (34). the overall concentration of NanoLuc was increased over 10-fold and reaction rates were increased by ~1.5-fold. Co-localization 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 pathways 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. Further, comparison of encapsulation and colocalization strategies, as in the 1,2-PD production pathway study, suggests that encapsulation is not always the most optimal spatial organization strategy (43, 58). To overcome these challenges, researchers can take advantage of computational models to guide selection of a spatial organization strategy. Modeling has the potential to 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 strategy for predicting which spatial organization strategy would most enhance metabolic pathway flux and how to best apply that strategy to a metabolic pathway. In an example of this, a

computational model was 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 1,2-PD utilization 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 the following parameters: cell membrane permeability to intermediates, Pdu enzyme kinetics, and external substrate concentrations. Another parameter that was considered was the permeability of the MCP shell, since shell permeability influences both pathway flux and intermediate retention. The interplay of these parameters dictated whether an open scaffold or full MCP encapsulation was predicted to be the optimal spatial organization strategy. Interestingly, in some cases it was predicted that no spatial organization would be most beneficial to pathway flux, indicating that spatial organization is not always necessary for enhancing pathway flux. The optimal organizational strategy can also differ depending on whether the goal of enzyme organization is to maximize pathway flux or to reduce intermediate leakage. This type of model has also been developed for a more general and simplified compartment system using Michaelis-Menten kinetics at steady state (93). Here, they also explored the relationship between permeability and enzyme rates to identify when a particular pathway would benefit from encapsulation. For additional details on how compartment models inform the field, we recommend Huffine et al. (38).

Another approach to model spatially organized systems is to use stochastic simulation such as the Gillespie method. Conrado et al. constructed a stochastic model of the biosynthesis of 1,2-propanediol (16). In this case, the stochastic model performed

545 similarly to mass-action kinetics based models when compared using a spatially
546 homogenous system. By then subdividing the reaction space, the authors could utilize
547 the spatial capabilities of stochastic modeling to model the increase in catalytic efficiency
548 when enzymes were confined to particular subdivisions, or compartments. There are also
549 other computational toolboxes, such as Smoldyn, that can model stochastic and spatial
550 detail to predict behavior about compartmentalization in a number of forms (2).

551 Additional aspects that should be considered within encapsulation strategies
552 include the geometry of the compartment strategy as well as diffusion limitations from the
553 encapsulation barrier itself, if it exists. For example, as discussed earlier, Mills et al used
554 kinetic modeling to compare the predicted performance of the microtubes as compared
555 to those of spherical compartments (69) While the diffusion across the compartment
556 barrier is limited by the permeability of the shell, the tubes have two ends that may or may
557 not have the same permeability. When diffusion through the ends was assumed to be the
558 same as free diffusion, there was an increased accumulation of the toxic intermediate,
559 rendering it more similar to no compartmentalization. However, when the diffusion
560 through the ends was more similar to the Pdu shell or blocked entirely, the performance
561 of the MTs was closer to the spherical compartments. Therefore, depending on the
562 system used, diffusion through uncapped tube ends could limit the utility of the
563 compartmentalization strategy. In this case, modeling helped explore the effect of this
564 feature of microtubes.

565 Hinzpeter et al. also explored how the size of a compartment as well as enzyme
566 density affects its productivity (37) In this study, they used carboxysomes as a guide for
567 parameter choices. They discovered that there is a critical compartment size to allow for

maximum productivity, as well as a switch between maximal enzyme packing to partial enzyme packing being the optimal strategy. The actual value for these critical radii also depend on the permeability of the compartment shell to the substrates and intermediates. This again emphasizes the role modeling can play in directing engineering efforts of compartment systems for a given pathway.

Additionally, different types of diffusive barriers may be desired depending on the cell environment that the metabolic pathway will exist in. 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 CO₂ (25) Using a reaction-diffusion model, Fei et al. determined that optimal function of the pyrenoid-based CO₂-concentrating mechanism required enough of a physical barrier against CO₂ leakage and proper enzyme localization. Additionally, at higher concentrations of CO₂, active transport was not required to have optimal function. This demonstrates that the external environment may not require maximum rates of substrate transport across a diffusion barrier for proper function.

