Cascades in Compartments: En Route to Machine-Assisted Biotechnology

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Biological compartmentalization is a fundamental principle of life that allows cells to metabolize, propagate, or communicate with their environment. Much research is devoted to understanding this basic principle and to harness biomimetic compartments and catalytic cascades as tools for technological processes. This Review summarizes the current state-of-the-art of these developments, with a special emphasis on length scales, mass transport phenomena, and molecular scaffolding approaches, ranging from small cross-linkers over proteins and nucleic acids to colloids and patterned surfaces. We conclude that the future exploration and exploitation of these complex systems will largely benefit from technical solutions for the integrated, machine-assisted development and maintenance of a next generation of biotechnological processes. These goals should be achievable by implementing microfluidics, robotics, and added manufacturing techniques supplemented by theoretical simulations as well as computer-aided process modeling based on big data obtained from multiscale experimental analyses.

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1. Introduction

Living systems have evolved complex networks of biomolecules which specifically interact with each other to control crucial functions of the cell, such as their metabolism, their propagation, or communication with their environment. Biological compartmentalization has evolved as a fundamental principle to orchestrate the innumerable interwoven molecular pathways. Compartmentalization is the general approach to spatially separate two or more active components of a system to prevent malfunctions from spreading as well as unproductive cross-talk. In biological systems, this is achieved by physically constrained cascades of multiple catalytically active proteins, such as those involved in metabolic and signaling pathways. Such cascades appear to be essential for the regulation of enzymatic activity or the signal transduction of receptors.[1–3] Inspired by this natural design principle, researchers in the fields of synthetic chemistry and biotechnology have long begun to explore and exploit biomimetic compartmentalization and multicatalytic cascade reactions for improving production processes. Spurred on by the advances in the past three decades, cascade reactions and compartmentalization are nowadays implemented as design principles for catalytic systems at an ever-increasing pace.[4–9]

This Review aims to provide a condensed overview on the current state of this technology for mimicking natural and realizing artificial multienzyme cascades. As depicted schematically in Figure 1, enzyme cascading can be realized in numerous ways, which differ in the level of control over the stoichiometry and the spatial arrangement of the involved enzymes as well as on the size and dimensions of the system. The simplest cascades can be realized by a mixture of different enzymes that catalyze multiple substrate conversion steps in a confined compartment, such as a cell or a synthetic reaction vessel (Figure 1 A). To improve the effectiveness of such millimeter- up to meter-sized systems, distinctive steps can be spatially separated into subcompartments, which are coupled to each other to enable efficient mass transport (Figure 1 B). Whereas these systems can be composed of freely diffusible biocatalysts, cascades immobilized on the surface of micrometer-sized cells or particles may contain the individual enzymes in either a random arrangement (Figure 1 C) or a directional order that follows the reaction pathway (Figure 1 D). The same principle can be realized on a smaller length scale of 10–100 nm when the enzymes are directly linked to each other through noncovalent or covalent means (Figure 1 E,F). This is typically the case, for example, in polyketide synthases or fatty acid synthase. Multienzyme complexes can also be assembled on nanometer-sized scaffolds which bear specific binding sites for the attachment of the individual catalysts (Figure 1 G,H). As we exemplify below, all such systems are realized in nature, and current research is devoted to mimicking, exploring, and exploiting these models for the improvement of technical production processes.

Although we use the classification given in Figure 1 to structure this Review, it should be noted that the different systems share many of the same aspects. Overarching themes concern the spatial dimensions of compartmentalization, the connectivity between compartments by diffusion or active transport, the nature of the structural scaffolds—ranging from small cross-linkers over proteins and nucleic acids to colloids and patterned surfaces—the catalytic properties of the

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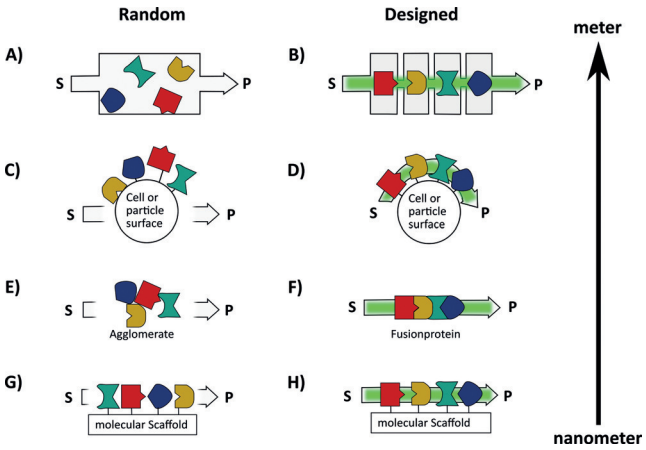

Figure 1. Overview of coupled reaction cascades. Sequential trans formation of a substrate (S) into a product (P) by, for example, four different enzymes can be carried out in compartmentalized reactors (A, B), on the surface of cells or particles (C, D), or by multienzymes, which are physically linked to each other directly (E, F) or by means of scaffold structures (G, H). The four systems involve different length scales and the individual catalytic entities can be combined in either a random or designed fashion with respect to the stoichiometry and spatial orientation. The directional cascading is thought to improve flux (green arrow) and thus increase reaction rates and minimize side reactions.

employed enzymes, and the role of theoretical studies for the further development of the field. To provide a condensed overview, the majority of aspects will be discussed through the use of previously published review articles on specific topics. Selected examples of original research papers will be presented to illustrate the most recent state of the art.

