Title: Bio-engineering of bacterial microcompartments – a mini review

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Abstract

Bacterial microcompartments (BMCs) are protein-bound prokaryotic organelles, discovered in cyanobacteria more than 60 years ago. Functionally similar to eukaryotic cellular organelles, BMCs compartment metabolic activities in the cytoplasm, foremost to increase local enzyme concentration and prevent toxic intermediates from damaging the cytosolic content. Advanced knowledge of the functional and structural properties of multiple types of BMCs, particularly over the last 10 years, have highlighted design principles of microcompartments. This has prompted new research into their potential to function as programmable synthetic nano-bioreactors and novel bio-materials with biotechnological and medical applications. Moreover, due to the involvement of microcompartments in bacterial pathogenesis and human health, BMCs have begun to gain attention as potential novel drug targets. This mini-review, gives an overview of important synthetic biology developments in the bioengineering of BMCs and a perspective on future directions in the field.

Introduction

Bacterial microcompartments (BMCs) are unique protein organelles with dedicated biochemical functions that can be thought of as the prokaryotic equivalent to lipid-bound eukaryotic organelles. These large (50-200 nm diameter) cytosolic structures are composed of a selectively permeable protein shell which encases a number of enzymes associated with specific metabolic activities[1–3]. The shell prevents escape of toxic and volatile intermediates into the cytosol, reduces loss of intermediates to side-reactions, increases local enzyme concentration and thus flux through the pathway and accommodates recycling of cofactors[4–6].

Operons encoding for BMCs are distributed across the bacterial kingdom, with many species encoding for multiple types of microcompartments[7,8]. BMCs are functionally diverse and carry out both, anabolic and catabolic processes. The only known anabolic BMCs are α- and β-carboxysomes in chemoautotrophic species and cyanobacteria. The carboxysome has major implications in global carbon fixation as it houses the enzymes carbonic anhydrase (CA) and Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), the latter being an enzyme of

the Calvin– Benson–Bassham cycle that fixes CO_2 (Figure 1)[9,10]. Catabolic BMCs are termed metabolosomes due to utilisation of organic compounds such as 1,2-propanediol (**p**ropane**d**iol **u**tilisation PDU), ethanolamine (**e**thanolamine **ut**ilisation EUT), ethanol (**et**hanol **u**tilisation ETU), choline (**c**holine **ut**ilisation CUT), fucose and rhamnose in heterotrophic bacteria (Figure 1)[11–16]. The catalytic cores of metabolosomes perform similar biochemical reactions catalysed by a signature enzyme, generating a toxic aldehyde, and a number of aldehyde-processing enzymes: an aldehyde dehydrogenase (AldDH), an alcohol dehydrogenase (AlcDH) and a phosphotransacylase (PTAC) (Figure 1). BMC genes are organised in superloci which include additional genes responsible for a variety of functions such as transcriptional regulation, cofactor synthesis, substrate transport into the cell and organisation of BMCs inside the cell[8]. Many BMCs, including PDU and EUT BMCs, utilise adenosyl cobalamin (B_{12})-dependant radical chemistry to convert the substrate into an aldehyde intermediate[17]. A recently discovered new class of BMCs, the glycyl radical microcompartment (GRM), uses B_{12}-independent glycyl radical enzymes (GRE) for these reactions and requires external electron sources to form a radical[7]. GRMs are a common but comparably understudied class of BMCs and can be divided into five subclasses (GRM1-5) that are metabolising different substrates such as choline and 1,2-propanediol (reviewed here[18,19]). Notably, GRM as well as EUT and PDU BMCs are encoded by many pathogenic gut bacteria[20–22]. There is now clear evidence that BMCs provide competitive fitness advantages and are implicated in bacterial pathogenic behaviour, not only in pathogens but also in otherwise harmless commensals[22,23]. Moreover, metabolism of choline has been linked with human cardiovascular disease such as arteriosclerosis[24].

BMC architecture and assembly

Shell architecture

Bioengineering of BMCs is based on the understanding of assembly principals of BMC structures. *In vivo* and *in vitro* protein interaction studies, X-ray crystallography and electron microscopy (EM) have given detailed insights into the building blocks and molecular details associated with shell assembly[25–27]. Crystal structures of more than 40 individual shell proteins and a high-resolution structure of the 6.5-MDa assembled BMC shell from *Haliangium ochraceum* are now available[3,27,28]. The shell of all microcompartments is built from three types of proteins: hexameric proteins (BMC-H), trimeric proteins (BMC-T) and pentameric proteins (BMC-P) (Figure 2A). BMC-H proteins contain a small single BMC domain of ~ 90 amino acids (Pfam00936 domain) consisting of an α/β-fold with four anti-parallel β-strands surrounded by small helices. BMC domains assemble into homo-hexameric disks with two

