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Advances and opportunities for the design of self-sufficient and spatially organized cell-free biocatalytic systems

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During the past decades, biocatalysis has made important contributions to chemical manufacturing by using both whole-cell and cell-free biotransformation reactions. More recently, multi-enzyme systems that can run step-wise reactions in one-pot with high selectivity are increasingly being developed. The use of multiple isolated enzymes to perform a series of reactions offers operational and process advantages over the use of living or resting cells, but such cell free processes need to be optimized to meet industrial productivity and titer requirements. Major advances have been made in enzyme discovery and engineering in order to access new activities and increase catalytic efficiency and stability. Yet, the efficient operation of multiple enzymatic reactions simultaneously requires new approaches for optimization. Inspired by the spatial organization of metabolic networks in cells, researchers have recently begun to exploit these mechanisms to increase the efficiency of multi-enzyme systems. This review highlights recent examples that adopt cellular enzyme co-localization mechanisms for multi-enzyme biocatalysis, which include enzyme attachment to preformed surfaces, enzyme clustering and enzyme encapsulation. Co-immobilization of multiple enzymes is achieved by merging tools from protein engineering and synthetic biology with approaches from material sciences.

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Introduction

Biocatalysis is a key technology that will enable the transition from our current petroleum centered economy to a bio-economy. Yet, industrial biomanufacturing of chemicals is still dominated by the production of relatively simple molecules (e.g. alcohols, amino acids, organic acids) derived via microbial fermentation. These products are expected to make up 11% of the worldwide chemical market by 2020 [1]. Any significant further increase in market share though will require expanding the current portfolio of bio-based chemicals to more complex molecules with diverse functionalities, even non-natural ones. These chemicals cannot be readily obtained by fermentation and need to be synthesized by artificial biosynthetic schemes involving cell-free multi-enzyme systems.

The past decade has witnessed rapid advances in discovery and engineering of enzyme catalysts for a wide-range of industrially relevant chemical manufacturing processes [2,3]. As a result, a diverse toolbox of enzymes and a framework of reaction parameters has been developed to run synthetic schemes that employ one-pot reaction cascades [4,5]. From these efforts, a new phase (dubbed the fourth wave of biocatalysis) of technology innovation is emerging that aims to develop complex, cell-free enzyme cascade reactions — analogous to cellular networks — with the ultimate goal of broadening the chemical palette of biomanufacturing processes [6].