2. Cascades in Mesoscaled Compartments

Application oriented research and development of com partmentalized systems in chemistry was motivated by the fact that sequential reactions could be conducted in a single vessel. Although this "one pot" approach brings advantages in terms of reducing solvents and processing time, intensive studies in the past 25 years have shown that the combination of two or more reactions in a single compartment is often hampered by adverse effects as a result of incompatible solvent requirements, deficient chemo and stereoselectivity of the catalyst, as well as unwanted cross reactivities.[10] Therefore, the development of one pot cascade reactions is still a topical area in the, nowadays, overlapping disciplines of biocatalysis[11] and synthetic organic chemistry.[12–14] To cir cumvent the problems arising from incompatible reaction conditions and cross reactivity, compartmentalization of sequential transformations by separation into individual reactions within fluidically coupled vessel systems has emerged (depicted in Figure 1B). This enables improved mass transport between the mesoscopic compartments along with opportunities to introduce additional products into reaction compartments or separate specific (side) products

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between two compartments. Furthermore, the general setup and particular flow conditions of the process can be optimized by theoretical simulations when specific reaction rates and diffusion coefficients are known. This approach, which is now established as "flow chemistry" in organic synthesis, is characterized by a high degree of machine assisted automa tion.[15,16] The aim is the fully automated synthesis of complex molecules, as illustrated by impressive recent examples. For example, many different types of small molecules have been produced by the "common building block" approach[17] and bioactive pharmaceutical compounds have been manufac tured by continuous flow technologies.[18,19]

The implementation of flow chemistry systems into biocatalytic transformation processes is far less developed. This is primarily due to the fact that flow through processes are usually conducted in the heterogeneous catalysis regime and the typical biocatalyst, namely, an enzyme, imposes much higher requirements on the surface immobilization procedure than conventional organo(metallic) catalysts.[20] Nevertheless, developments in this direction are underway. For example, Liese and co workers have emphasized that established biotransformations with enzymes and cells, and even their combination with chemocatalysis, will largely benefit from continuous flow systems to overcome fundamental compat ibility issues of heterogeneous catalysis.[21] The majority of the continuous flow systems use macroscopic systems, which handle reaction volumes in the milliliter to liter regime.[14] As a consequence of their great versatility, such systems are currently explored at the fundamental level.[14,21] This is illustrated, for example, by the combination of asymmetric organo and biocatalytic reactions in organic media to produce chiral 1,3 diols[22] as well as by the recent imple mentation of a computational simulation and prediction of a linear three step enzymatic cascade in a fed batch reactor to yield insights into the kinetics of the synthesis of ε caprolactone.[23]

2.1. Cell-Based and Cell-Free Systems

It is important to realize that two different approaches are used to harness the unrivaled chemo and stereoselectiv ity of biocatalysts for cascade transforma tions. They either employ cell free sys tems or whole microbial cells. In the latter, the natural enzymatic machinery of living cells is reconfigured by genetic incorporation of designed enzyme var iants, often selected by directed evolution strategies to facilitate an unnatural trans formation.[24] For example, Reetz and co workers recently exploited directed evo lution to create whole cells of *E. coli* for regio and stereoselective cascade sequences.[25] The so called "metabolic engineering" approach often goes along with concomitant alterations of one or more metabolic pathways by adjusting the expression rate and turnover of related enzymes to avoid bottlenecks in substrate availability or to realize effective secretion of the product and minimal disruption by unwanted side products. Metabolic engineering is, nowadays, often accompanied by quantitative flux analyses to reveal how the organic building blocks of the (ideally cheap and widely available) substrates are converted into the spectrum of biogenic products produced by the microbial host. The implementation of such flux analyses in whole cell biocatal ysis has opened the door to the design of effective producer strains for a wide range of chemicals,[26,27] including fatty acids and fatty alcohols,[28] amino acids and their derivatives,[29,30] as well as succinic acid[31] (Figure 2).

Whole cell biocatalysis reaches its limitation when it comes to issues such as product inhibition, toxicity of heterologous products, limited solubility of substrates, inter mediates, or products and their transport across the cell membrane, as well as unwanted side reactions by interference with the metabolism of the host and/or during product workup. Therefore, this approach is complemented by the methods of cell free biotechnology. In these artificial multi component systems, all the reaction conditions, such as concentrations, pH value, and temperature, can be optimized independently of a surrounding cell. Furthermore, non biological cosolvents as well as non natural cosubstrates and cofactors can be easily incorporated to expand the scope of