distinct faces, a convex and a concave face. Both, the N and C termini are generally located on the concave side of BMC proteins[29,30]. BMC-Ts assemble into pseudohexamers, similar in size to BMC-H. Here two BMC domains are located in tandem in the same polypeptide chain and three of these molecules form a trimer (Figure 2A). BMC-P contains a different domain (Pfam03319) that forms a five-stranded anti-parallel β-barrel and oligomerises into pentamers occupying the vertexes of microcompartments[31]. An icosahedral shell will only require 12 pentamer copies. Consequently BMC-P is the least abundant protein in the shell. The faces of the BMC are constructed from BMC-H and BMC-T which form interactions via key amino acid residues at the interfaces[32–34]. Selective transport across the shell is mediated via circular pores at the symmetric centre of the hexamers, trimers and pentamers. Small, charged pores (~6 Å diameter), typically found in BMC-H enable the transport of small substrates and products across the shell, e.g. 1,2-propanediol enters through the pores of PduA[6,33]. Larger cofactors like HS-CoA and NAD^+ are thought to enter the shell through large specialised pores (12–15 Å) found in BMC-Ts via a gated mechanism. It has been proposed that the pore opens and closes, controlled by molecule ligands[6,28,35]. Some BMC-H and BMC-T proteins contain an Fe–S centre in the pore, and have been proposed to facilitate electron transfer between the lumen of the BMC and the cytosol for oxidoreductive reactions[36–38]. A much debated question is the orientation of the shell proteins[39]. Two models are currently under debate: The “concave-side-in” model favours the N and C termini of the BMC protein facing the lumen of the BMC. This is based on computational and experimental interaction studies[32,40,41] suggesting that the C-terminal tails of major shell proteins are important for interaction with enzymes in the lumen of BMCs. In the “concave-side-out” model the concave surface of BMC-H faces towards the cytosol consistent with structural evidence of the *H. ochraceum* microcompartment shell[27] and predictions from *in silico* modelling on *in vitro* assembled nanotubes and empty PDU microcompartments[42]. Until shell protein orientation is conclusive shell-core interactions and the orientation of pores remain somewhat ambiguous and constrain rational design of BMC proteins.

Interaction between enzymes and shell

Enzymes residing in the lumen of the shell carry short sequences, ~20 amino acids, termed encapsulation peptides (EP). These are located on the N or C termini or internal regions of the encapsulated enzyme[43]. EPs bind to the shell proteins during BMC assembly (Figure 2B). The affinity of the EP-shell interaction is foremost determined by the primary amino acid sequence, the amphipathic nature and the alpha-helical shape of the EP. In PDU BMCs EPs can be found on the propionaldehyde dehydrogenase PduP[40,43,44], the medium subunit of the diol dehydratase PduD[3,45], and the phosphotransacylase PduL[4] (all N-terminal). Analogous extensions are present in other BMC systems, for example in the ethanolamine lyase small

subunit of EutC[46] in the EUT BMC (N-terminal), and in CcmN, a protein required for assembly of the β- carboxysome[47] (C-terminal). Some glycyl radical enzymes (GRE) contain putative intra-protein EPs, an indicator that EPs could be engineered between protein domains[48]. To date no structural information of an EP-shell protein complex is available. Experimental studies and computational predictions[49] suggest that the C-terminal region of the hexameric shell proteins PduA and PduJ interacts with the N-terminal EPs of cargo protein PduP[40,41]. PduP has also been observed to interact with shell protein PduK[44]. Furthermore, an N-terminal putative helical region of the PduB shell protein is believed to be critical in binding the entire microcompartment core to the shell[50].

BMC Assembly

Two BMC assembly mechanisms have been discovered: "Inside out assembly" where an enzyme core forms followed by the shell assembling around it, and "simultaneous assembly" where enzymes and shell proteins interact simultaneously. Evidence for the two mechanisms has been derived from biogenesis studies of carboxysomes[51–57]. β-carboxysomes form through the condensation of the interior enzymes to a so-called "pro-carboxysomal" body. This is facilitated through aggregation of RubisCO by the protein CcmM which exists in two forms: a full-length M58 which contains a carbonic anhydrase-like domain followed by so-called Rubisco small subunit-like (SSUL) modules connected by unstructured linkers, and a short M35 protein that lacks the carbonic anhydrase like domain[3,54,58–60]. The SSULs are homolog to the small subunit of RubisCO and link RubisCO molecules in a unique biophysical mechanism to form condensates[54,55]. CcmM also recruits carbonic anhydrase (CcaA) via high affinity interaction[61]. The N-terminal domain of CcmM interacts with the CcmN protein which brings the shell proteins to the enzyme core via its C-terminal EP[47,52]. Recent work has shown that CcmM is not solely concentrated around the core as previously proposed[52] but it is also present deep within the enzyme core[62]. This poses future questions about how RubisCO condensation is coordinated with shell recruitment.

In contrast, α-carboxysomes co-assemble cargo and shell at the same time by concurrent recruitment of RuBisCO clusters and shell via interactions with the scaffold protein CsoS2[53]. CsoS2 is composed of three distinct regions, the N-region which recruits the shell proteins, a middle (M-region) that coalesces with RuBisCO through the RuBisCO small subunit (CbbS), and the C-region that anchors the growing sheet of assembled shell proteins[53]. Furthermore, there is evidence that a short helical motif in the N-terminal domain of the large subunit of RuBisCO (CbbL) is essential for the encapsulation of the enzyme by interaction with the major shell protein[56].

