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Review Article

Structural organization of biocatalytic systems: the next dimension of synthetic metabolism

Tobias J. Erb[1,2]

[1]Department of Biochemistry and Synthetic Metabolism, Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch-Str. 10, D-35043 Marburg, Germany; [2]LOEWE Center for Synthetic Microbiology (Synmikro), Marburg, Germany

Correspondence: Tobias J. Erb (toerb@mpi-marburg.mpg.de)

In natural metabolic networks, more than 2000 different biochemical reactions are operated and spatially and temporally co-ordinated in a reaction volume of <1 μm^3. A similar level of control and precision has not been achieved in chemical synthesis, so far. Recently, synthetic biology succeeded in reconstructing complex synthetic *in vitro* metabolic networks (SIVMNs) from individual proteins in a defined fashion bottom-up. In this review, we will highlight some examples of SIVMNs and discuss how the further advancement of SIVMNs will require the structural organization of these networks and their reactions to (i) minimize deleterious side reactions, (ii) efficiently energize these networks from renewable energies, and (iii) achieve high productivity. The structural organization of synthetic metabolic networks will be a key step to create novel catalytic systems of the future and advance ongoing efforts of creating cell-like systems and artificial cells.

The power of natural metabolic networks

Nature is an incredible chemist. Photosynthesis uses the energy of light to convert inorganic carbon into the central building blocks of life. These are further diversified and give rise to more than 200 000 different chemical compounds that have been purified from various organisms to date [1]. All this is achieved in an incredibly dynamic, self-regulated, self-optimized and synchronized fashion. For instance, there are more than 2000 different reactions that can take place at the same time in one *Escherichia coli* cell [2,3] with a volume of ~1 μm^3, corresponding to 10^{-15} l [4].

The synthetic capabilities of Nature are unmatched by humankind. Even though chemists have achieved exquisite control over individual chemical reactions [5], synthetic organic chemistry is to a large extent still pursued in individual, sequential steps, which makes the build-up of a chemical structure a tedious and multi-step process (Figure 1). This modus operandi requires several follow-up steps between individual reactions, such as separation, purification, and sometimes even crystallization. This, in turn, needs additional resources, such as solvents, and energy and most importantly also time, which makes stepwise chemical synthesis very often a cost-intense, wasteful undertaking [6,7]. As an example, the total synthesis of vitamin B12 (cobalamin) requires more than 70 steps at a total yield that 'never should be asked for' (A. Eschenmoser) [8], probably below 0.01‰ [9]. In contrast the same compound can be produced by the bacterium *Pseudomonas denitrificans* at 200 mg l^{-1} over a 180 h fermentation [10,11].

Limitations of natural metabolic networks

So, can we not simply harness the power of natural metabolism to create a more efficient, sustainable and integrated chemistry? For more than three decades, biotechnology and in particular metabolic engineering has striven to program cells into chemical factories that produce a wanted compound from a given starting material, very often a renewable carbon source. Impressive progress was made; there are yeast strains that are able to produce complex chemical structures at 25 g l^{-1} [12] and there