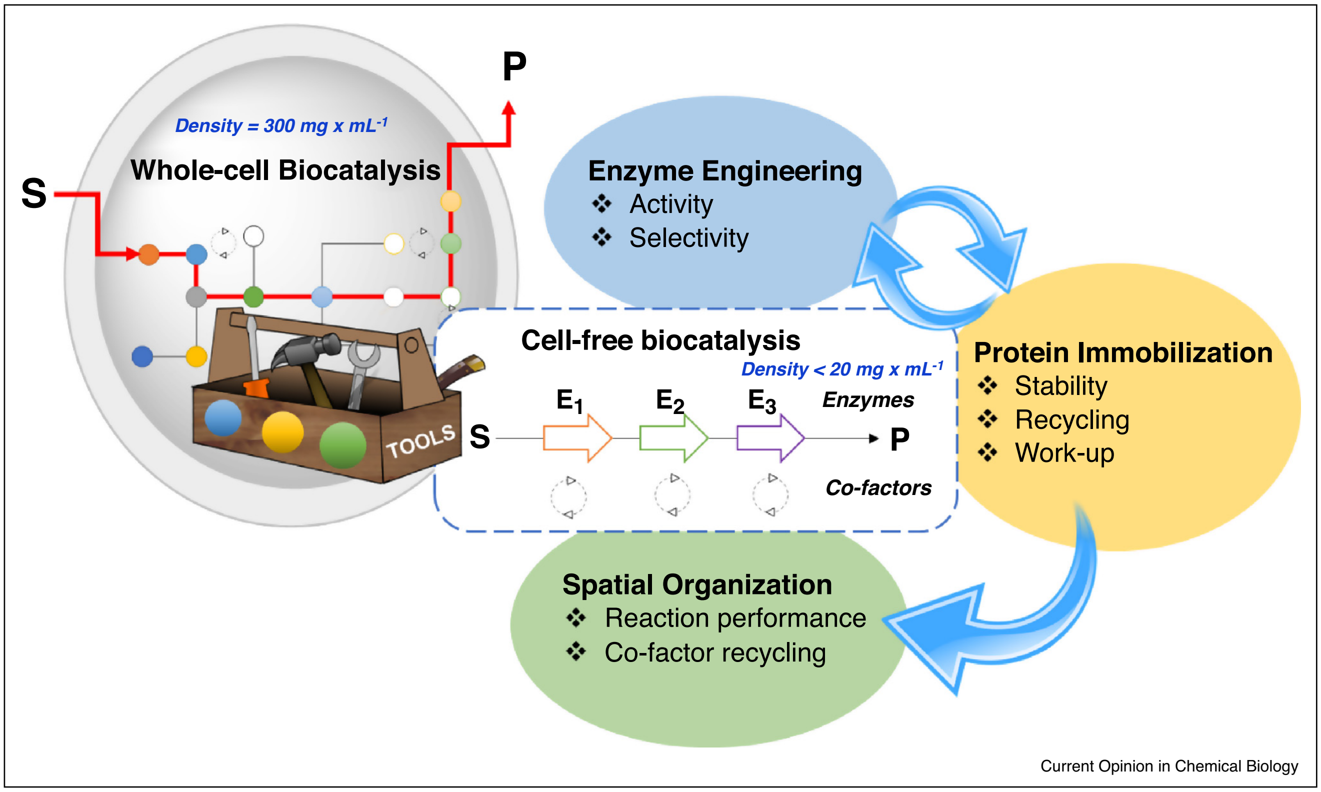
Despite these advances, the scale-up of cost-efficient cell-free biocatalytic processes that ideally operate in one-pot with concurrent co-factor recycling remains challenging [7]. In order to meet the techno-economical requirements for industrial processes, the design of cell-free multi-enzyme systems must address several cost-driving factors to achieve economical space-time-yields (productivities) [8]. These include for example low enzyme production costs, robust biocatalysts that can operate under 'non-biological' conditions, high reaction efficiency and recovery of biocatalysts, co-factors and products [9].

Enzyme engineering [10] and immobilization [2,11] of enzymes on solid carriers are commonly used to stabilize biocatalysts and increase their reaction efficiency. Besides, immobilization also allows for biocatalyst recycling and easy enzyme integration into flow-reactors.

However, co-immobilization of multiple enzymes on the same support is challenging [12] and enzyme engineering can be a laborious process, especially if multiple enzymes require optimization for cell-free operation. Biological systems on the other hand, operate simultaneously and with high efficiency a multitude of complex enzymatic cascade reactions. Unlike current *in vitro* multi-enzyme systems, *in vivo* enzyme cascades are spatially (and temporally/dynamically) organized [13] and operate in a crowded environment with significantly higher protein concentrations [14] (Figure 1). In cells, clustering of enzymes via attachment to surfaces, scaffolding into biomolecular structures and aggregation or confinement into compartments increase the overall efficiency and control flux of metabolic pathways [15,16]. The mechanisms responsible for rate enhancement are not well understood yet, and thus, are still under debate. Recent studies suggest that the specific microenvironment created by enzyme co-localization in confined and/or crowded spaces is a major determinant for increased reaction efficiency [17–19], rather than often-cited substrate channeling induced by spatial proximity of enzymes. In those microenvironments, the intermediates are likely 'pseudo'-trapped through reversible interactions (i.e. ionic, Van der Waals, hydrogen bonds) that hamper their dilution into the reaction bulk. The colocalization of multi-enzyme systems across micro or nanometric areas increases the effective concentration of both enzymes and intermediates within those confined microenvironments. As consequence, the intermediates transport between the different active sites is maximized having positive effect on the overall performance of the multi-enzyme systems. This mechanism is known as 'microdomain' effect and resembles the enzyme clustering found in some biosynthetic pathways rather than the direct substrate channeling found in megasynthases (polyketide synthases, fatty acid synthases, tyrosine synthase . . .), where the intermediates are physically trapped into protein tunnels connecting different active sites through protein-protein interactions [16].