According to phylogenetic analysis, the β-carboxysome shell is structurally more closely related to metabolosomes than to α-carboxysomes[10]. Interestingly, metabolosomes have been found to contain repeat domains similar to SSULs in β-carboxysomes. For example, glycyl radical microcompartments contain some enzymes with extensions that mimic a part of the enzyme but are not catalytically active themselves[15,63]. These domains could form interactions to bring enzymes together. In metabolosomes, multiple enzymes can oligomerise via coiled coil interactions of their EPs to generate a "pro-metabolosome" prior to encapsulation[64]. Enzymes without EPs are "piggybacking" onto enzymes with EPs to ensure enclosure into the shell. Such protein-protein interactions have been observed for a number of enzymes[37,65], for instance the key PDU enzymes, aldehyde dehydrogenase (AldDH containing EP) and alcohol dehydrogenase (ADH lacking EP)[5]. Despite a growing understanding of these interactions metabolosome biogenesis is still to be determined.

Engineering of BMCs

Recombinant expression of whole operons

BMC operons are in essence metabolic islands that are horizontally transferrable between bacterial species[66]. This has been demonstrated by the first attempt of BMC engineering where the entire PDU BMC operon associated with 1,2-propanediol utilization from *Citrobacter freundii* was transferred into *Escherichia coli* to generate fully functional PDU BMCs[67]. These compartments were found to be similar in composition, size, shape and mechanical properties to wild type PDU BMCs[68]. Since then other transfers of BMC operons, predominantly into *E. coli*, have been undertaken[69] (Table 1). One example is the expression of a complete α-carboxysomal gene cluster of the Gram negative bacterium *Halothiobacillus neapolitanus* in the Gram positive biotechnological species *Corynebacterium glutamicum* which led to the formation of functional carboxysome-like structures[70]. Remarkably, carboxysome genes have also been expressed in the chloroplasts of the plant *Nicotiana benthamiana*[71] demonstrating that transkingdom expression is possible. Furnishing higher plants with carboxysomes is highly desirable to enhance carbon fixation and productivity in crops.

Synthetic empty BMCs

BMCs are suitable for compartmentalisation of synthetic multi-enzyme pathways due to their capacity to encapsulate hundreds to thousands of molecules. They have a significantly larger internal volume than natural protein cages or current *de novo* capsids [72–74]. The successful generation of "empty" BMC variants[26,45,46,68,75–77] including PDU compartments

from *C. freundii*, EUT compartments from *Salmonella enterica*[78,79], a microcompartment of unknown function from *H. ochraceum*[26] and a synthetic β-carboxysome shell from the cyanobacterium *Halothece* sp. PCC 7418[77] marked the first crucial steps towards generation of novel nano-bioreactors (Table 1). Notably, empty BMCs are seemingly smaller than native BMCs[75,77], indicating that the enzyme core controls the shell dimensions. Evidence for modularity of BMC proteins has been provided by combining shell proteins from α- and β-carboxysomes to form chimeric shells[80] or, in another example, by integrating an α-carboxysome shell protein from *H. neapolitanus*, a PDU shell protein from *S. typhimurium*, and the a RubisCO large subunit from *H. neapolitanus* into a native β-carboxysome[81]. While mixing and matching of components from different compartments is possible because shell protein homologs share the same essential residues at the edges of proteins[92] the result may not always be functional. This was demonstrated by co-expressing closely-related EUT and PDU BMCs which resulted in non-functional hybrid BMCs due to incorrect interactions among PDU and EUT BMC domain shell proteins[82].

Recruiting enzymes into BMCs

Non-native enzymes can be directed into the BMC shell by genetic fusion of the protein of interest with a native encapsulation peptide. It is possible to modify the affinity of the EP-shell interaction by amino acid changes in the EP as long as the amphipathic property of the encapsulation peptide is conserved[84]. The general interaction mechanism enables encapsulation peptides from different BMCs to be fused to heterologous proteins which in turn can be localised in other types of BMCs. For example, putative EUT and glycyl radical enzyme EPs have been used to direct proteins into a PDU BMC[85]. While this provides useful modularity to a recombinant system, EPs seem relatively inefficient at recruiting cargo, mainly due to low control over stoichiometry, competition with other EPs, and the lack of specificity. Alternative interaction approaches include rational design and library-based screening of *de novo* encapsulation sequences[86]. In a different approach, orthogonal shell-cargo interaction pairs were used instead of EPs[87,88]. As a result of attaching one coil of a *de novo* coiled coil pair to the N terminus of shell protein PduA* and the cognate coil to a fluorescent protein, the protein was recruited to the PDU shell[39]. Furthermore, directional targeting to either the cytoplasmic side or the lumen of the shell was demonstrated. The latter was facilitated by a permuted version of PduA*. Here, the native N and C termini were connected and a new N terminus, facing the lumen of the PDU BMC, was created on the opposite face of the PduA tile [39]. The work assumes that the N and C termini of PduA (concave side) face the cytoplasm, as seen in the crystal structure of the synthetic *H. ochraceum* BMC[27,28]. However, as discussed earlier, the orientation of the shell proteins in wild type BMCs is still under debate[40,41,50]. Regardless, the work by Lee *at al.* is an important step towards the design of

