Cell PRESS

Special Issue – Synthetic Cell Biology

Designing biological compartmentalization

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Intracellular organization is a key factor in cell metabolism. Cells have evolved various organizational systems to solve the challenges of toxic pathway intermediates, competing metabolic reactions, and slow turnover rates. Inspired by nature, synthetic biologists have utilized proteins, nucleic acids, and lipids to construct synthetic organizational systems that mimic natural systems. Many of these systems have been applied to metabolic pathways and shown to significantly increase the production of industrially and commercially important chemicals. Further engineering and characterization of synthetic organizational systems will allow us to better understand native cellular strategies of spatial organization. Here, we discuss recent advances and ongoing efforts in designing and characterizing synthetic compartmentalization systems to mimic natural strategies and increase metabolic yields of engineered pathways.

Compartmentalization benefits natural and engineered systems

Biological complexity requires varying degrees of organization. Cells require spatial organization to perform the various enzymatic reactions and processes necessary to sustain life [1]. This is achieved through compartmentalization, the physical separation of biological reactions. Examples of compartmentalization include membrane-bound organelles, bacterial microcompartments [2,3], multienzyme complexes, and others [4,5].

Inspired by nature, synthetic biologists have recently devised strategies to mimic cellular organizational systems. These synthetic systems have been predominantly designed toward metabolic engineering of pathways, harnessing the capability of cells to produce industrially [6,7] or pharmaceutically useful [8,9] compounds.

In this review, we describe the various difficulties faced by the cell when performing metabolic reactions and natural compartmentalization systems that solve these problems. We review recent advances in designing synthetic compartments that provide modular solutions to overcome these same challenges. These systems capture the benefits of spatial organization and apply them to engineered pathways. This has also recently been reviewed in [10–12]. Our review will discuss the latest progress and challenges in designing compartmentalization, especially in building bacterial microcompartments and RNA scaffolds. We also analyze the degree to which these mimic natural systems and discuss how they aid in our understanding of the biological organization of the cell.

The need for intracellular organization

Cells face many challenges that benefit from compartmentalization (Figure 1a). First, some enzymes, such as ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCO) [13], suffer from slow turnover, which results in flux imbalances or bottlenecks in pathways. Reliance on such enzymes may require establishing local concentration gradients of substrates. This would increase reaction rates to support adequate pathway flux [14]. Second, diffusion of volatile intermediates through the cell membrane results in their loss from the cell [15]. Third, biosynthetic pathways can generate toxic intermediates that inhibit growth, such as hydrogen sulfide accumulated during bacterial sulfur metabolism [16]. Finally, metabolites can participate in multiple competing reactions, reducing their availability for any single pathway. An example of this is malonyl-CoA, an intermediate that is consumed in fatty acid and phospholipid production but is also used in the biosynthesis of polyketides and flavonoids [17].

Nature's solutions

To deal with these challenges, nature has evolved compartmentalization strategies (Figure 1b), such as large enzyme complexes [10,18,19] and organelles [2,20], to spatially organize metabolism. In eukaryotes, compartmentalization in the form of membrane-bound organelles is common. The peroxisome, for example, encapsulates reactions that generate or consume hydrogen peroxide, a toxic intermediate from the breakdown of organic substrates in oxidative reactions [21].

Until recently, prokaryotes were generally thought to lack internal organization [22]. However, researchers have recently discovered different types of bacterial microcompartments that partition the internal space of the bacterial cell for specialized functions [2,23]. In cyanobacteria and other autotrophic prokaryotes, carboxysomes encapsulate RuBisCO and carbonic anhydrase, enzymes involved in the rate-limiting step of the Calvin cycle [24,25] (Figure 2a). These proteinaceous microcompartments are the primary 'carbon-concentrating mechanism' in these bacteria. They

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Keywords: compartmentalization; synthetic scaffolds; bacterial microcompartments; biological organization.

 0962-8924/$ – see front matter © 2012 Elsevier Ltd. All rights reserved. http://dx.doi.org/10.1016/j.tcb.2012.07.002