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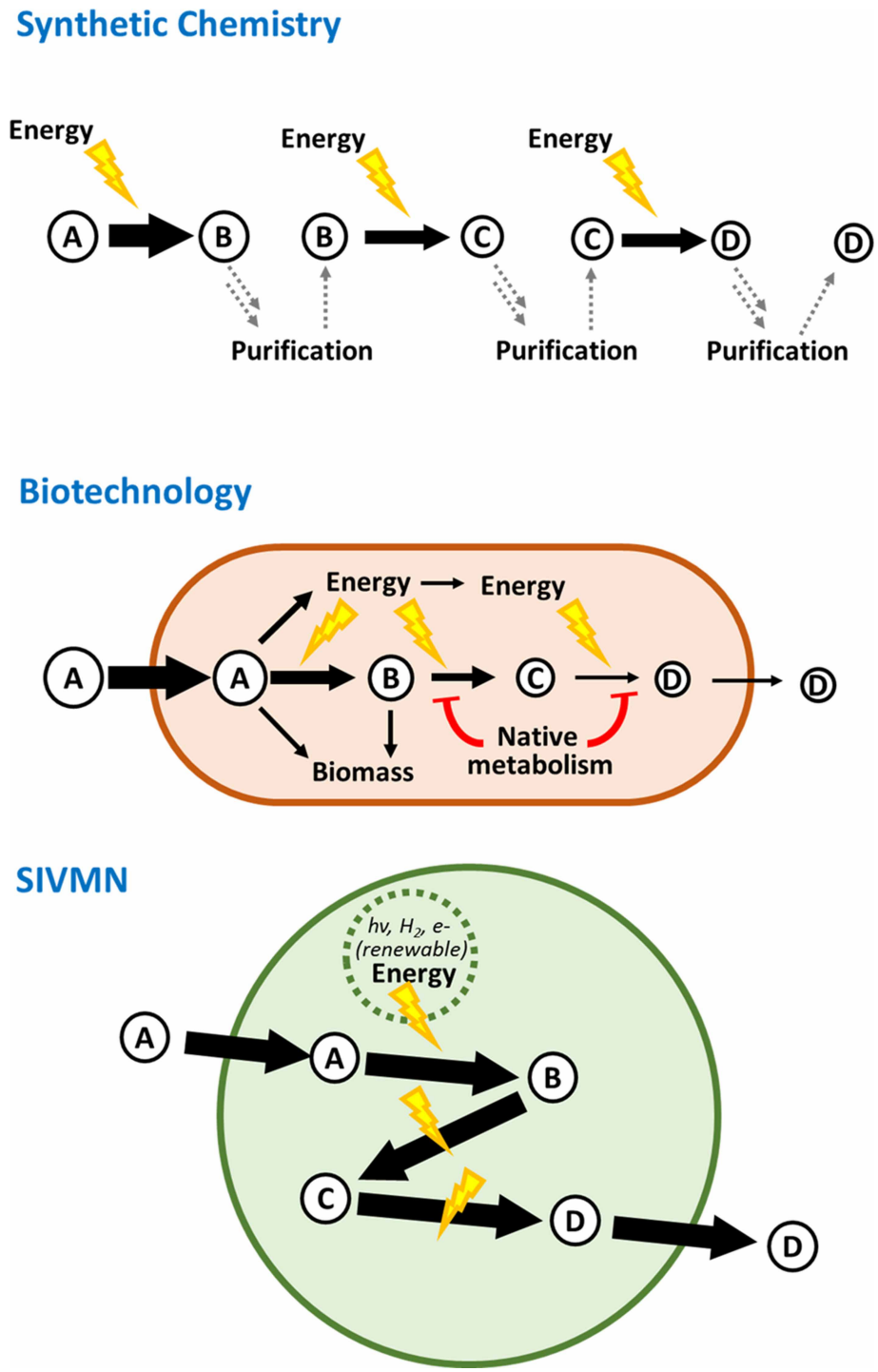

Figure 1. Comparison of synthetic chemistry, biotechnology and SIVMNs. Part 1 of 2

In synthetic chemistry, the synthesis of compound D from substrate A is carried out in separate steps, which requires the separation and purification of intermediates between the different steps. This is a time- and resource-intense process. The overall yield is lowered due to losses during synthesis and purification. Biotechnology is an integrated production process, however, some of the substrate is used by the cell to provide the energy for the synthesis and some of the substrate is drained into biomass to allow the cells to grow and divide, which leads to an overall decrease in yield. Interactions of the metabolites with the native metabolic network of the cell make engineering of the biotechnological process a complicated task. SIVMNs ideally combine the advantages of both systems. SIVMNs are defined multi-reaction networks that are assembled in a

Figure 1. Comparison of synthetic chemistry, biotechnology and SIVMNs. Part 2 of 2

bottom-up fashion, which do not drain metabolites into other processes. External energy from renewable sources or even electricity might be used to power the catalytic network.

is an ever-growing number of complex molecules that can be derived through metabolically engineered cells [13,14]. Vitamin B12, for instance, can now be produced at 300 $\mu g\ g^{-1}$ cell dry weight within 24 h in a recombinant *E. coli* strain after introduction of 28 additional genes [15].