the outer and inner surface of BMCs. Encapsulation via covalent linkage (EnCo) has recently been achieved using the SpyTag/SpyCatcher split bacterial adhesin system[89]. This allowed multiple proteins to be introduced into the shell at defined ratio[90]. Other potential systems for covalent attachment may be the utilisation of split inteins[91] or incorporation of non-natural amino acid residues.

Redesign of a BMC for novel functions

Multiple GFP variants[26,43,45,78,80] have been targeted to BMCs via fusion to native or synthetic EPs to provide evidence of successful encapsulation and to study encapsulation efficiency[84]. The first proof of concept for an encapsulated non-native metabolic pathway was provided by introduction of an alcohol dehydrogenase (AdhB) and a pyruvate decarboxylase (Pdc) from *Zymomonas mobilis* into an empty *C. freundii* PDU shell and which resulted in increased ethanol production in *E. coli* [44]. Further examples of re-purposing recombinant PDU BMCs include: (i) polyphosphate accumulation for bioremediation[92], (ii) enhanced recombinant expression of a toxic protein[93] and (iii) encapsulation of various enzymes. The latter demonstrated that multimeric or cofactor dependent enzymes retain their activity inside a BMC shell whilst being protected from the cytoplasmic environment[94] (Table 1). These examples[44,92,94] suggest that a range of small non-native substrates and cofactors can cross the shell, except for lipophilic compounds whose diffusion seems restricted[94]. However, since there is the possibility that recombinant BMCs are not completely closed the promiscuity of shell proteins for non-native substrates remains ambiguous. If it is necessary to tailor BMC diffusion properties for a pathway of interest the following approaches towards pore design can be used: (i) Modification of the residues lining the pores to mimic pores of other native shell proteins[83,95] and (ii) insertion of shell proteins from one BMC system into another BMC system[80]. In the future, these may be applied for a more rational design of custom BMCs.

Assuming complete insulation of a pathway or enzyme is not required, it may not be necessary to fully encapsulate a pathway. Condensing enzymes within the cell in absence of the shell has been shown to increase the metabolic activity of a synthetic pathway of four enzymes. Enzymes fused with native PDU EPs formed a catalytically active protein aggregation in the cytoplasm of *E. coli*, converting glycerol into 1,2-propanediol with productivity increased by 245 % compared to enzymes free in solution[96]. Presumably, aggregation is facilitated through coiled coil interactions of the encapsulation peptides.

Ex vivo and in vitro assembly

While most studies on BMCs have been carried out *in vivo* major advancements have been made on *in vitro* assembly[90,97,98]. Since BMC proteins self-assemble *in vivo*, individual shell

proteins will not readily be available for purification and *in vitro* assembly. Reduced self-association has been observed when fusing shell proteins with affinity purification tags such as the hexa-histidine tag (His_6). This permitted *in vivo* production of His_6-PduA and His_6-PduB, followed by purification and *in vitro* assembly at low salt conditions after His_6-tag removal[98]. In an alternative approach by Hagen *et al.,* individual shell proteins of the *H. ochraceum* shell[26,27], a β-carboxysome and a single BMC-H protein (BMC-H_{Rmm}) from the aminoacetone catabolising Rhodococcus and Mycobacterium microcompartment (RMM) in *Mycobacterium smegmatis* were translationally fused with a short ubiquitin-like modifier (SUMO) "protecting group". This prevented the formation of macromolecular structures *in vivo*[97]. The proteins were purified, the SUMO tag cleaved and the shell components subsequently mixed in a test tube to assemble three types of supramolecular architectures: a metabolosome shell, a carboxysome shell and a BMC protein-based nanotube. The same technique was applied to build a shell with a positively charged inner surface which enabled electrostatic interactions with a charged protein[97]. The HO synthetic BMC (without SUMO) was also produced *in vivo* without the BMC-P vertex protein, then capped *ex vivo* by adding purified BMC-Ps tagged with an affinity tag which was subsequently used for BMC purification[90]. Furthermore, it was possible to load cargo protein *ex vivo* by adding protein to uncapped compartments. This promises titration of cargo of any species able to transit the 47 Å diameter pentamer gap.