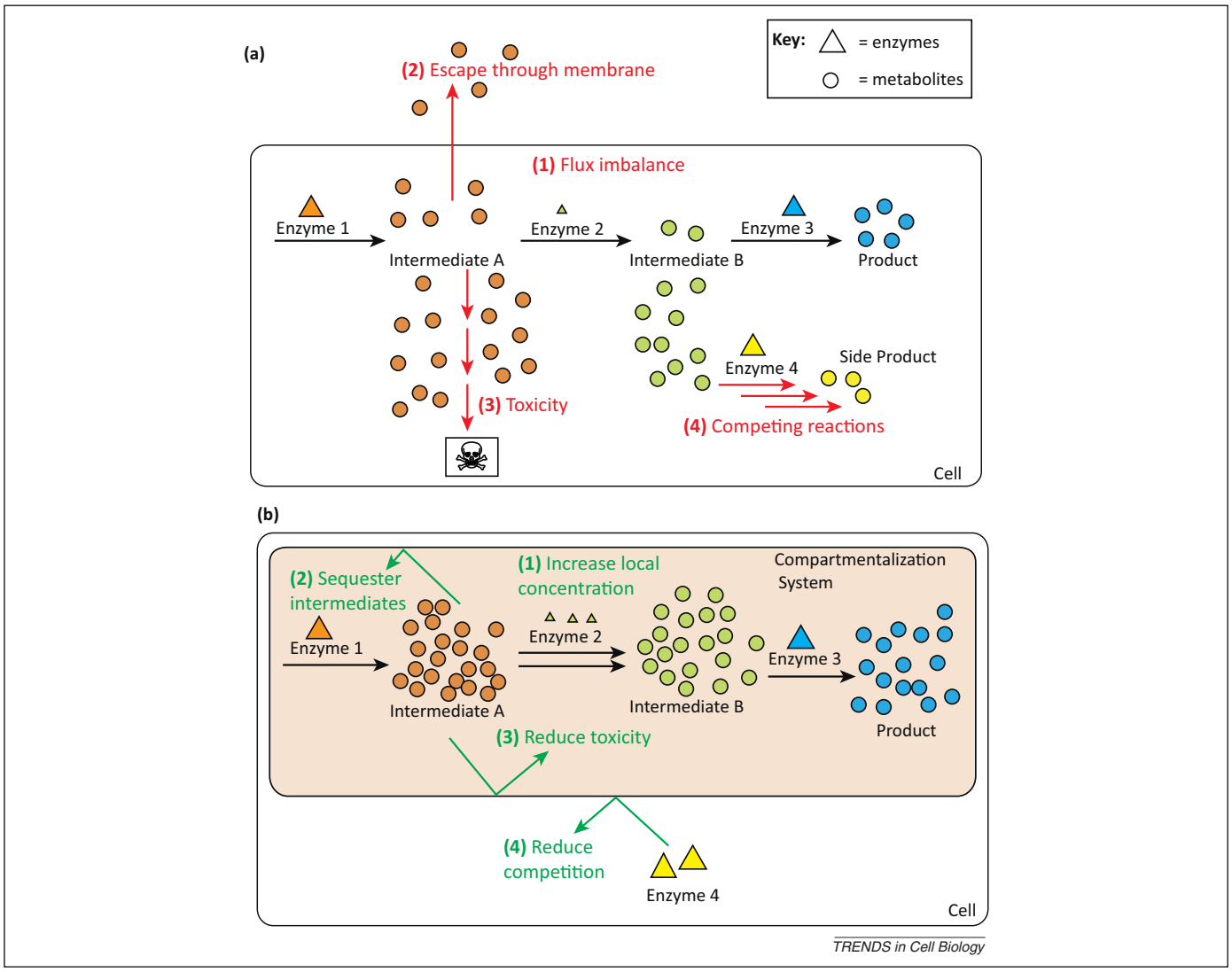

Figure 1. Compartmentalization: nature's solution to various challenges. **(a)** Nature faces many challenges when conducting the chemical reactions of the cell. (1) Differing enzyme kinetics may result in flux imbalances. (2) Intermediates may be lost through the cell membrane. (3) Toxic intermediates can result in growth inhibition. (4) Competing reactions can divert flux through undesired pathways. **(b)** Compartmentalization systems specifically solve challenges 1–4, respectively, by: (1) creating areas of local concentrations to favor reaction kinetics; (2) sequestering intermediates; (3) reducing toxicity; and (4) reducing competition. (Adapted from [11].)

are proposed to help overcome the slow turnover rate of RuBisCO by providing a high local concentration of carbon dioxide to the enzyme [26,27].

Two other bacterial proteinaceous microcompartments protect the cell from toxic aldehyde intermediates. The ethanolamine utilization (Eut) microcompartment sequesters acetaldehyde, a volatile and toxic intermediate of the ethanolamine utilization pathway [28]. Likewise, the 1,2-propanediol utilization (Pdu) microcompartment encapsulates propionaldehyde, minimizing its toxicity [29]. These and numerous other bacterial microcompartments have been found in approximately 400 microbial genomes [2].

Another method of compartmentalization found in nature is multienzyme complexes, which directly link enzymes involved in a given pathway. Ideally, this results in substrate channeling, the process by which intermediates are directly transferred between the active sites of two enzymes that catalyze sequential reactions in the pathway [30]. Substrate channeling prevents the loss of intermediates and minimizes competing cross-reactions. A classic example is tryptophan synthase, a multienzyme complex that catalyzes the last two reactions in the biosynthesis of L-tryptophan [18,31]. The intermediate, indole, is channeled from one active site to the next without being released into the surrounding environment. This is advantageous for the cell not only because indole is reactive and easily lost through the cell membrane, but also because, in the absence of indole, tryptophan synthase catalyzes dehydration of serine to pyruvate at 5% the rate of tryptophan formation [32]. Other multi-enzyme complexes found in nature include polyketide synthase [33], carbamoyl phosphate synthetase [34], and cellulosomes [35], which may function similarly by increasing reaction kinetics and reducing the loss of intermediates.