However, the biosynthetic reaction space of cells is limited by the number of naturally existing enzymes, as well as the narrow reaction conditions that can be operated inside of the cell (pH, salts, ionic strengths) [16]. Also, the engineering of novel biosynthetic processes very often requires complex manipulations of the regulatory and metabolic networks of a cell. As a simple exercise, imagine the introduction of five new non-native reactions and metabolites into an *E. coli* strain. There are potentially 5×2000 interactions with the native metabolic network of the cell possible (e.g. in form cross-reactivities, degradation, side reactions, inhibitory and allosteric interactions). This number even increases, when considering the potential interactions of these new metabolites with the regulatory network of the cell (i.e. several hundred different transcription factors, regulators, etc.). Lastly, the fraction of substrate carbon that can be channeled into a desired biosynthetic product is very often constrained, because of the many parallel operating reaction networks inside of a living cell, which overall reduces the possible yield and atom efficiency of a given process (Figure 1). Overall, these facts limit the possibilities to replace synthetic routes by biotechnological processes.

The vision of synthetic *in vitro* metabolic networks

From one — arguably provocative — point of view, the use of biotechnology can be understood as an effort to overcome our current technical incapability to achieve a similar level of precision, coordination, as well as temporal and spatial control of catalysis in technical systems. Ideally, instead of the laborious re-wiring of metabolism and dealing with the disadvantages of cellular systems, it would be more desirable if we were able to build catalytic networks that allow the multi-step synthesis of the desired compound at high yields and atom efficiency (Figure 1). An important step towards achieving this goal is the successful assembly of (bio)catalytic reaction networks in a bottom-up fashion *in vitro* with purified proteins [17].

Several recent examples demonstrated that it is possible to construct and operate complex multi-enzyme reaction cascades outside of a cellular context. These synthetic *in vitro* metabolic networks (SIVMNs) are drafted based on physical and chemical principles, assembled from individual enzymes and further optimized in several rounds [17,18]. Examples are the formation of polyhydroxybutyrate from glucose through a 18-enzyme network [19], a synthetic pathway that converts glucose in more than 20 steps into geranyl pyrophosphate, which in turn is used to prenylate different compounds [20], as well as a synthetic pathway ('CETCH cycle') for the continuous capture and conversion of CO_2 that was constructed from 17 different enzymes, including three re-engineered enzymes [18].

Although these systems are fascinating examples of what can be achieved today, they are only the first steps on the way of building more efficient catalytic systems in the future. Automation will allow fast prototyping to determine optimal concentrations of individual components of a given SIVMN. Enzyme engineering will extend the range pf possible transformations that can be used in SIVMNs. Lastly, the prospects to operate SIVMNs at non-physiological reaction conditions, include new-to-nature materials and even synthetic-chemical catalysts will help to further expand the solution space of SIVMNs beyond the realm of natural metabolism. In other words, the real potential of SIVMNs lies not in the efforts to establish naturally existing metabolic routes outside of a living cell, but in the possibility to create completely new bio-inspired catalytic networks that outcompete naturally evolved metabolism in efficiency and productivity.

The challenges of building SIVMNs

What are the challenges in building and operating SIVMNs? One particular challenge is that many enzymes show inherent promiscuity [21], which becomes a problem for the assembly of complex networks and scales with the number of reaction steps as well as the diversity of the biological origin of the individual components used. Deleterious side reactions and unfavorable interactions between different metabolites and enzymes of a

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network can cause the accumulation of unwanted metabolites and ultimately the breakdown of SIVMNs [22]. To prevent this from happening, the construction of synthetic *in vitro* metabolic networks very often requires the engineering and/or replacement of problematic enzymes, or the introduction of metabolite proof-reading strategies that recycle dead-end metabolites [18,23].

An alternative mitigation strategy is to spatially separate problematic reactions, as happens in natural systems, which show a high degree of structural organization. A yeast cell, for instance, features several organelles that serve as separate reaction compartments in which complicated and incompatible chemistries can be operated in parallel (Figure 2). The discovery of phase separation as an active principle to create distinct non-membrane bound compartments is another example for the spatial organization of biological components inside living cells. Within microbial cells, organelle-like structures can be found, too. Examples are bacterial microcompartments, which serve as carbon concentrating and/or detoxification chambers or encapsulins (Figure 2). Lastly, spatial confinement can also be achieved on the single enzyme level (Figure 2). The recent report of a dynamically regulated multi-catalytic reaction chamber that sequesters a toxic metabolite illustrates the elaborate design of such naturally evolved protein nanoreactors [24,25].