Self-assembly of shell proteins to form higher architectures

Shell proteins can form diverse higher-order architectures ranging from flat sheets[99,100], tubes [45,101,102], filaments[67,103] to swiss-role like structures[45] (reviewed here[104]). The shape and size of the structures has been shown to be modifiable by changes in pH, ionic strength[102] and mutation of key amino acid residues in the hexamer-hexamer interface[101]. These structures have enormous potential as biomaterials and for the scaffolding of enzymes. In a recent study by Lee *et al*. filamentous tubes formed of the PDU shell protein variant PduA* have been utilised as a "cytoscaffold" that was decorated (via coiled coil interactions) with two enzymes for ethanol production[105]. Furthermore, localisation of the scaffold to the inner membrane of *E. coli* was possible, which allowed for the spatial organisation of scaffold and enzymes within the cell.

Practical considerations

Despite impressive advances in the design and production of recombinant BMCs, a number of challenges need to be considered. Preparation of BMCs is not trivial because heterologous expression often leads to heterogenous and mis-assembled particles. Improved particle formation frequently requires optimisation of expression levels and efficient loading of enzymes of interest. This has been achieved, for example, by adjusting relative timing of BMC

formation and cargo expression[106] and by addition of a small ssrA degradation tag [64,76,84] to degrade proteins that are not encapsulated. Fusing native EPs to enzymes can severely affect their structure, activity and solubility and as a result can cause aggregation[64]. Novel approaches to improved encapsulation, e.g. via synthetic interaction pairs, have been discussed above. But while synthetic fusions to the shell and cargo can be successful, this might not be a universally applicable method as it may impair hexamer-hexamer interactions or affect enzyme activity.

Expression of BMC proteins reportedly has effects on the host cell by changing cell morphology, cell growth and protein interactions with the cytoskeleton[45,67,70,101]. This effect is understudied because most recombinant expression studies used *E. coli*. Therefore, it is not well understood how readily synthetic BMCs express and assemble in other hosts. A recent study addressed this issue by screening for the ability of diverse bacterial species to produce functional BMCs by insertion of a broad host plasmid carrying a whole *S. enterica serovar Typhimurium pdu and cob/cbi* operon into these hosts: *S. Typhimurium* Δ*pdu*, *E. coli, Salmonella bongori, Klebsiella pneumoniae, Cronobacter sakazakii, Serratia marcescens*, and different *Pseudomonas* species[69]. Such studies may help to identify robust hosts for the expression of synthetic BMCs and may be relevant for synthetic ecology investigations. Many studies use plasmid-based expression systems. Plasmid systems are often unstable due to large numbers of BMC-encoding genes and unsuitable for long-time strain storage. Improved stability might be achieved by genome integration and genomic expression. Alternatively, novel genome editing methods such as CRISPR-Cas9 could be used in future to modify native BMCs *in vivo* and repurpose for new functionalities.

Finally, industrial scale use of BMCs for biotechnological or medical applications requires a robust bioprocess. To our knowledge, all studies so far have been carried out on laboratory bench scale. It remains to be seen whether recombinant strains can readily be cultured at high-density and at scale without impairing BMC expression and assembly. Implications of cost-effective downstream processing and product recovery will also have to be considered.

Perspective

(i) Importance of BMCs

- Bacterial microcompartments are modular, programmable protein bioreactors with a large capacity for enzymes. These properties make BMC architectures attractive as nanotechnology platforms with applications in metabolic engineering and biomedicine.

(ii) Current understanding and challenges

- A variety of BMC architectures can be produced via heterologous expression of the components and functionalised with non-native proteins via non-covalent and covalent interactions. The complexity of BMCs is presenting challenges such as non-optimal protein ratios and inefficient assembly in non-native hosts that will need to be overcome.

(iii) Future directions (Figure 3)

- Rational design of novel BMCs and high-throughput screening via cell-free expression systems should allow for the production of "à la carte" BMCs in prokaryotic and eukaryotic hosts. For industrial applications, scale-up and bioprocess development will be required.

Conflict of interest

The authors declare no conflict of interest.

Abbreviation list

BMC= Bacterial microcompartment

EP=Encapsulation peptide

SE= Signature enzyme

GRM= Glycyl radical microcompartments

GRE= Glycyl radical enzymes

RubisCO= Ribulose-1,5-bisphosphate carboxylase/oxygenase

Ccm= Carbon dioxide-concentrating mechanism

Figures and tables

Figure 1. Metabolic pathways and organisation of BMCs. Sketches representing generic features of α- and β-carboxysomes (left) and metabolosomes (right) with their associated metabolic pathways. Shell proteins are represented as coloured squares forming hexagonal structures within which enzymes and products are shown with the following nomenclature : CA = carbonic anhydrase; 3-PGA= 3-phosphoglycerate; RuBP = ribulose 1,5-bisphosphate; the CBB cyle= Calvin-Benson-Bassham; SE= signature enzyme; AldH = aldehyde dehydrogenase ; AlcDH= alcohol dehydrogenase ; PTAC = phosphotransacylase; R-OH = alcohol product; R-CHO= aldehyde; R-P= phosphorylated product.
The metabolic pathways within BMCs are here briefly described. Carboxysomes encapsulate CA which provides CO_2 to RuBisCO, a key step of the CBB cycle, where the BMC's shell prevents the loss of CO_2 into the cytoplasm. Metabolosomes use a range of substrates (listed on the left). Once transported into the metabolosome lumen, these substrates are converted via so-called signature enzymes into aldehyde, a toxic intermediate. Signature enzymes are specific to certain types of BMCs. For example, the signature enzyme for the substrate ethanolamine is ethanolamine ammonia lyase and the enzyme produces acetaldehyde and ammonia[8]. It is important to highlight that the metabolosome shell prevents these toxic compounds being released into the cytoplasm. The aldehyde is subsequently converted into R-CoA or R-OH. Conversion to R-CoA requires NAD^+, which is provided by AldDH.