Synthetic compartmentalization

The goal of metabolic engineering is to optimize a given biosynthetic pathway to increase production of a particular substance [7,36]. Many of these pathways present the same challenges of toxic intermediates, competing reactions, and flux imbalances found in nature [10,14]. Therefore,

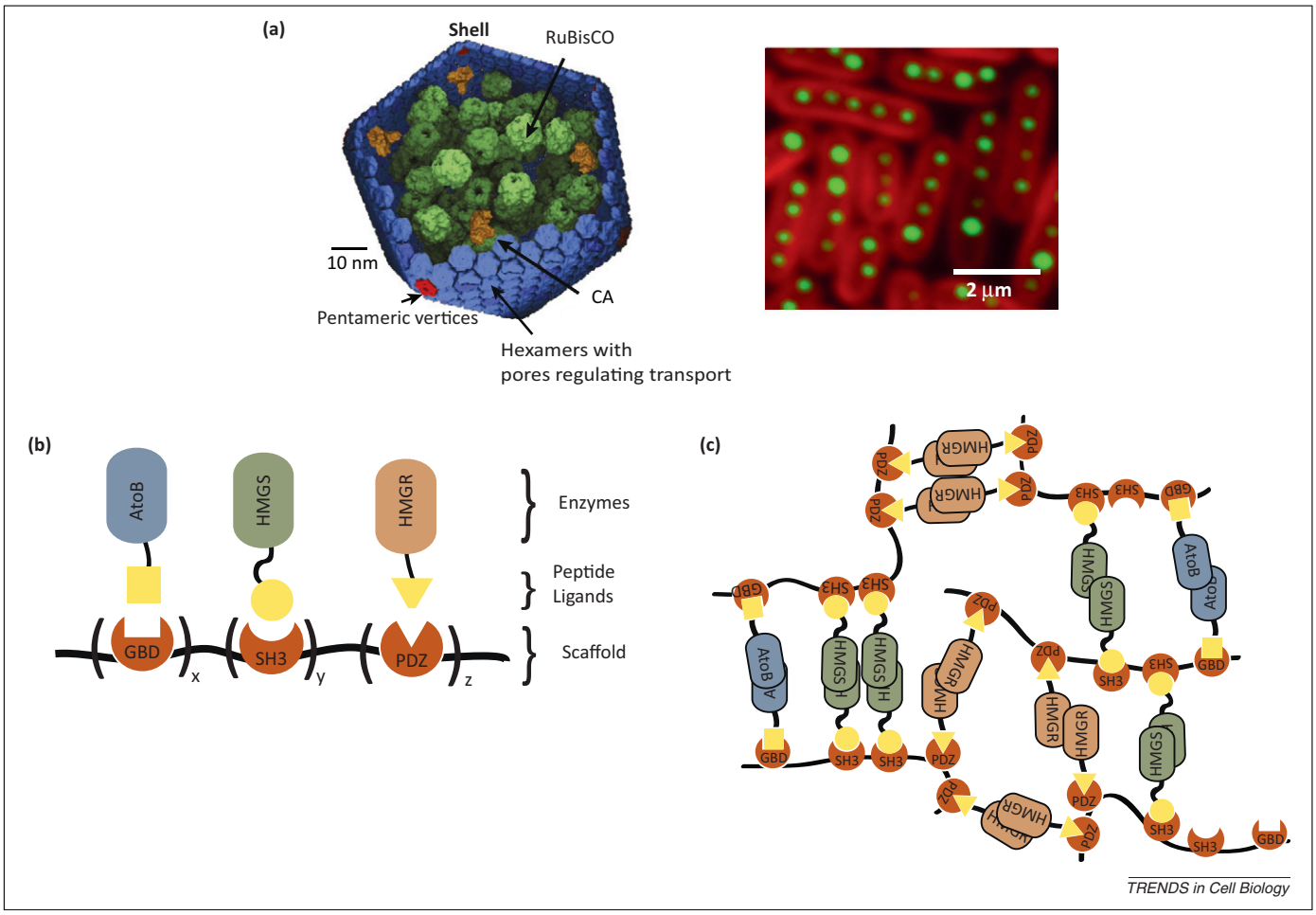

Figure 2. Protein scaffolds: utilizing nature's building material for functional complexes. Nature compartmentalizes reactions via enzyme complexes. Proteins often serve as scaffolds, bringing together pathway enzymes and increasing flux. **(a)** Bacterial microcompartments are protein structures that encapsulate reactions [2]. Carboxysomes contain ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCO) and carbonic anhydrase. The shell comprises pentamers and hexamers, some of which contain pores that are thought to regulate substrate transport (left). Carboxysomes spatially align in the cell [51] (right). Heterologous expression of microcompartments and further engineering of targeting and transport has the potential for making microfactories that may be capable of encapsulating various reactions. (Left: reprinted from [46] with permission from the authors. Right: image courtesy of David F. Savage, Berkeley, CA, USA). **(b)** Inspired by natural functional complexes, synthetic protein scaffolds were created [40]. Eukaryotic protein–protein interaction domains (GBD, SH3, PDZ) were expressed as a single polypeptide, creating a synthetic scaffold. Modular protein domain–ligand interactions were used to colocalize three enzymes of the mevalonate pathway: AtoB, HMGS, and HMGR. The stoichiometries of the enzymes could be controlled by changing the numbers (x, y, z) of each of the scaffold domains. Scaffolds balance metabolic flux, sequester a toxic intermediate, HMG-CoA, and create a high local concentration of intermediates, effectively mimicking nature's strategies of compartmentalization. **(c)** Scaffolds are hypothesized to oligomerize into large complexes that resemble metabolite microdomains, trapping intermediates in the interior and quickly converting them to product before they escape [11].

synthetic biologists have drawn inspiration from nature to design synthetic compartmentalization systems.

Compartmentalization by tethering using protein scaffolds

Nature uses proteins and protein–protein interactions to build functional multienzyme complexes. In some cases, non-catalytic scaffold proteins are used to assemble these complexes. One example of such a scaffold protein is Ste5, which selectively brings together MAPKK Ste7 and its substrate MAPK Fus3, promoting phosphorylation in this signaling cascade and disfavoring competing substrates [37]. Another example is scaffoldins, which organize protein subunits into the cellulosome and assist in the breakdown of cellulose [35]. Bacterial microcompartments, large assemblies comprising thousands of protein subunits, also function as organizational devices in the cell. Attempts to mimic natural protein scaffolding mechanisms have included crosslinking enzymes [38], enzyme immobilization on solid substrates *in vitro* [39], and direct enzyme fusion [10].