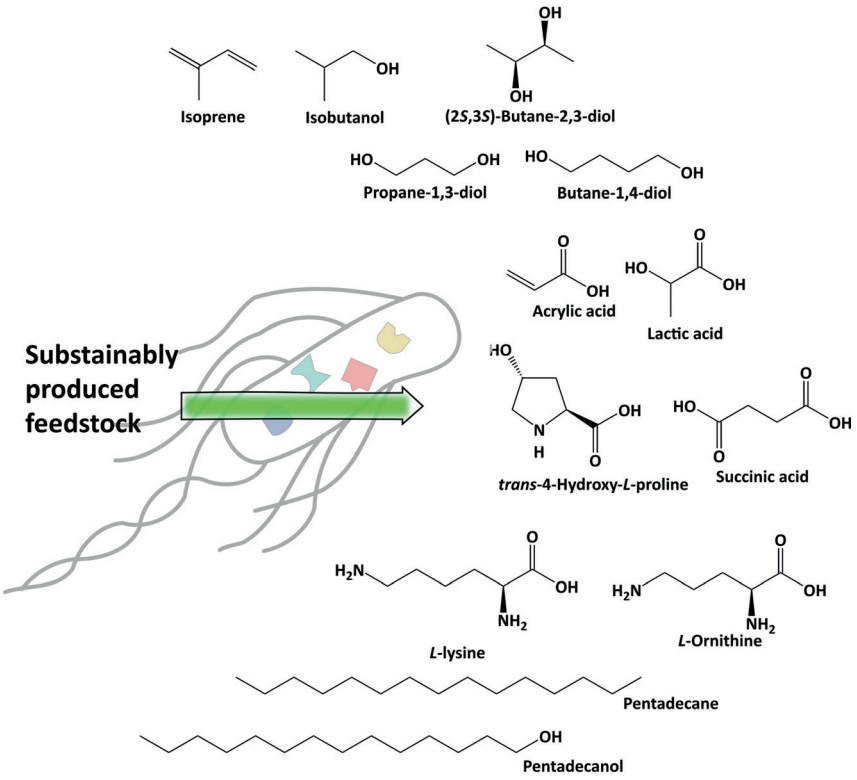

Figure 2. Metabolic engineering of whole cell biocatalysts for the production of biotechnolog ically relevant chemicals. The engineered cells ideally live on sustainably produced feedstocks, such as cellulose.

the production process.[10,32–34] However, when these "classical" enzyme cascades are used in homogeneous solution in simple one pot reactions, problems may occur as a result of insufficient compatibility with, for example, the pH value, temperature, demands for cofactors, and unwanted cross reactivities. The development of compartmentalized meso and macroscopic flow reactors aims to circumvent these obstacles.[14,21] However, the large vessel size and volumes often lead to inefficient mixing, thus making it difficult to establish homogeneous environmental conditions and to optimize mass transport between different catalytic species.

2.2. Microfluidic Enzyme Reactors

The use of microfluidics to perform multistep cascade reactions (compare Figure 1 A and B) would bring significant advantages. The high level of control over temperature profiles and mixing efficacy[35] would simplify the adjustment of process parameters to optimize product turnover. Furthermore, reaction kinetics can benefit from the micrometer dimensions, because they enable fast diffusion between heterogeneous interfaces, which enhances the collision rates of the reactants.[36] Consequently, microstructured flow reactors for enzymes are being developed,[37] but their applications are currently focused on biosensing[38] or proof of concept demonstrations regarding, in particular, protein immobilization techniques.[39] For example, surface photoactivation and enzyme immobilization inside porous polymer monoliths has been used to create compartmentalized microfluidic reactors containing different enzymes.[40,41] These studies, however, are limited to stable enzymes that withstand the photolithographic procedures and have, so far, been primarily carried out with model enzymes, such as glucose oxidase (GOx), horseradish peroxidase (HRP), and alkaline phosphatase (AP) to investigate the kinetic parameters of coupled reactions.[41]

As an alternative to photolithographic immobilization, microbeads carrying the enzyme of interest can be placed into distinctive compartments of a flow through microreactor. For example, highly delicate human cytochrome P450 enzymes have been immobilized on magnetic microbeads to create packed bed compartments, which were combined in a modular microfluidic system to emulate human phase I/phase II metabolism.[42] Similar packed bed microreactors have been used for the synthesis of polyketides[43] and the multistep synthesis of 2 aminophenoxazin 3 one with silica immobilized enzymes.[44] As an alternative to beads, homogeneous coating of capillary surfaces through the interaction of Ni^{2+} NTA (NTA = nitrilotris(acetic acid)) with hexahistidine has been used to establish a transketolase transaminase pathway in a microfluidic reactor for the synthesis of chiral amino alcohols.[45] Surface coating with the adapter protein (strept)avidin was used to selectively load compartments with biotinylated enzymes to assemble enzymatic cascades, for example, for GOx/HRP based detection (Figure 3 A,B).[46] An innovative contribution to this strategy of compartmentalization takes advantage of self assembling biocatalysts. To this end, synthetic DNA molecules can be immobilized on the surface of specific compartments and then used as address tags to specifically bind enzymes bearing the complementary oligonucleotide (Figure 3 C).[47] This approach is highly versatile because the physicochemical stability of DNA allows various methods to be used to microstructure and pattern the fluidic systems.[48–50] However, further advances will critically depend on improved access to semisynthetic DNA conjugates, even from sensitive enzymes.[51]