Inspired by the organization of metabolic reactions in cells, different systems have been developed to co-localize *in vitro* multi-enzyme cascades by merging tools and approaches from synthetic biology, enzyme engineering, biocatalysis, material sciences and heterogeneous catalysis (Figure 1). We will discuss the most recent advances in spatial organization of biocatalysts towards the goal of creating self-sufficient, robust and flexible systems suitable for future industrial production of diverse chemicals. Examples will be described in the context of co-localization strategies inspired by biological systems that

Figure 1



Design of self-sufficient enzyme cascades for cell-free biocatalysis. Adopting nature's strategies for spatial organization of enzymes to achieve macromolecular crowding and confinement as a mechanism to increase cascade performance, robustness and recycling.

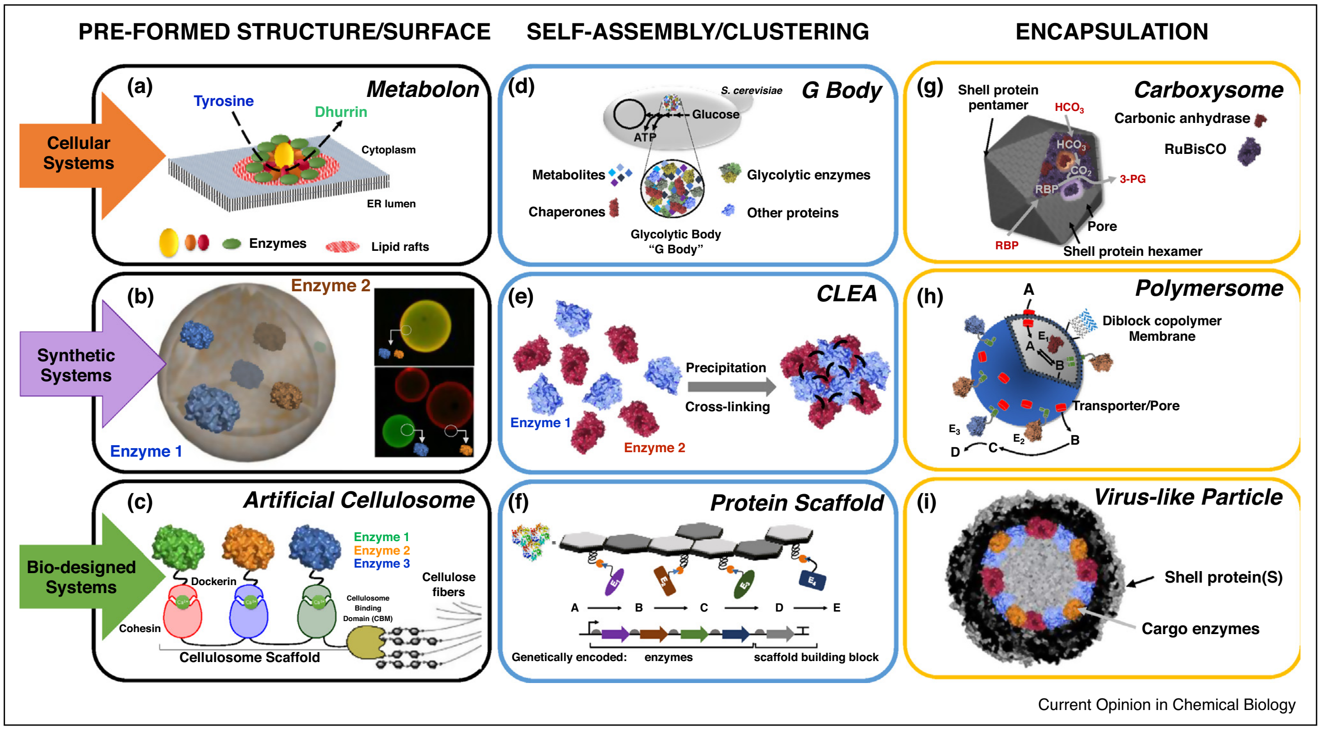
influence the design of artificial cell-free systems. Compartmentalized reactions in macroscopic systems such as reactors or bi-phasic systems [20–22] will not be discussed in this review.