Inspired by naturally existing enzyme complexes, a synthetic protein scaffold, capable of colocalizing enzymes and increasing product titers, was produced [40] (Figure 2b). Eukaryotic protein–protein interaction domains were fused to form scaffolds. Each of the domains selectively recruited enzymes fused to cognate peptide ligands, colocalizing them to the scaffold.

Protein scaffolds have been tested on various pathways and increases in titers were shown. The mevalonate pathway, for example, produces a toxic intermediate and suffers from flux imbalance due to differing enzyme turnover rates. Scaffolding increased product yields by 77-fold [40]. The glucaric acid pathway was also scaffolded, and 5-fold improvement was seen over a high baseline titer of 0.6 g/L [41]. This pathway requires high levels of intermediate myoinositol to drive myoinositol oxygenase kinetics. A final example is biological hydrogen production, which suffers

from competing reactions and requires close contact of the two enzymes involved. This pathway benefited by about fivefold from protein scaffolding [42]. In principle, these synthetic protein scaffolds can replicate natural compartmentalization strategies found in multienzyme complexes, thereby alleviating the problems sometimes found in engineered metabolic reactions.

The extent of the increase in yields due to scaffolding depends on scaffold stoichiometry. Nature uses gene duplication events and evolution of protein–protein interactions, events that operate on a long timescale, to change the ratio of each of the enzymes in multienzyme complexes. Synthetic scaffolds similarly enable us to build and test many different enzyme stoichiometries, but in a short period of time, to determine the optimal ratio for production of a compound of interest. Varying the number of each interaction domain in the scaffold was shown to dramatically affect product titers [40]. In this way, designing stoichiometries and geometries of synthetic protein scaffolds may result in more effective compartmentalization mechanisms.

Although the exact mechanism by which protein scaffolds increase yields remains unknown, it has been hypothesized that they may form higher order complexes due to enzyme oligomerization [11] (Figure 2c), similar to naturally occurring metabolite microdomains, such as Ca^{2+} or cAMP microdomains [43,44]. If true, intermediates produced within the complex would be quickly consumed before they could escape into the cellular milieu, thereby reducing their toxicity. This would allow protein scaffolds to more closely approximate bacterial microcompartments.

Compartmentalization using protein encapsulation

Bacterial microcompartments are relatively closed to the surrounding environment with protein-based pores that likely regulate transport of substrate into and out of the proteinaceous shell (Figure 2a) [45]. This strategy effectively isolates reaction intermediates from the rest of the cellular milieu and could create high local concentrations of substrates, thereby increasing product yields.