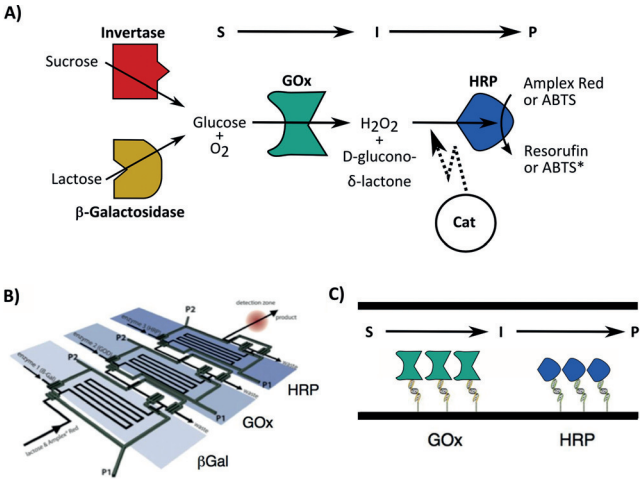

Figure 3. Compartmentalized microfluidic reactors. A) Schematic overview of the most commonly used model cascade comprised of glucose oxidase (GOx) and horseradish peroxidase (HRP). This two step system can be supplemented with upstream enzymes, such as invertase (Inv) or β galactosidase (βGal), which produce glucose as the substrate for GOx. Note that the hydrogen peroxide intermediate, produced by GOx and consumed by HRP, can be scavenged by catalase (Cat). B) Serial array of avidin activated microcompartments, each bearing an individual biotinylated enzyme, βGal, GOx, or HRP (from Ref. [46]). ABTS 2,2′ azino bis(3 ethylbenzthiazoline 6 sulfonic acid). C) Self assembled formation of microcompartments in a pseudo 1D fluidic channel using DNA address tags to specifically bind enzymes bearing the complementary oligonucleotide (adapted from Ref. [47]).

3. Cascades on Particles

Instead of using enzyme functionalized particles to create fluidically coupled compartments, as discussed above, several different enzymes of a cascade can also be co immobilized on the surface of synthetic beads or on the surface of cells[52,53] that have typical dimensions in the upper nanometer to low micrometer range (Figure 1 C,D). An early example, reported by Mosbach and co workers,[54] concerned a three enzyme system of malate dehydrogenase, citrate synthase, and lactate dehydrogenase, which were immobilized on various bead materials to mimic the NADH utilizing system of mitochondria. Continuous determination of the rate of citrate production from malate, NAD^+, and acetyl CoA revealed an up to 400 % increased activity compared to the soluble system. The authors attributed this increase to shorter diffusion paths and thus higher diffusion rates of the reaction intermediates.

The Travis research group has developed a particle based approach which mimics the assembly of glycolytic enzymes on cytoskeletal elements in mammalian spermatozoa. To this

end, hexahistidine tagged recombinant glycolytic enzymes were immobilized in an oriented fashion with respect to the surface of Ni^{2+} NTA modified agarose beads. Initial studies with two[55] or three[56] different enzymes demonstrated that surface tethered pathway components indeed show sequen tial enzymatic activities. Very recently, this approach was extended to a 10 step sequential enzymatic reaction of glycolytic enzymes, which converted glucose into lactate.[57] It was observed that, although the efficiency of the individual enzymes was higher in solution, the efficacy of the 10 step pathway was significantly higher when the enzymes were tethered on the particle surface (Figure 4). The authors suggest that a channeling of intermediates within the hydra

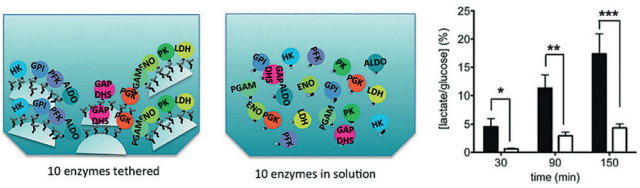

Figure 4. Cascades aranged on microparticles. Sets of 10 different glycolytic enzymes were immobilized on Ni^{2+} NTA modified particles and compared to a mixture of the enzymes in solution. The glucose to lactate conversion was much higher for the particle bound enzymes (black bars) than for the enyzme mixture in solution (white bars). Reprinted from Ref. [57]. The stars indicate statistical significance.

tion layer of proteins on the bead interface and a concomitant reduced diffusional loss of an intermediate product might be a reason for the increase in production efficiency. Alterna tively, weak attractive interactions between the substrate molecules and the bead surface even might create a "virtual compartment" which can accelerate the throughput.[58] Indeed, these hypotheses are in agreement with earlier reports of scaffold immobilized enzyme cascades,[59] and of the increased overall reaction rates of GOx/HRP cascades co immobilized on particles in a packed bed flow through reactor.[60]

Randomly oriented assemblies of enzymes on surfaces have also been explored for biosensing. For example, Dronov et al. co adsorbed oppositely charged redox proteins cyto chrome *c* (Cyt *c*) and sulfite oxidase (SOx) through layer by layer deposition of both enzymes onto electrode surfaces. The layered assemblies showed a remarkably efficient electron transport from the substrate in solution to the electrode over long distances in the *z* direction.[61] Similar work with electro active multilayer assemblies of redox proteins, carried out by the Lisdat research group,[62] led to the development of nano biomolecular multiprotein clusters on electrodes for the formation of a switchable cascade, wherein two different enzymes (laccase and cellobiose dehydrogenase) were con nected with Cyt *c* by means of carboxy modified silica nanoparticles.[63] The system was used as a sensor for the measurement of lactose and oxygen.