Figure 2. Shell proteins and BMCs assembly. Schematic representation of the structures of the shell proteins BMC-H (blue), BMC-T (pink), and BMC-P (green) and core enzymes are shown in panel A. BMC-T can be found in the single trimer form (BMC-T^s) and as two trimers dimerising along the concave face which is referred to as double BMC-T (BMC-T^d). Pfam domain identification numbers (Pf00xxx) of the corresponding shell proteins are indicated. The EP (when associated with core enzymes) is represented as a black helix, a scaffolding enzyme such as CcmM is shown in brown, enzymes without EP are shown in green. B, Sketch representing the generic assembly process of BMCs: the core enzymes/encapsulation peptide can assemble first or together with shell proteins to form fully assembled BMCs. EM images (right) showing purified PDU metabolosomes from *C. freundii* (modified from[68]).

Figure 3. Overview of future approaches of BMC engineering. A, a library of individual or multiple BMC "parts" can be produced *in vivo* or *in vitro*. The parts can be further assembled in order to engineer "à la carte" BMCs. Red extensions denote affinity tags for BMC

purification. Blue triangles indicate protecting groups that stop shell proteins from self-assembling. B, A representative strategy to use BMC components as scaffold building blocks is shown. Here BMC shell proteins are genetically fused with synthetic interacting parts (coiled-coil peptides or covalent pairs) and produced together with enzymes of interest that are fused with interacting counterparts. This would allow physical proximity of the enzymes with controlled stoichiometry and enhance flux and ultimately, product formation. Letters A-D are substrates/products of a designed pathway.

Figure 1

Figure 2

Figure 3

Table 1: Representative examples of BMC engineering

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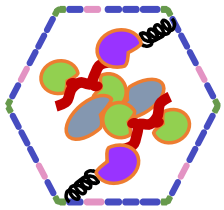
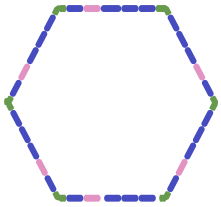
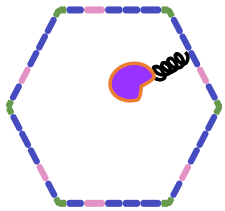
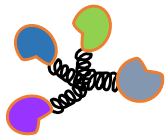
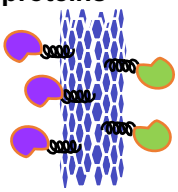
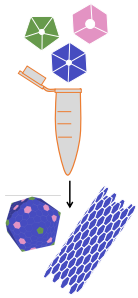
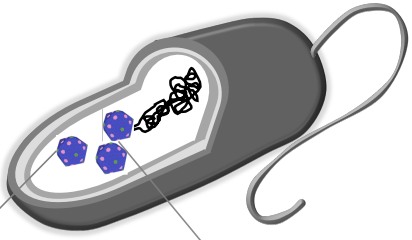
[106] Jakobson CM, Chen Y, Slininger MF, Valdivia E, Kim EY, Tullman-Ercek D. (2016) Tuning the Catalytic Activity of Subcellular Nanoreactors. J. Mol. Biol. [Internet]. **428**, 2989–2996. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0022283616302510