Spatial organization of enzymes via preformed structures and surfaces

Co-localization of metabolic enzymes by using existing cellular structures and surfaces such as, for example lipid membranes or cytoskeletal filaments is a widely used strategy by cells to organize enzymes into metabolons to increase pathway flux [13,18]. Examples include the oxidative phosphorylation pathway in the inner membrane of mitochondria [23], clustering of the dhurrin pathway at the outer membrane of the ER [24] (Figure 2a) and the spatial and temporal organization of glycolytic enzymes via attachment to actin filaments [25]. Immobilization of enzymes to synthetic matrixes or surfaces has been extensively used in industrial biocatalysis for process scale-up and intensification [26]. Typically, one or at most two enzymes are immobilized onto solid materials in a stochastic manner without any targeted positioning of the biocatalysts. Only recently have researchers begun to explore approaches by which to organize multiple biocatalysts on synthetic surfaces.

Microfluidic channels offer the most straightforward way of organizing multi-enzyme cascades onto surfaces by sequential immobilization of catalysts at predefined positions across the surface of the microfluidic system [20,27]. A similar level of organization is significantly more difficult when multiple enzymes are immobilized through chemical bonds on other types of surfaces, such as porous beads or hollow nanofibers. Precise control over the spatial arrangement of several fluorescent proteins across the surface of porous agarose microbeads has recently been achieved by tuning individual immobilization rates [28]. Controlling the immobilization rates of a diamine oxidase onto porous agarose beads led to an optimized biocatalytic system for the degradation of biogenic amines (e.g. putrescine) with concomitant *in situ* elimination of H_2O_2 [29•]. Notably, co-localization of both enzymes at a submicrometer distance in the same bead increased the deamination reaction efficiency sevenfold over reactions with beads where the two enzymes were further apart from each other and thus, delocalized within the same bead (Figure 2b). Close proximity between the two enzymes is hypothesized to result in efficient *in situ* removal of H_2O_2 by catalase, which surrounds the H_2O_2-generating diamine oxidase, and the creation of a favorable O_2 gradients for the oxidase [29•]. Likewise, several recent examples report the selective attachment of

Figure 2



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Overview of cellular and orthogonal enzyme co-localization strategies. Enzymes can be co-localized on preformed structures **(a)–(c)**, via aggregation or self-assembly **(d)–(f)** and through encapsulation into compartments **(g)–(i)**. Natural systems (top row) inspire the development of orthogonal systems using synthetic (middle row) or biological components (bottom row) for the spatial organization of enzymes.

enzymes to either the outside or inside of electro-spun hollow nanofibers. In the latter case, encapsulation inside the nanofiber increased catalytic performance and stability of the encapsulated biocatalysts. This effect was attributed to the confinement of enzyme and substrate and the microenvironment provided by attachment to the luminal surface of the fiber [30,31].

With the goal of creating enzymatic systems suitable for cost-efficient industrial scale biocatalysis, self-sufficient redox cascades have been co-immobilized in both porous microbeads and hollow nanofibers. In these systems, NADH-dependent alcohol dehydrogenases are co-immobilized with NADH and co-factor recycling enzymes to allow for *in situ c*o-factor regeneration [31–33]. Herein, the phosphate groups of the cofactor ionically interact with the cationic groups (i.e. amine groups) of the porous material, establishing an association/dissociation equilibrium within the pores. This equilibrium determines the cofactor concentration available for the immobilized enzymes as well as the amount of cofactor lixiviated to the bulk during the operational process. Therefore, the availability of cofactors within the pores, and consequently the activity of the co-immobilized enzymes, relies on the equilibrium constant of that association/dissociation equilibrium. Hence, tuning the different parameters (pH, ionic strength, nature of cationic groups . . .) that rule that equilibrium, the enzyme activity can be maximized whereas the cofactor lixiviation minimized. In principle, this concept can be expanded to any phosphorylated cofactor. Having in mind that 15 out 29 natural cofactors are phosphorylated, this approach can be applied for a large number of cofactor-dependent enzymes (UDP-dependent glycosyl transferases, ATP-dependent kinases, flavin-dependenent oxidases . . .).