The surface confinement of enzymatic cascade reactions can also be realized on soft matter particles, such as vesicles. This approach has been used to study mechanistic aspects of the HRP catalyzed polymerization of aniline.[64] However, in addition to vesicles, water in oil microemulsions, liposomes, nanometer sized protein cages, and other hollow capsules can also be used for volume confined enzymatic reactions. Nature harnesses such physical compartments for pathway seques tration.[65] Their mimicry for technical exploitation is strongly dependent on basic research in the fields of supramolecular chemistry and enzymology, as discussed in the excellent review by Küchler et al.[7]

Nature has evolved more sophisticated methods for decorating extracellular or intracellular surfaces with coop erating enzyme systems. A prime example is the extracellular complex of the so called cellusome (Figure 5 A),[66] wherein various cellulose digesting enzymes are organized at the outer cell membrane of cellulolytic bacteria such as *Clostridium thermocellum* to enhance the availability of sugar. This multienzyme assembly offers a high degree of enzymatic modularity, which can adapt to the changing properties of a diverse range of natural substrates, such as cellulose.[67] Mimicking the cellusome system is considered a promising strategy for engineering multienzyme systems on cells, and

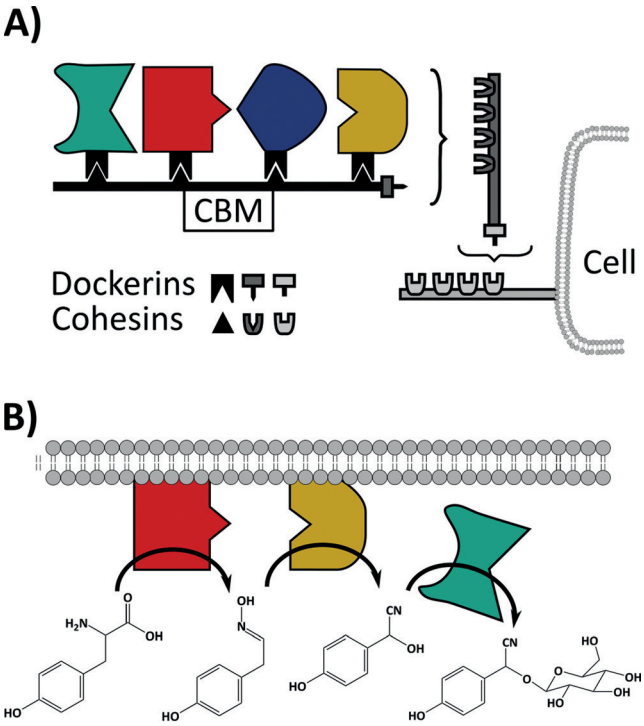

Figure 5. Natural enzyme cascades arranged on cell membranes. A) Dockerin Cohesin based assemblies of cellulose degrading enzymes, adapted from Ref. [66]. A main element is the scaffoldin, which is made up of several cohesin domains and terminates in a dockerin domain. Starting at the cell surface, an anchoring scaffoldin (light gray) fixes the cellusome to the cell surface. Adaptor scaffoldins (dark gray) act as branching points to enable the binding of the primary scaffoldins (black), which carry the catalytic enzymes and contain CBMs (cellulose specific carbohydrate binding modules). B) Membrane associated enzyme assembly of the dhurrin biosynthetic pathway. Starting from the amino acid tyrosine, two membrane anchored P450 enzymes generate the compound *p* hydroxymandelo nitrile, which is then further modified to the cyanogenic glucoside dhurrin by a soluble UDP (uridine diphosphate) glucosyltransferase.[70]