Table 1: Representative examples of BMC engineering

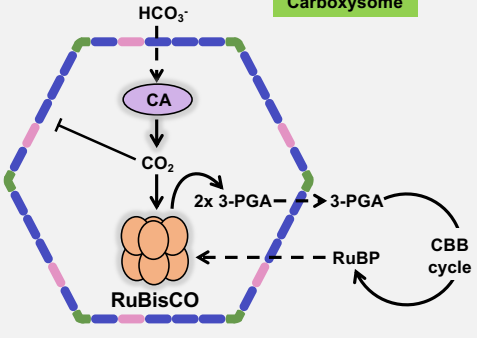
Purpose	Description of components	Expression host	References
Recombinant BMCs 	*pdu operon* of *Citrobacter freundii*	*Escherichia coli*	Parsons et al. 2008[67]
	α-carboxysome regulon from *Halothiobacillus neapolitanus*	*E. coli*	Bonacci et al. 2012[107]
	β-carboxysomes from *Synechoccus. elongatus* PCC7942	*Nicotiana benthamania* chloroplasts	Lin et al. 2014[71]
	α-carboxysome gene cluster of *H. neaplitanus*	*Corynebacterium glutamicum*	Baumgart et al. 2017[70]
	12 β-carboxysome genes of *S. elongatus* PCC7942	*E. coli*	Fang et al. 2018[81]
	Broad host plasmid carrying a whole *S. enterica serovar Typhimurium pdu and cob/cbi genes* operon	Many different Gram negative hosts	Graf et al. 2018[69]
Empty shell 	PDU shell proteins from *C. freundii*	*E. coli*	Parsons et al. 2010[45]
	EUT shell proteins from *Salmonella enterica*	*E. coli*	Choudhary et al. 2012[46], Quin et al. 2016[78]
	Shell of BMC of unknown function from *Haliangium ochraceum*	*E. coli*	Lassila et al. 2014[26]
	Synthetic β-carboxysome shell from *Halothece* PCC7418	*E. coli*	Cai et al. 2016[77]
	PDU shell proteins from *C. freundii*	*C. glutamicum*	Huber et al. 2017[108]
Encapsulation of heterologous proteins 	PduP$^{1-18}$-eGFP and PduP$^{1-18}$-GST fusions	*S. enterica*	Fan et al. 2010[43]
	PduC and PduD from *C. freundii* fused to GFP targeted to recombinant PDU shell	*E. coli*	Parsons et al. 2010[45]
	EutC$^{1-19}$-eGFP, EutC$^{1-19}$-ß-galactosidase targeted to empty EUT shell (*S. enterica*)	*E.coli*	Choudhary et al. 2012[46]
	H. ochraceum aldehyde dehydrogenase (full length or EP) fused with GFP, targeted to empty *H. ochraceum* shell	*E. coli*	Lassila et al. 2014[26]
	Increased ethanol production: alcohol dehydrogenase (AdhB) and a pyruvate decarboxylase (Pdc) from *Zymomonas mobilis* fused with PduP and PduD EPs from *C. freundii* and targeted into empty PDU shell	*E. coli*	Lawrence et al. 2014[44]

	EutC$^{1-19}$-eGFP and EutE$^{1-21}$-eGFP interact with same shell protein EutS in empty EUT shell	*E. coli*	Quin et al. 2016[78]
	β-carboxysomal CcmN$^{211-258}$ fused to the C terminus of GFP, targeted into synthetic carboxysome shell	*E. coli*	Cai et al. 2016[77]
	PduP$^{1-18}$-PKK1 (polyphosphate kinase) targeted into empty PDU shell	*E. coli*	Liang et al. 2017[92]
	Enhanced recombinant expression of lysis protein E from bacteriophage φX174: EPs of PduD, PduP, EutC fused with lysis protein E and targeted into EUT or PDU shell	*E. coli*	Yung et al. 2017[93]
	Esterase Est5 from soil metagenome, β-galactosidase and NADH-dependent glycerol dehydrogenase (GldA) from *E. coli*, targeted to PDU BMC shell, protection against external pH stress	*E. coli*	Wagner et al. 2017[94]
Shell-free aggregates 	Increased 1,2-propanediol production by enzyme aggregation via coiled coil interaction of native EPs: glycerol dehydrogenase, dihydroxyacetone kinase, methylglyoxal synthase and 1,2-propanediol oxidoreductase fused with PduP and PduD EPs	*E. coli*	Lee et al. 2016[64]
Scaffolding of heterologous proteins 	Citrine, mCherry and alcohol dehydrogenase, pyruvate decarboxylase from *Z. mobilis* attached to a PduA* filament using coiled coil interactions, increased production of ethanol compared to non-scaffolded enzymes	*E. coli*	Lee et al. 2018[105]
***In vitro* assembly & *ex vivo* cargo loading** 	*In vivo* production of shell proteins from *H. ochraceum* shell, β-carboxysome and single BMC-H protein (BMC-H$_{Rmm}$) from *Mycobacterium smegmatis* fused with protecting group (SUMO), SUMO tag cleaved, shell components subsequently mixed in tube to assemble a metabolosome shell, a carboxysome shell, and a BMC protein-based nanotube	*E. coli/ in vitro*	Hagen et al. 2018[97]
	H. ochraceum shell produced *in vivo* without the BMC-P vertex protein, then capped *in vitro by adding* BMC-Ps, fluorescent cargo protein loaded *ex vivo* by adding proteins to uncapped compartments, then capped and purified.	*E. coli/ ex vivo*	Hagen et al. 2018[90]

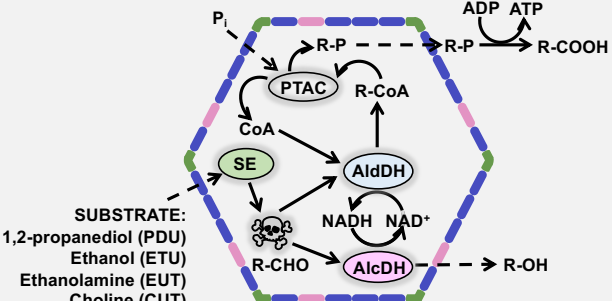
References are included in the main document