Applying these sorts of models is a key first step toward using 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 also be used to guide selection of ideal enzyme ratios for a given pathway. Thus far, many enzyme encapsulation and scaffolding studies have largely focused on demonstrating that a given metabolic pathway is functional upon

encapsulation or scaffolding. However, precise control and consideration of enzyme stoichiometries is 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. This would potentially enable more efficient use of design-build-test cycles for pathway organization.

Pathway encapsulation in MCPs should be further studied to realized their use as a spatial organization method

MCPs represent a promising pathway-encapsulating platform, as 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 non-native pathways is poorly understood. To this end, encapsulation of heterologous pathways with features that allow for 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 non-native 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 of 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 intermediate from diffusing freely in the cell.

Protein scaffolds have been used in a variety of settings and constructions to increase yields and stability of enzymes in metabolic pathways. While this 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

636 still open to the environment, so if the pathway of interest produces a toxic intermediate,
637 an encapsulation strategy may be more desirable.

638 There are many spatial organization strategies that provide a diffusive barrier
639 between the external environment and pathway enzymes. These often take the form of a
640 capsid, especially in the case of VLPs and encapsulins. These have mostly been
641 demonstrated to improve the stability and longevity of single enzymes, though multi-
642 enzyme pathways could potentially be incorporated depending on the encapsulation
643 strategy used. Looking towards encapsulating multi-enzyme pathways, MCPs are
644 emerging as another system for spatial organization. As they natively encapsulate
645 pathways that generate a toxic intermediate and have a kinetic mismatch between
646 pathway enzymes, MCPs are a promising candidate for use with other metabolic
647 pathways that suffer from the same limitations. Several heterologous pathways have
648 been encapsulated in MCPs; however their full utility has not been extensively explored
649 to date.

650 Further studies should be carried out to help enumerate which types of pathways
651 will benefit the most from spatial organization. Kinetic modeling has helped in answering
652 this question but needs to be further refined to properly account for the complexity that
653 comes with implementing spatial organization methods. In this way, a combination of
654 modeling and experimental studies will help to expand the toolkit of understanding for
655 spatial organization of metabolic pathways, increasing their utility across many desired
656 applications.

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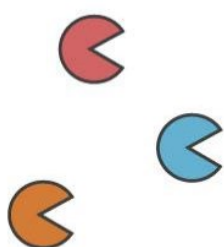
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Table Captions

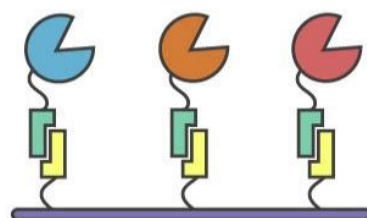
Table 1 Examples of enzyme organization using array, encapsulation, and condensate strategies