Biopolymer-based templating of spatially organized enzyme systems

Unlike synthetic and preformed solid carriers, biopolymers like proteins, peptides, nucleic acids and sugar polymers allow for a much more facile and precise organization of multiple enzymes on a nanometric scale. Because of their biological origin, selective binding mechanisms such as nucleotide base pairing, peptide-peptide interactions or protein binding domains can be used to attach multiple enzymes to biopolymer derived structures and scaffolds. Nucleic acids are the most widely explored biopolymers for the design of sophisticated nanostructures-based one Watson-Crick base pairing. A number of examples describe enzymatic processes templated on nucleic acid structures, being the co-localization of horseradish peroxidase and glucose oxidase the most extensively used system as model [34]. Because DNA structures allow spacing of enzymes at precisely defined distances, a major objective of these studies has been the demonstration that catalyst proximity enhances cascade performance [35]. This proximity effect, however, was later disproved by Hess and co-workers who showed that conjugating the two enzymes had no effect on cascade activity while the DNA scaffold surface instead provides a microenvironment that enhances cascade performance [36].

In addition to nucleic acid-based nanostructures, enzyme systems have also been attached to self-assembled β-sheet peptide nanofibers [37•] and to sugar polymers [38]. Cellulosomes represent an example of a protein-based scaffold to which cellulose degrading enzymes are attached via calcium-driven interactions between cognate cohesin and dockerin domains [38]. This scaffold has been engineered for the co-localization of enzymes (Figure 2c). For example, a synthetic scaffold composed of two cohesin domains and an oleosin domain was constructed to co-localize enzymes for ethyl acetate synthesis, anchoring the scaffolded cascade to lipid droplet membranes via its oleosin domain. The spatially organized pathway was twofold more efficient than the non-scaffolded pathway [39••].

Co-localization of multi-enzyme systems by induced aggregation

In cells, metabolic enzymes aggregate into dynamic metabolons in response to nutritional or stress conditions in order to enhance activity of a pathway [40]. Examples include the ‘purinosomes’ made up of enzymes required for *de novo* purine biosynthesis [41,42] or the ‘Glycolytic (G) bodies’ formed under hypoxic stress in yeast [43] (Figure 2d). In analogy, enzyme aggregates formed by covalent cross-linking (CLEAs for cross-linked enzyme aggregates) of precipitated proteins have long been used to create robust biocatalysts for manufacturing of chemicals [44] (Figure 2e). This technology, originally implemented by the Sheldon laboratory in the 90’s, has been exploited for numerous enzymes, multi-enzyme systems (combi-CLEA) and hybrid systems composed of chemical and biological catalysts for diverse applications. For example, hybrid-CLEAs made of cross-linked lipase CalB and Pd nanoparticles were used for the selective kinetic resolution of sec-alcohols. This chemo-enzymatic and heterogeneous catalyst could be recycled up to five times without loss of activity and selectivity [45••].

More recently, hybrid organic-inorganic enzyme aggregates have been produced with metal phosphates as inorganic glue to form catalytic nanostructures [46]. Because the preparation of these structures is simple and cheap, various architectures using different metals have been reported and used for biocatalysis, bioremediation and biosensing. Co-immobilization of a His-tagged ω-transamanise and its pyridoxal-5′-phosphate (PLP) on cobalt-biominerals afforded a self-sufficient deamination reaction system that tolerated drastic pH changes during the reaction because the material itself functioned as a solid buffer [47].