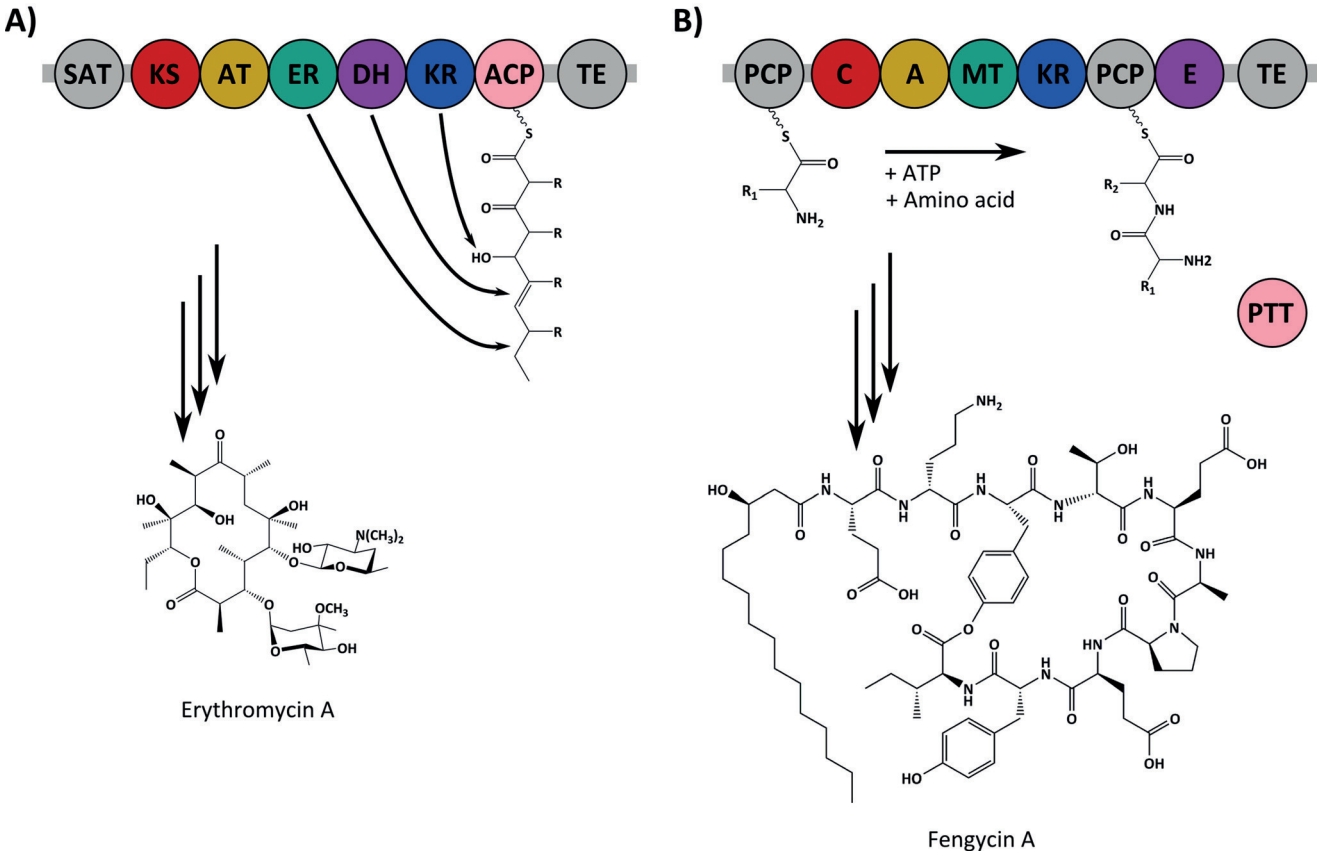

Figure 6. Representative PKS and NRPS assembly lines. A) General arrangement of PKS domains. SAT: Starter ACP transacylase, AT: acyl transferase, ER: enoyl reductase, DH: dehydratase, KR: β ketoacyl reductase, ACP: acyl carrier protein, TE: thioesterase. A prominent example is the erythromycin synthase, which is composed of 28 domains organized into 7 modules on 3 polypeptides. B) General arrangement of NRPS domains. PCP: Peptide carrier protein, C: condensation domain, A: adenylation domain, MT: methyltransferase, KR: β ketoacyl reductase, E: epimerization domain, TE: thioesterase. The 4′PP transferase (PPT) is usually encoded separately. A well described example is the fengycin synthetase, which comprises 35 domains organized into 10 modules on 5 polypeptides. Adapted from Ref. [74]. The acyl and peptidyl carrier moieties in PKS and NPRS act as a type of swinging arm linker to enable facilitated transport of the intermediate (see also Figure 7 B).[75, 79]

has already been used to create scaffolding materials for the conversion and immobilization of biomass.[68, 69] The enzyme arrangements on the surfaces have their counterparts inside the cell, where they are often arranged as compact agglom erates or individual multienzyme complexes (Figure 1 E,F). Such arrangements of sequential enzymes are often dubbed as "metabolons", which can dynamically assemble at the inner cell surface (Figure 5 B). For example, a dynamic metabolon that catalyzes the formation of the cyanogenic glucoside dhurrin, a defense compound produced in sorghum plants, has recently been discovered.[70]

4. Multienzyme Complexes, Fusion Proteins, and Substrate Channeling

Multienzyme complexes have been studied for a long time.[71] The most prominent examples include fatty acid synthase (FAS),[72] polyketide synthases (PKS), and non ribosomal peptide synthetases (NRPS).[73, 74] These multi enzyme complexes use an "assembly line" strategy, wherein hundreds of catalytic centers conduct stereospecific trans formations on a growing substrate. The substrate is often covalently anchored to a "swinging arm" linker in a central position of these so called "megaenzymes".[75] Each individ ual step in the metabolite synthesis is facilitated by an independently folded domain. The domains are clustered into functional modules, and the modules are arranged on the polypeptide chains in the order in which they act (Figure 6). In the case of the bacterial PKS and NRPS, this leads to an enormous diversity of bioactive small molecules, and inten sive research has been devoted in the past two decades to reengineer these systems as a tool in drug discovery. The simplicity of the assembly line logic suggests that artificial multienzymes could be created by a modular "LEGO ization" of gene fragments.[76] However, it appears that the numerous protein protein interactions within the megaen zymes, which are essential for functionality and which are poorly understood,[77] are critical and difficult to control to render this approach suitable for routine synthesis of libraries of bioactive compounds. An up to date overview can be found in the themed issue on biosynthetic assembly lines.[78]

Although the chemical nature of the intermediates during PKS and NRPS mediated syntheses, as well as the kinetic