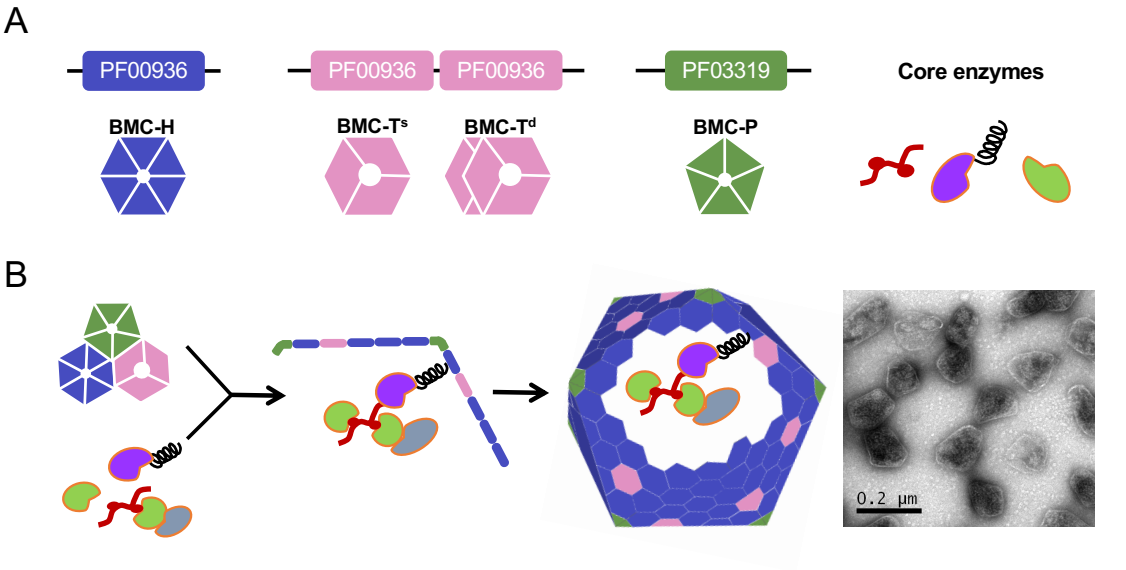


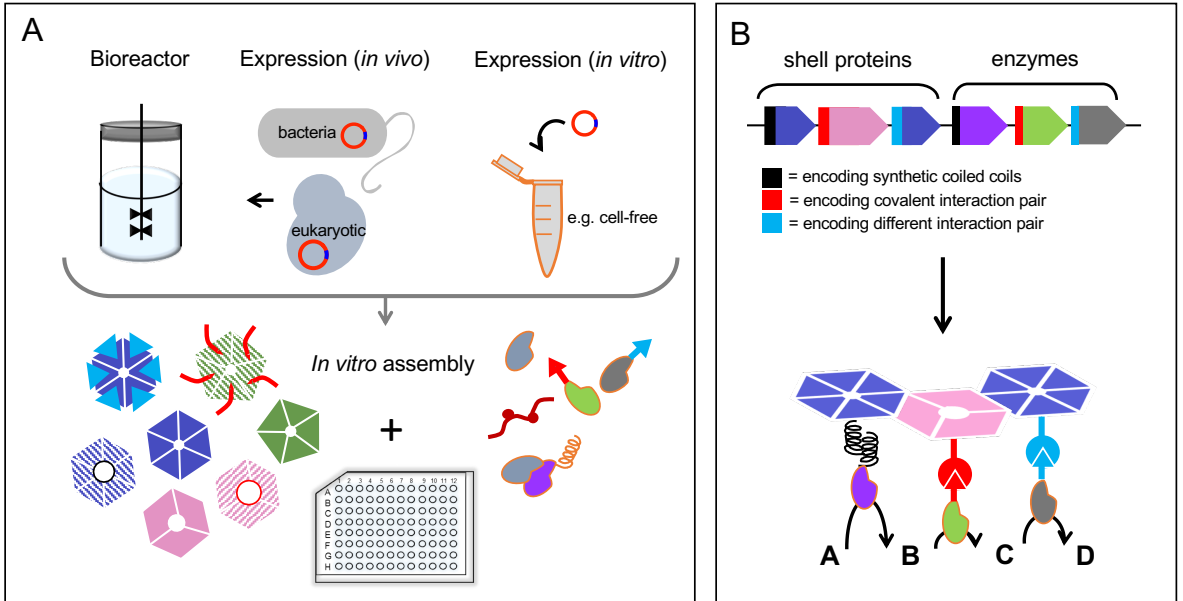
Carboxysome



Metabolosome




A
PF00936
BMC-H
PF00936
PF00936
BMC-T^s
BMC-T^d
PF03319
BMC-P
Core enzymes
B
0.2 μm


A
Bioreactor
Expression (in vivo)
Expression (in vitro)
bacteria
eukaryotic
e.g. cell-free
In vitro assembly
+
B
shell proteins
enzymes
= encoding synthetic coiled coils
= encoding covalent interaction pair
= encoding different interaction pair
A
B
C
D