Protein-protein interaction driven self-assembly of multi-enzyme systems

Protein-protein interactions drive the self-organization of higher ordered structures in biological systems. Such interactions can be engineered to create nanoarchitectures for the organization of multi-enzyme systems. For example, by exploiting the self-assembling properties of the EutM shell protein from the ethanolamine utilization bacterial microcompartment (Eut BMC) from *Salmonella enterica*, it was possible to recombinantly produce robust protein scaffolds for the covalent attachment of cargo proteins. Fusion of a Spy-Catcher domain to EutM and a short Spy-Tag to desired cargo allowed for simple and fast co-immobilization of enzymes (Figure 2f) for redox neutral chiral amine synthesis [48••]. The scaffolded system improved cascade performance by stabilizing the catalysts attached to the scaffold, affording the same conversion yields in 24 hours rather than 48 hours as with the unattached, free system [48••]. The scaffold itself appears to provide a microenvironment that is beneficial for the catalytic efficiency of the enzyme cascade. Consequently, a toolbox of EutM homologs has therefore been built and characterized for the production of robust scaffolds with different architectures and electrostatic surface properties for their use in cascade biocatalysis [49].

In a different approach, the self-assembling properties of the heterotrimeric *Sulfolobus solfataricus* proliferating cell nuclear antigen (PCNA) was exploited to build controlled supramolecular assemblies for the co-localization of a cytochrome P450 monooxygenases with its two electron transfer partners [50]. This system was subsequently immobilized on magnetic beads allowing enzyme reuse for up to 10 cycles with negligible loss of catalytic performance [51].

Instead of forming CLEAs via precipitation and cross-linking of isolated enzymes, catalytically active enzyme aggregates can also be produced recombinantly via translational fusion of a coiled-coil domain to enzymes, inducing tetramerization and inclusion body formation in *E. coli* [52]. A simple isolation method was used to obtain recombinant aggregates of a benzaldehyde lyase and an alcohol dehydrogenase to sequentially synthesize (*1R*,*2R*)-1-phenylpropane-1,2-diol in one-pot [53].

Encapsulation of enzymes into synthetic compartments (liposomes and polymersomes)

Inside living cells, many metabolic reactions and pathways are compartmentalized into lipid-based (e.g. mitochondria, peroxisomes) [54] or protein-based (e.g. carboxysomes, bacterial microcompartments) organelles [55] (Figure 2g). Such subcellular compartmentalization optimizes metabolic processes by providing optimal pH or redox environments, recycling co-factor or substrate pools, retaining toxic intermediates and by creating crowded reactions environment.

Artificial vesicles based on lipids (liposomes) or polymers (polymersomes) have been used for a range of applications in biotechnology, including enzyme catalysis and more recently, the design of proto-cells [56]. Random encapsulation of enzymes into synthetic vesicles represents the simplest way of compartmentalizing multiple enzymes where substrates diffuse passively across the artificial membrane. More sophisticated vesicle systems have been built with multiple stacked compartments where vesicles inside a larger vesicle perform ‘organelle’ like functions [56]. For example, a compartmentalized polymersome has been designed for a cascade involving a Bayer-Villiger monooxygenase, lipase and alcohol dehydrogenase to synthesize a fluorescent product [57].

Synthetic vesicles can be endowed with cell-like functionalities such as membrane attached proteins or membrane transporters to control transport across vesicle membranes [58] and separating, potentially incompatible reactions, from each other [59]. The latter was shown for a three-enzyme cascade encapsulated into polymersomes, where one enzyme was localized at the lumen and the other two attached to the outside of the vesicle to prevent cross-inhibition [60•] (Figure 2h). Selective transport of substrates across polymersome membrane was achieved via channel proteins, resulting in a compartmentalized cell-free system twofold more efficient than its free counterpart [60•].

The creation of truly self-sufficient, cell-like biocatalytic systems would require the incorporation of energy producing systems. Light-driven processes are therefore explored to generate chemical energy for biocatalytic processes [61,62]. Towards the design of such a light-driven synthetic cell, photosystem II, proteorhodopsin and ATP synthase were successfully combined into light-switchable photosynthetic organelles, which upon encapsulatation in large vesicles drive ATP-dependent polymerization of actin filaments to form a cytoskeleton [63••].