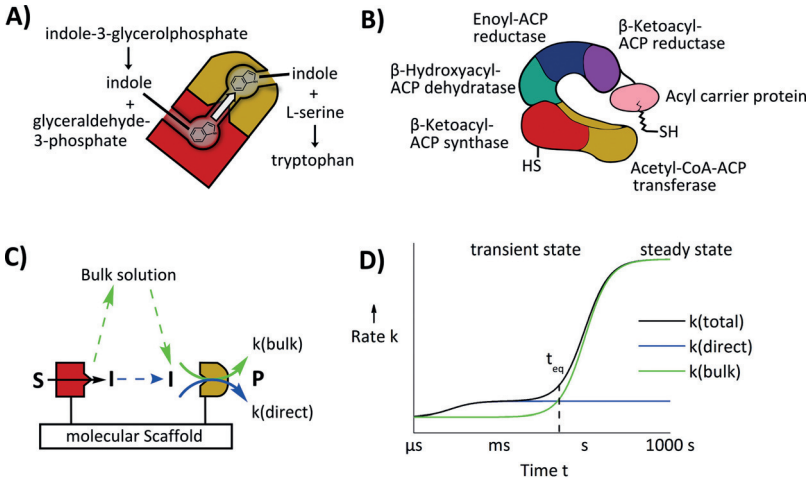

Figure 7. Substrate channeling, also described as "facilitated transport", can occur through quasi 1D channels, as exemplified by the tryptophan synthase complex (A) or "swinging arms" (B) that immobilize the intermediate during a multienzymatic trans formation, as depicted schematically for the fatty acid synthase (FAS) complex, where the thiol group in the ACP acts as a swinging arm. Graphic adapted from Ref. [153]. The graphic in (C) shows the two enzyme diffusion model (not drawn to scale), which illustrates that an intermediate I produced by the first enzyme (red) can either diffuse (dashed arrow) into and back from the bulk solution to be converted (solid arrow) by the second enzyme (yellow) into product P with an associated rate *k*(bulk) or, alternatively, by direct diffusion to the second enzyme and conversion with an associated rate *k*(direct). The time dependent production rate of P, *k*(total) (black), resulting from *k*(direct) (blue) and *k*(bulk) (green) is illustrated in (D). Note that *k*(bulk) becomes dominant after a very short time. C, D) Modified from Ref. [89].

parameters of catalysis by individual domains, have been described, the dynamics of monomer activation and chain elongation in the context of a larger assembly are still not well understood.[73] In general, it is assumed that a spatial prox imity of the catalytic centers decreases the time necessary for diffusional transfer of intermediates. This so called "facili tated transport" or "substrate channeling" also reduces the risk that an intermediate can escape from a multicatalytic protein complex by random diffusion into the bulk solution, thereby leading to a significantly reduced formation of side products. The tryptophan synthase complex, which catalyzes two sequential reactions to produce tryptophan, provides an illustrative example for substrate channeling.[80] It is thought that the metabolic intermediate indole diffuses from the first to the second active site through a physical hydrophobic channel (Figure 7 A). This prevents it from freely diffusing out of the complex and increases the reaction rates by one to two orders of magnitude compared to the free, uncomplexed subunits.[81]

In addition to spatial confinement through a pseudo 1D tunnel,[82] facilitated transport can also be mediated by dimensionally limited diffusion (2D instead of 3D) across physical surfaces and/or hydration layers of proteins, mem branes, or particles (see Section 3). It can also be aided by physical fixation of the intermediate through carrier proteins and "swinging arms", which are flexible linkers located at central positions inside the multicatalytic complex, as is the case, for example, in FAS (Figure 7 B) or PKS and NRPS (Figure 6). It is not questioned that channeling reduces the concentration of reactive and/or toxic inter mediates in the bulk medium, since it can be measured experimentally by, for example, transient time analysis, inhibition studies, or competing enzymes in the bulk solution.[83,84] Moreover, channeling also allows the flux of matter within distinctive biochemical path ways to be controlled. However, the acceler ated conversion is still the subject of contro versial debate.

Proximity channeling: Motivated by the idea that proximity alone could suffice to increase the rate of multistep conversion, researchers have studied synthetic bienzyme complexes produced by chemical cross linking or genetic fusion (compare Figure 1 E,F). The Mosbach research group was amongst the first to systematically investigate this issue,[85] for example, by generating a fusion of β galacto sidase (ßGal) and galactokinase (GalK) for the two step conversion of lactose into gal actose 1 phosphate.[86] The results obtained by Mosbach and co workers and several other research groups were contradictory. Theory predicts that minimization of the distance between two catalytic centers leads to a max imization of the collision probability. This was demonstrated, for example, by simulations of Brownian motion, which indicated that an increased overall reaction probability occurs only within very close distances of 0.1 1 nm between the active sites of two cooperating enzymes.[87] Extended simu lations with regard to the relative orientation of active sites suggested highest probabilities for a 0° (face to face) orientation. Indeed, these predictions were experimentally confirmed recently by a set of chemically linked mannitol dehydrogenase/formate dehydrogenase conjugates with defined active site arrangements. The defined orientations were realized by utilizing site specific incorporation of reactive non natural amino acids (NNAAs) into the enzymes followed by bioorthogonal enzyme to enzyme conjugation through strain promoted azide alkyne cycloadditions or inverse electron demand Diels Alder reactions. The studies revealed a fourfold relative enhancement for the 0° config uration in the initial phase of the reaction ($t \leq 1$ min).[88]

Since substrate channeling is tightly connected with the spatial distance between the catalytic centers and the dimensions of the bulk container, it is important to realize that the mean time of diffusion between two points in space increases as the square of the distance between the two points. For example, a small molecule requires about 15 ms to diffuse over distances of 10 µm. This corresponds to the average distance between two enzymes in a homogeneous solution at a concentration of 1 pM, as estimated from reported data.[90] Since typical turnover times of enzymes, such as GOx or HRP, are in the range of 50 ms,[91] bulk diffusion is much faster than the chemical conversion, even under these diluted conditions. However, since diffusion occurs randomly in 3D space, this assumption is too simple.