Microcompartments have been heterologously expressed and could be used to encapsulate foreign pathways. As such, these microcompartments have the potential to increase pathway flux, making them useful tools for metabolic engineering. For example, carboxysomes from cyanobacteria (Figure 2a) were heterologously expressed in *Escherichia coli* and the encapsulated RuBisCO was functional in *in vitro* assays [46]. The Eut and Pdu microcompartments from *Salmonella* have also been expressed in *E. coli* [47,48].

Many challenges remain before heterologously expressed microcompartments can be utilized in metabolic engineering. Targeting of foreign enzymes to microcompartments is an area of ongoing research and localization sequences have been found for the Pdu and Eut microcompartments [47,49]. However, although associations between carboxysome shell proteins and other protein components have been shown [50], targeting novel enzymes to the carboxysome remains not well understood [46]. Another challenge is elucidating the mechanism of substrate transport across the microcompartment shell. In carboxysomes, diffusion of small molecules across the shell is likely, but pores that suggest active regulation of substrate crossing have also been revealed in crystal structures [45]. Heterologously expressed microcompartments, isolated from their native cellular environment, may help us better understand these mechanisms.

Spatial organization of microcompartments can be regulated within a cell, providing the cell with another mechanism to optimize metabolic processes. For instance, carboxysomes are aligned along the major axis of the cell [51] (Figure 2a, right) and the bacterial cytoskeletal component ParA is required for proper alignment to occur. The exact interaction between the cytoskeleton and the bacterial microcompartment remains unknown and is an active area of research. This knowledge would enable the design and construction of a novel synthetic cytoskeletal scaffolding device. By inducing polymerization and depolymerization of cytoskeletal proteins in response to substrate availability or other environmental signals, we may be able to control the spatial organization and number of compartments tethered to this synthetic cytoskeleton. This may allow dynamic regulation of metabolic reactions in synthetic microcompartments.

Another bacterial microcompartment-like encapsulation device was recently engineered. A lumazine synthase capsid from *Aquifex aeolicus* was heterologously expressed in *E. coli* [52]. Lumazine synthase, an enzyme catalyzing riboflavin synthesis, forms icosahedral assemblies that do not naturally encapsulate other enzymes. However, electrostatic interactions were engineered, enabling it to encapsulate a toxic enzyme, HIV protease. Directed evolution was used to select for a capsid with higher loading capacity. In addition, the assembly state of lumazine synthase was controlled through engineered point mutations, which changed the quaternary structure of the capsid, thereby changing the size and structure of the capsid, without disrupting the secondary or tertiary structure [53]. The ability to insulate toxicity from the cytoplasm using a designable compartmentalization system is important for metabolic engineering. It allows the expression of heterologous pathways that may otherwise be toxic, or even lethal, to the cell.

Overall, protein assemblies have been used effectively to organize metabolic reactions and increase product yields. Thus far, however, such increases have been low. Therefore, the present challenge is to further increase yields to levels high enough for industrial applications. To do so, the mechanism of scaffold action must be further elucidated. In addition, scaffolds have been applied to only a few select pathways as proof of principle. Generality and scalability of scaffolds will require a better understanding of localization to synthetic scaffolds.

Compartmentalization using nucleic acids

DNA and RNA nanotechnology is a field of research that has many promising applications in medicine and industry [54–56]. Short strands of DNA or RNA can fold into various structures or assemble into dimer or multimer building blocks (Figure 3c). These building blocks can then polymerize into various 3D structures *in vitro*, including simple structures such as sheets, as well as more complex