Encapsulation of enzymes into protein-based compartments

Virus and phage capsids are the archetypical examples for protein-based compartments. Other, lesser known, naturally occurring protein shells include the bacterial microcompartments (including the CO_2-fixing carboxysomes Figure 2g) [64], the encapsulins [65] and lumazine synthase [66]. Shell composition, transport across shells via pores, self-assembly and cargo loading can be manipulated by engineering the genetic components encoding the protein shell. One can therefore create a variety of genetically programmable and self-sufficient nanoreactors for cell-free biocatalysis.

Although viruses and microcompartments are typically made up of multiple different shell proteins, engineered shells have successfully been assembled from only one shell protein [67,68] similar to the smaller shells formed by encapsulins and lumazine synthase. Encapsulation of cargo proteins into the lumen protein shells is a directed process and the translational fusions of a short targeting peptide or scaffolding domain to a cargo enzyme is required to localize it to the luminal side of the assembling shell. In some examples, cargo proteins may be directly fused to shell protein building blocks. Native [69] or designed targeting mechanisms [70] can be used to localize multiple cargo proteins into protein compartments.

Most efforts have so far focused on engineering protein compartments for *in vivo* applications with the goal of engineering metabolic functions. The exceptions are the protein cages formed by lumazine synthase and virus like particles (VLPs) assembled from bacteriophage P22 capsid coat protein monomers; which have been engineered for *in vitro* use. The lumazine synthase from *Aquifex aeolicus* self-assembles into a dodecahedron formed by twelve pentamers of enzyme monomers. This naturally occurring protein cage was found to be exceptionally amenable to engineering for a range of *in vitro* applications [66], including the encapsulation of RuBisCO and carbonic anhydrase to construct an artificial carboxysome [71]. Yet, despite achieving high enzyme densities inside the protein cage equivalent to the crowded environment in a bacterial cell ($\sim$ 4 mM or 200–300 mg mL^{-1} protein), no kinetic advantage was obtained by encapsulation, suggesting that the natural carboxysomes must have evolved functional benefits not provided by the artificial cage *in vitro*. Similarly, no kinetic advantage or proximity induced channeling was observed when a two or three-enzyme cascade for the conversion of lactose to galactose- and/or glucose-6-phosphate was encapsulated within VLPs (Figure 2i) [72]. Encapsulation into VLPs, however, shielded and protected enzyme cargo [73] and enabled the formation of higher-order, modular architectures, dubbed ‘superlattice materials’, for cascade reactions [74]. More recently, encapsulins have been engineered as another protein-based nanocompartment for *in vitro* encapsulation of enzymes [75]. As with the VLP and lumazine synthase systems, the encapsulin stabilized the enzyme cargo while allowing unimpeded molecular transport through the shell.

Given the recent advances made in engineering synthetic protein assemblies, it may become possible to design diverse protein compartments with electrostatic surface and molecule transport properties tailored for their enzyme cargo just like natural metabolic microcompartments.

Conclusions

Spatial organization of multi-enzyme systems has until recently largely been ignored by the field of industrial biocatalysis as researchers focused on optimizing individual enzyme properties. The push towards the design of complex enzyme cascades capable of operating efficiently and self-sufficiently in one-pot has required the adoption of new strategies to meet industrial production requirements. Inspired by the efficiency of metabolic pathways and networks in cells, researcher and engineers are combining approaches in enzyme engineering, material sciences and synthetic biology to develop artificial architectures and systems that mimic spatial enzyme assemblies found in nature. Although these efforts are just at the beginning stage, spatially organized multi-enzyme assemblies have already demonstrated promising increases in performance, robustness and recyclability for future integration and upscaling into industrial processes. At the same time, these studies have revealed that there is still much to be learned about the fundamental mechanisms that govern the operation of spatially and temporally organized enzyme cascades in cells. With this knowledge and by taking advantage of the rapid progress made in synthetic cell and *de novo* protein design, it may become possible in the future to design from the bottom-up synthetic cell-like systems dedicated for the self-sufficient, cost-efficient manufacturing of diverse chemicals.

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