Altogether, these examples emphasize the importance of spatially structuring individual reaction sequences in natural metabolic networks and show the great potential of this organizational principle to achieve improved and robust operation of SIVMNs (Figure 2). While the community has started to re-purpose and re-design spatially organized enzymes [26], protein complexes and reactors, application of these principles remains still to a large extent underexplored in the design of SIVMNs. Most promising are current efforts to engineer naturally existing microcompartments for new cargo [27–29] or to build and load protein cages *de novo* [30,31]. Further progress in protein scaffold design will expand our possibilities of spatially structuring catalysis in SIVMNs the future [32–34].

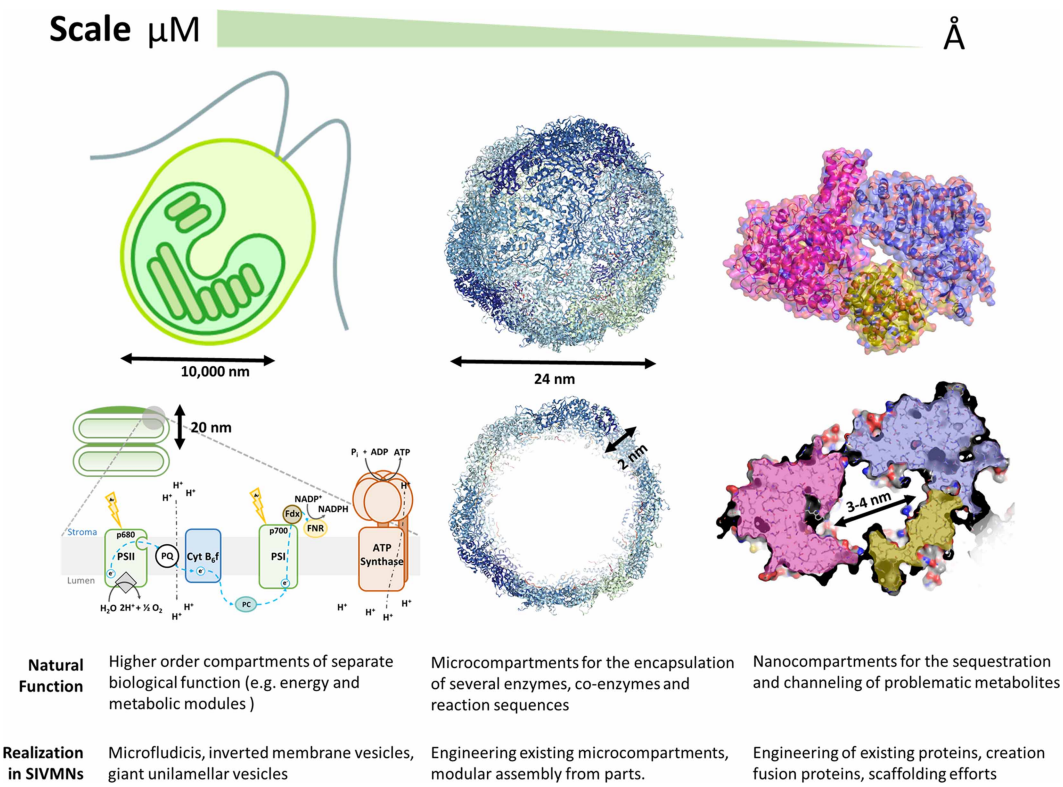

Figure 2. Spatial organization of metabolism in natural systems and potential realization in SIVMNs.
Natural organization spans several orders of magnitude. From organelles, such as the chloroplast and its thylakoids that convert light into chemical energy, bacterial microcompartments and encapsulins that encapsulate various proteins, metabolic enzymes and cofactors, to single protein nanoreactors, such as propionyl-CoA synthase, that sequester toxic metabolites. Data and pictures from [24,51,52]. Thylakoid scheme obtained with permission from Miller T. Building an artificial chloroplast drop-by-drop. Lecture presented at Engineering Life; 2019; Dresden.