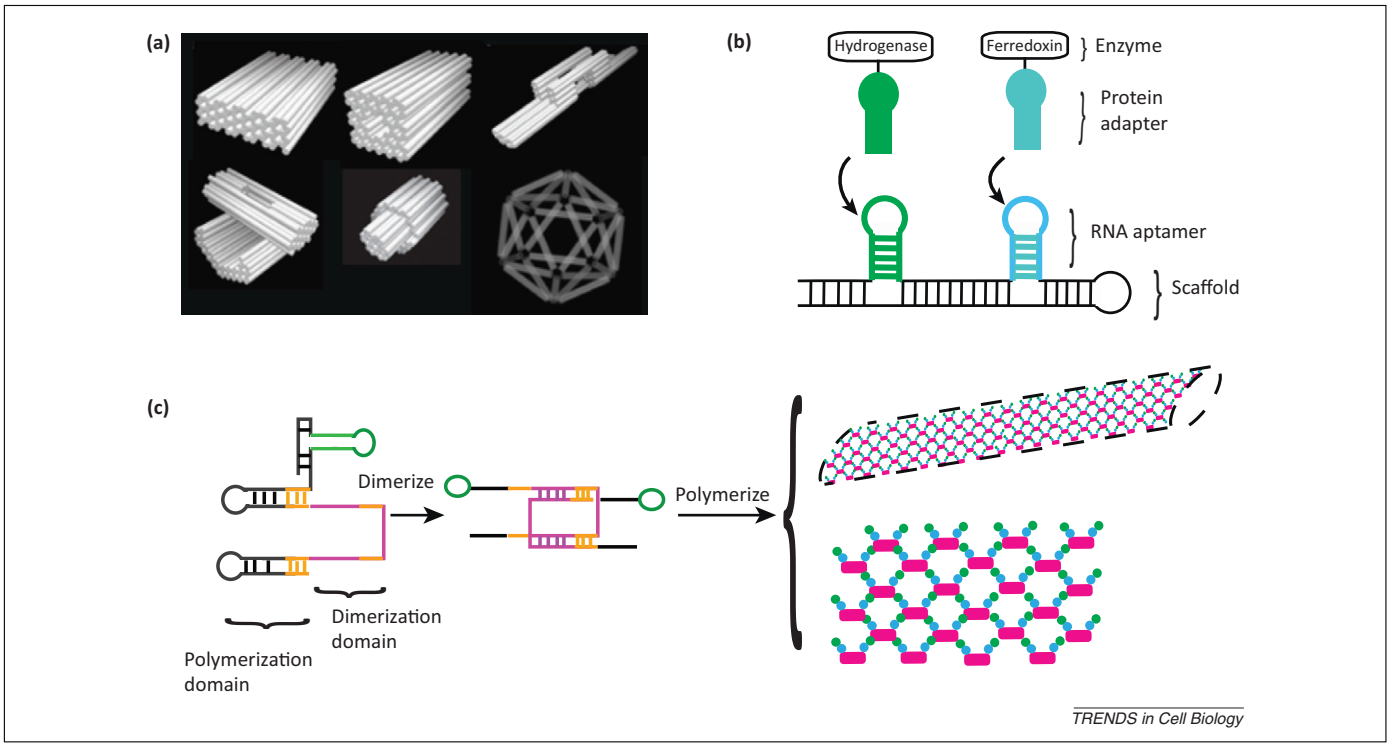

Figure 3. Nucleic acid scaffolds: mimicking the diverse geometries found in nature. (**a**) DNA and RNA nanotechnology can produce many different structures *in vitro*, including tubes and sheets [86]. Although nucleic acids are not frequently used in nature for scaffolding, the ease with which synthetic biologists can design and build these structures make them a versatile tool for building synthetic scaffolds that can mimic various natural compartmentalization strategies of different geometries. (Adapted, with permission, from [86].) (**b**) Synthetic RNA scaffolds were designed *in silico* and expressed *in vivo* [75]. Binding between RNA aptamers and protein adapters facilitated recruitment of enzymes to the scaffold. Hydrogenase and ferredoxin, components of a hydrogen production pathway, were scaffolded and pathway output was increased. (**c**) Single strands of RNA with aptamers dimerize and these building blocks polymerize into 2D sheets and nanotubes. Polymerization domains are protected with hairpins before dimerization to prevent self-binding and assure order of assembly. The versatile architectures of RNA nanostructures *in vivo* allow the mimicking of various natural compartmentalization systems.

structures such as tubes and capsules [57,58] (Figure 3a). In this way, DNA and RNA nanotechnology can be used to build complexes that structurally mimic natural organizational systems, such as enzyme channels and bacterial microcompartments.

Nucleic acid structures can also functionally mimic natural organizational systems. That is, proteins can be targeted directly to the scaffolds. Several multienzyme systems have been successfully organized using nucleic acids *in vitro*. NADH-flavin mononucleotide (FMN) oxidoreductase and luciferase, enzymes that catalyze two consecutive reaction steps, were assembled onto DNA scaffolds using streptavidin–biotin linkages and coupled enzymatic activity was increased relative to unscaffolded enzymes [59]. Glucose oxidase (GOX) and horseradish peroxidase (HRP) were immobilized through covalent conjugation to DNA oligonucleotides and an increase in reactivity was seen in both scaffolded systems compared with unscaffolded enzymes alone [60]. Other enzyme cascades have been organized using large supramolecular DNA scaffolds [61] and DNA origami [62] and reactivity was found to depend on relative enzyme positioning on the scaffold.

Although traditional *in vitro* assembly of DNA or RNA nanostructures relies on physiologically unsustainable temperatures and slow cool down to denature and anneal designed strands correctly [63], isothermal strategies for both DNA and RNA were recently developed [64–66]. This paved the way for *in vivo* applications of RNA and DNA scaffolds.

A DNA scaffold, in the form of a plasmid, was constructed. It recruited enzymes via zinc finger domains that bound to specific motifs on the scaffold [67]. Several enzymes were tested using this system, including pathways for the production of resveratrol, 1,2-propanediol, and mevalonate. Yields were found to increase as a function of scaffold architecture, similar to protein scaffolds.