Type of Enzyme Organization Strategy Employed	Organization System	Number of Different Enzymes Involved	Attachment Strategy	Kinetic Enhancement Factor (fold change)	Citation
Arrayed		2	Genetic Fusion of enzymes	3.32	Fukuda et al. 2018 (27)
		2	Genetic Fusion of enzymes / Zinc finger protein guided assembly	4.8 / 8.8	Rahman et al. 2014 (81)
		2	Genetic fusion of enzymes	317	Wang et al. 2011 (96)
		3	Cohesin docterin domain interactions	8	Liu, Banks, and Chen 2013 (63)
		3	Cohesin docterin domain interactions	1.37	Kim and Hahn 2014 (52)
	Sequentially Arrayed	3	BH3, GBD, and PDZ domain interactions	77	Dutber et al. 2009 (21)
		2	Col-coi domains attached to PduA nanotubes	2	Lee et al. 2018 (59)
		1	SpyCatcher/SpyTag and SnoopTag/SnoopCatcher attached to EufM	N/A	Zhang et al. 2019 (99)
		2	SpyCatcher/SpyTag and SnoopTag/SnoopCatcher attached to EufM	1.5	Chen et al. 2022 (11)
		2	SpyCatcher/SpyTag and SnoopTag/SnoopCatcher attached to EufM	1.24	Liu et al. 2022 (64)
		2	Staphylococcus aureus Sortase A pentamutant biotinylation to the T13-21 protein nanocage	2.7	McConnell et al. 2020 (54)
	Arrayed onto Msh	3	BH1 domain interaction	97	Price et al. 2015 (79)
Encapsulated	VLP	1	Capid reassembly	N/A	Comellas-Aragones et al. 2007 (16)
	VLP	1	Fusion of the scaffolding protein C-terminus to protein of interest	Variable depending on P22 VLP shape	Patterson, Prevettis, and Douglas 2012 (77)
	Encapsulin	1	Fusion of C-terminus of encapsulin targeting peptide	N/A	Lau et al. 2018 (56)
	Vault Protein	1	Fusion of the INT domain to the C-terminus of the protein of interest	N/A	Kickhoefer et al. 2005 (51)
	Vault Protein	1	Fusion of the INT domain to the C-terminus of the protein of interest	3	Wang et al. 2015 (96)
	Vault Protein	1	Fusion of the INT domain to the C-terminus of the protein of interest	2 to 3	Wang et al. 2019 (97)
	HO MCP	3	SpyCatcher/SpyTag and SnoopTag/SnoopCatcher attached to T1 of the HO compartment	N/A	Kirst et al. 2022 (54)
	Pdu MCP	2	Pdu MCP signal sequences genetically fused to the enzymes of interest	1.53	Lawrence et al. 2014 (57)
	Pdu MCP	4	Pdu MCP signal sequences genetically fused to the enzymes of interest	2.45	Lee et al. 2018 (59)
	Carboxysome	1	C-terminus of Gds2 fused to enzyme of interest	6	Li et al. 2020 (60)
Condensate		2	Genetic fusion of the FUSIN domain to enzyme of interest	6	Zhao et al. 2019 (100)
		1	Genetic fusion of the ROG domain to enzyme of interest	1.5	Guan et al. 2021 (34)

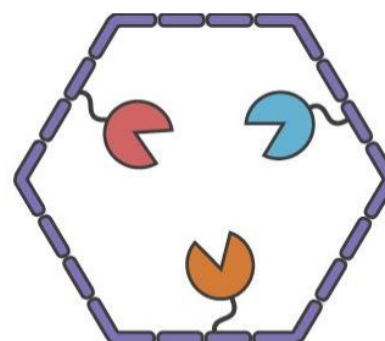
Figure Captions



Free Floating



Open Scaffold



Closed Shell

Figure 1. Spatial Organization Strategies - This review will specifically cover spatial organization using open protein scaffolds and encapsulation within a protein shell and their benefits when compared to free floating enzymes.