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Energizing SIVMNs

Another challenge is how to power SIVMNs. In some cases, the supply of reducing power and chemical energy is deliberately designed into the network to create a stochiometrically balanced process [19]. An example is the production of bioplastics from glucose. However, in many cases, this is not possible, so that the required reducing power and chemical energy either needs to be balanced with purge valves [35] and molecular rheostats [36] or provided through dedicated external energy modules [18]. Well-known are polyphosphate- and creatine phosphate-based ATP regeneration systems [37–40] and regeneration systems for NADH and NADPH based on formate-, glucose- or alcohol-dehydrogenase. So far, all these regeneration systems require the supply in the form of chemical energy, which is very often provided from petrochemical or biological sources, representing an indirect way to energize SIVMNs.

In the long run, hydrogen-, light- or even electricity-based systems could be used to provide an alternative, renewable ways to energize *in vitro* metabolic networks directly. Again, the three-dimensional organization of these systems will become important for these efforts, especially if membrane-bound regeneration systems featuring photosynthetic or ATP-synthesizing complexes are to be used. Recent work has shown that naturally isolated and synthetically created vesicle systems are indeed able to provide energy and reducing equivalents [41–43]. The further development of efficient microfluidics-based tools for the creation and manipulation of droplets and vesicles [44], as well as the use of coacervates and polymers [45] will open the way to assemble higher organized systems in which SIVMNs are powered by compartmentalized energy modules, resembling the architecture of a natural cells with its organelles [46].

Structural confinement to achieve high yields

Lastly, the productivity of SIVMNs is still an issue that needs to be solved, for any long-term operation, upscale or ultimately commercial application of these systems. Strategies to enhance productivity will include increasing protein stability, introducing effective metabolite detoxification and proof-reading systems (see above), but also protection against oxidative stress and damage. The latter task can be achieved by introducing enzymes to the SIVMN that maintain a reducing reaction environment or lower the concentration of oxygen and reactive oxygen species. Structural elements can augment these efforts and provide alternative solutions, e.g. by creating protein- or membrane-bound reaction compartments for the SIVMN that would allow operating the network in a protected environment.

Note that encapsulation provides another advantage. Introducing a physical barrier that separates a reaction compartment from the environment automatically creates a source-sink situation. This will, in turn, allow the establishment of a vectorial gradient, if the barrier (capsule or membrane) is specifically permeable for a given metabolite. In other words, if the product of a given SIVMN can be specifically exported to the outside of the reaction compartment, this would allow longer operation of the SIVMN by keeping the system in an out-of-the-equilibrium state and removing any product feedback inhibition loops, plus, it would enable an easier separation of the product from the SIVMN. While the introduction of such selective membranes is a technical challenge that needs to be solved, it would allow the creation of a similar situation that is found in naturally existing cells, which features specific exporters and importers. Also, it should be mentioned that the possibility to use non-biological materials, such as polymers or coacervates [47] could open new options for building selectively permissive compartments.

Controlling SIVMNs

Power is nothing without control. While this review has explored the chances of structurally organizing SIVMNs, it should not go unmentioned that temporal organization and control of metabolic networks are as important, especially when we aim at creating robust catalytic reaction networks that are resilient against perturbations and stochastic fluctuations [48,49]. This challenge, however, will be discussed by another article in this mini-review series [50].

Conclusions

SIVMNs have the potential to provide several advantages compared with chemical and biotechnological synthesis processes. However, to overcome the current limitations of SIVMNs it will be essential to spatially organize SIVMNs, which will allow improvement of their performance by suppressing deleterious interactions between enzymes and metabolites, coupling them to renewable energy modules and increasing product yield. The

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implementation of the spatial design into SIVMNs will be a necessary prerequisite to leverage their full potential and move catalytic networks into the next dimension.

Summary

- Natural metabolic networks, provide synthetic routes to many different chemical compounds, but are limited in malleability and productivity.
- Synthetic chemistry is currently unable to build, operate and control complex catalytic networks.
- Synthetic *in vitro* metabolic networks (SIVMN) provide a new approach to build novel biosynthetic routes in a rational fashion, thus bridging the natural and the synthetic world.
- To increase the performance of SIVMN, structural organization of the enzymes and components will be required.
- The structural organization of SIVMN will be a key step to create catalytic systems of the future and advance ongoing efforts of creating cell-like systems and artificial cells.

Abbreviations

SIVMNs, synthetic *in vitro* metabolic networks

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Competing Interests

The Author declares that there are no competing interests associated with this manuscript.

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