Scaffolds with more complex geometries were constructed using designed non-coding (nc)RNA (Figure 3b,c). As a building material, RNA has several advantages. Because the base-pairing interactions in RNA (and DNA) are well studied [68,69], secondary and tertiary structures of nucleic acids can be predicted *in silico* [70–72] with nanometer precision [57] and are relatively easy to design [55]. RNA can also be expressed in large amounts by engineered cells and can stably exist outside the nucleus in eukaryotic cells, making it portable to a wide range of hosts. Additionally, RNA can recruit proteins fused to adapters via RNA aptamers, which can be evolved to bind with high specificity to various ligands [73,74].

A single molecule of RNA (approximately 100 bases) can fold into a linear discrete scaffold that mimics multienzyme complexes in nature [75] (Figure 3b). The RNA molecule comprises multiple different aptamer motifs, which can recruit proteins, as well as complementation regions, which form the scaffold (Figure 3b). This scaffold is relatively easy to build and characterize and is suitable for expression of specific stoichiometries of enzymes in a chain [75]. Scale up, which may allow production at industrially

feasible titers, is also possible; numerous RNA aptamer domains can be connected in a single molecule of RNA. In this way, almost 100 proteins were recruited in a chain for visualization experiments [76], although this has not yet been adapted for metabolic engineering purposes.

An RNA nanotube was built using short single-stranded building blocks that polymerized *in vivo* [75] (Figure 3c). Here, the directionality of enzyme loading was not specified; that is, enzymes were tethered to both the interior and the exterior of the nanotube. However, loading enzymes selectively to the interior has been shown *in vitro* [77]. If such selective loading could be accomplished *in vivo*, a modified version of this 1D scaffold could act like a pipe, preventing intermediates from escaping and increasing product yields. Another scaffold architecture, a 2D sheet, was constructed using a similar strategy and product yield was highest when using this scaffold (Figure 3c). Although there is no directly analogous system in nature, this 2D-scaffold is likely to function by increasing the number of neighbors any enzyme has in close proximity, allowing exploration of the quantitative effect of colocalizing additional proteins in two dimensions; for example, by recruiting them to a membrane.

As a demonstration of feasibility, [FeFe]-hydrogenase and ferredoxin, enzymes involved in the biological production of hydrogen [78], were recruited to RNA scaffolds. This pathway is a useful test bed because it suffers from competing reactions and requires the enzymes to be in close proximity [42]. Hydrogen output was found to increase as a function of scaffold architecture.

DNA and RNA scaffolds allow metabolic engineers to mimic many different strategies implemented by nature for substrate channeling and reducing competition to increase pathway flux. *In vitro* work provides a wide array of tested architectures and functionalities that mimic various natural compartmentalization systems in different ways. This ability to design precise scaffold geometry is a major advantage of nucleic acid scaffolds over protein and lipid compartmentalization systems, where structure prediction and design remain difficult. However, the *in vitro* to *in vivo* transition is a major challenge for nucleic acid scaffolds and is not always possible due to many constraints, such as isothermal assembly. Specific control over scaffold architectures and the ability to design customized shapes with RNA and DNA isothermally *in vivo* are crucial next steps in utilizing this technology to increase yields. If successful, RNA and DNA may even be used to build scaffolds in architectures not found in nature. Although the effects of such structures on the cell's metabolic load and the crosstalk with native systems may be problematic, it raises interesting possibilities for metabolic engineers to exceed nature's capabilities to produce useful compounds.

RNA scaffolds currently have an added advantage over DNA and other types of scaffold in that the amount of scaffold expression can be controlled. However, the effect of expressing high amounts of nucleic acid on cell growth and viability is not well understood and titration of scaffold expression has yet to be performed.

The generality of nucleic acid scaffolds remains to be demonstrated, because they have been used in only a few select pathways. Further application of nucleic acid scaffolds will require generation of additional orthogonal RNA aptamers or zinc finger domains. The application of RNA scaffolds, with its diverse architectures, to various pathways will not only allow further improvement of the technology, but will also allow testing of the mechanism of scaffold action. For example, the effect of scaffolding is hypothesized to be greater for non-diffusible intermediates compared with diffusible intermediates if local high concentration of intermediates is an important contributing factor. Overall, RNA scaffolds are capable of becoming versatile, designable scaffolds that can mimic natural systems of differing geometries, compartmentalize various metabolic pathways, and increase yields, possibly beyond nature's capabilities.

Compartmentalization using lipids

Lipids, often in the form of membranes, are widely used to encapsulate reactions in nature. Lipid vesicles and oil emulsions have been used to perform a wide variety of reactions *in vitro*, such as gene expression [79], sequencing, and evolution of new enzymes [80]. Even self-replicating lipid systems have been created: protocells that are capable of catalyzing RNA reactions and processing 'food' micelles [81]. Although these systems are effective at encapsulation of reactions, targeting specific enzymes to synthetic lipid vesicles remains difficult because localization signals or other specific targeting mechanisms to lipid vesicles are unknown.