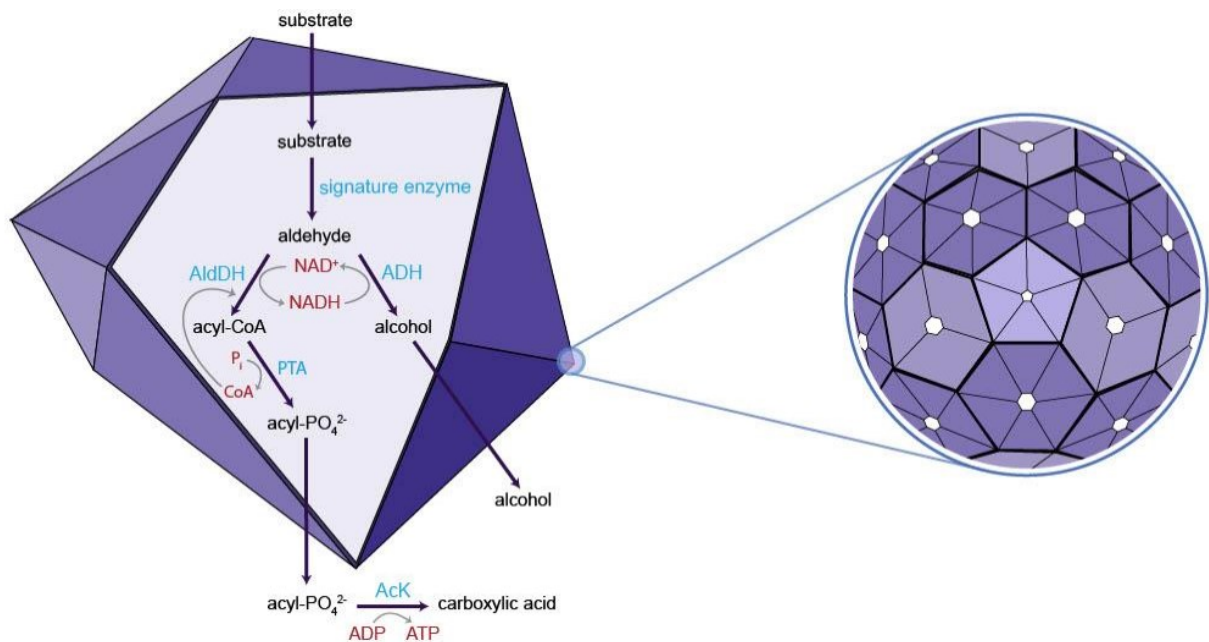
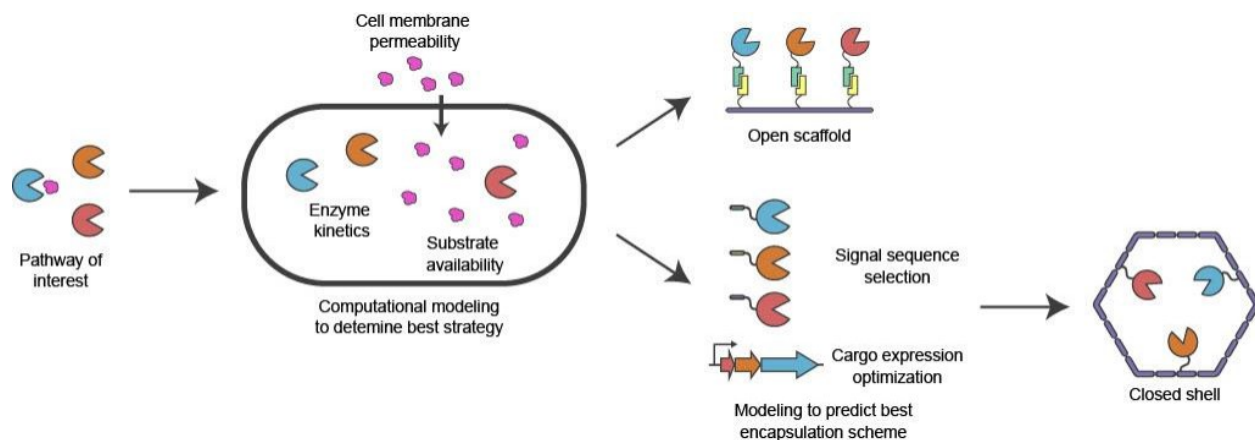


Figure 2. Bacterial microcompartments encapsulate metabolic pathways inside a proteinaceous shell – Bacterial microcompartments are polyhedral proteinaceous organelles consisting of shell proteins that self-assemble into hexamers, trimers and pentamers to form the compartment shell. Bacterial microcompartments natively encapsulate pathways that follow a signature form as shown. AldDH: aldehyde dehydrogenase, ADH: alcohol dehydrogenase, PTA: phosphotransacylase, AcK: kinase.



968

969 Figure 3. Spatial Organization Pipeline – To spatially organize heterogeneous pathways,
 970 modeling should be employed to determine the best spatial organization strategy. If the
 971 best strategy is pathway encapsulation inside of an MCP, modeling may be used to
 972 predict the most optimal encapsulation scheme for the pathway of interest.