Another compartmentalization strategy utilizes naturally existing membrane-bound organelles. Unlike lipid vesicles, many membrane-bound organelles have well studied localization mechanisms [82]. One study targeted methyl halide transferase to yeast vacuoles to increase methyl iodide production [83]. The increases seen were likely to have been due to added access to a cofactor and sequestration of halogenated products. Also, targeting of terpenoid production pathway components to the mitochondria significantly increased yields [84]. The advantage of this system is that it allows engineering of eukaryotic cells such as commercially important yeast.

Lipid compartmentalization systems remain a relatively unexplored area of synthetic biology, because they have many weaknesses compared with nucleic acid or protein scaffolds. For example, controlling the architecture of compartments and stoichiometries of encapsulated enzymes is challenging. Other major challenges for *in vivo* use of lipid compartmentalization devices include secretion of final products and, in the case of native organelle utilization, potential interactions with the host metabolism. A better understanding of these factors and studies in natural lipid compartmentalization systems may yield results that can inform the development of synthetic lipid compartments.

Using synthetic scaffolds to understand the limitations of natural systems

Studying engineered scaffolds may give us new insight into the function and design principles of natural compartmentalization strategies. For example, scaffold architecture and enzyme stoichiometries were found to be important

to pathway output [40,75]. Through scaffolding pathways with known stoichiometries and differing architectures, it may be possible to probe the limitations of native systems and even improve on them.

Knocking out a native compartment, replacing it with a synthetic one, and studying the resulting effects on the cell may allow us to decouple biological functions. This will help elucidate the functions and limitations of the native system. Conversely, studying heterologous expression of natural compartments and targeting will allow us to determine the components that are necessary and sufficient for proper function. Mechanisms of localization, assembly, and substrate gating may be elucidated from such studies.

In addition, the well-studied examples of natural compartmentalization systems have comprised primarily proteins and lipid membranes. Synthetic systems have shown, for example, that nucleic acids can function as scaffolds. However, RNA and DNA are not commonly used as materials for scaffolding in nature. Yeast telomerase is one of a few known examples of RNA serving as a flexible scaffold for protein subunits [85]. Given findings of the capabilities of a synthetic RNA scaffold, it may be that an as yet to be discovered function of ncRNA, which constitutes 80% of total RNA, is scaffolding. Synthetic scaffolds, in this case, may help us elucidate the organizational function of the material from which the scaffold was made.

Concluding remarks

Cells have evolved strategies for regulating and maintaining proper metabolic flux. Synthetic biologists have taken inspiration from nature and developed synthetic systems that solve some of the same problems. From direct mimicry using proteins to more novel solutions using nucleic acids, synthetic biologists have devised promising strategies for increasing metabolic production of industrially useful chemicals. However, some major challenges remain (Box 1). For example, scaffolds must be applicable to various pathways outside the test pathways chosen in the proof-of-principle studies conducted so far. Further increases in yields must be obtained before scale up to industrially relevant quantities can be achieved. Utilizing multiple types of scaffold in the same system, if feasible, may help accomplish this, because improvements may be made orthogonally by each scaffold. In addition, the effect of such high levels of production on cell growth must be minimized. To overcome these challenges, a better understanding of scaffold mechanism of action and enzyme localization must be achieved. If these efforts prove successful, scaffolds can be widely applied as an orthogonal novel method of increasing yields in metabolic engineering efforts. In addition, implementing and studying synthetic systems will help us understand the native biological organization of the cell.

Acknowledgments

The authors thank D. Ducat, C.J. Delebecque, J.E. Dueber, G. Sachdeva, D.F. Savage, J. Torella, and G.C. Wu for discussions and helpful suggestions. This work is supported by the National Institutes of Health (Grant #GM080177) and a Graduate Research Fellowship from the National Science Foundation to A.H.C. and by the Wyss Institute for Biologically Inspired Engineering and Department of Defense to P.A.S.

Box 1. Outstanding questions

- What is the mechanism by which scaffolds operate to increase yield? Do they form higher order structures?
- How can we predictably engineer scaffold architecture and control assembly?
- For what pathways are different scaffolds most useful? Is there a pathway size limitation? Does the diffusability of intermediate substrates influence the effectiveness of scaffold action?
- How do substrates enter and exit synthetic compartments? How are enzymes localized?
- How can we further improve yields using synthetic scaffold technologies? How do we scale up the existing technologies to industrial scale production?
- Are there other undiscovered natural scaffolding systems that utilize proteins, nucleic acids, and lipids? How does biological organization function natively?

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