Idan and Hess have discussed the physics of diffusive transport processes relevant for such reaction diffusion systems.[89] They modeled a spatially ordered enzyme pair inside a container, which represents either a microcompartment or can be interpreted as the average free space in a homogeneous solution of enzyme pairs. In their model (Figure 7C), the reaction output of the first enzyme is divided into two diffusional streams, which either flow directly to the second enzyme or into the bulk solution. The ratio of the two fluxes was simulated using a random walk algorithm and depends on the distance between the two enzymes and the size of the second enzyme. The direct flux of the intermediate leads to product formation at a constant rate k(direct), while the second flux leads to a linear increase in the concentration of the intermediate within the bulk solution. The overall production rate k(total) can thus be divided into three different phases. In the first phase (< 1 ms), the direct diffusion sharply increases the reaction rate. In the second, transient state phase, intermediates fill up the bulk container, thereby leading to a linear increase in the reaction rate k(bulk), because the turnover of the second enzyme depends on the concentration of the intermediate ([I]). At t_{eq}, both streams contribute equally to the product formation at the second enzyme. The rate then continues to grow until the steady state is reached on a timescale of seconds to minutes. Therefore, the proximity effect in a system lacking limited diffusion through tunnels or hydration layers is only temporary. The characteristic time scale at which proximity boosts the overall production is given by the ratio of the surrounding container volume to the product of the intermediate diffusion coefficient and the interenzyme distance, and it is on the order of milliseconds to seconds, depending on the particular enzyme systems.[58] In other words, the benefit of the scaffold is lost as soon as [I] in the bulk is high enough to drive the second enzyme. This is consistent with results of Brownian motion calculations for steady state conditions, which indicated no significant accumulation of [I] within 1 μm of the first enzyme, even for high generation rates and slow diffusion coefficients.[6] It was also found by stochastic simulations that, in the steady state, a channeled reaction cannot be faster than the non channeled reaction.[92]

Consequently, proximity only leads to a permanent boost of overall production when the intermediate remains at low concentrations in the bulk solution, which is the case for systems where there is a loss of intermediates to a large volume or side reactions.[58,89] The latter can be verified experimentally when the intermediate is sequestrated by alternative reactions. In the case of the GOx/HRP cascade (Figure 3A), this can be easily achieved by adding catalase (Cat), which readily converts the intermediate hydrogen peroxide into water and molecular oxygen. Interestingly, such experiments led to complete suppression of product formation by enzyme pairs in homogeneous solution,[91] whereas in heterogeneous reactions, where GOx/HRP cascades were assembled on surfaces, complete suppression was not observed.[93] These results support the notion of a channeling of intermediates by dimensionally limited diffusion through the hydration layer at the interface between a solid surface and the solution phase (see discussion in Section 3).

As detailed in the review by Conrado et al.,[81] the studies on simple fusion proteins did not provide distinctive evidence for a mechanism which involves the channeling of intermediates. This notion is supported by recent studies by Hess and co workers, who demonstrated that proximity does not contribute to activity enhancement in the GOx/HRP model cascade,[91] as discussed in Section 5.2 in the context of DNA arranged multienzymes. Further evidence was provided by Castellana et al., who combined experimental studies with reaction diffusion modeling to investigate whether enzyme clustering might be effective in regulating flux division at metabolic branch points.[90] They focused on a fundamental branch point in *E. coli*, where carbamoyl phosphate synthetase (CarB) synthesizes carbamoyl phosphate. This intermediate can then be used by aspartate carbamoyltransferase (PyrB) for the biosynthesis of pyrimidine or by ornithine carbamoyltransferase (ArgI) for the biosynthesis of arginine. They found that the simple genetic fusion of CarB to PyrB does not induce channeling, whereas channeling occurs when CarB and PyrB forms larger clusters through inherent protein protein interactions. A quantitative model demonstrated that clustering into compact agglomerates accelerates the processing of intermediates and yields the same efficiency benefits as the direct physical channeling described above. Importantly, the theoretical model predicts the optimal size (about 260 nm) and separation (about 6.5 μm) of coclusters to maximize metabolic efficiency. The work also emphasized the importance of achieving a maximum density, but also the relative unimportance of internal organization of the cluster.[90]

The highest possible enzyme concentrations are reached in the so called “cross linked enzyme aggregates” (CLEAs). As estimated from literature data[90,94] for a globular protein with a molecular weight of 30 kDa and a radius of 2 nm, concentrations can be as high as 25 mM (700 mg mL^{-1}). CLEA systems can be produced in a nondirected fashion, for example, by means of glutaraldehyde mediated unselective cross linking to form amorphous CLEAs of one enzyme or combi CLEAs of two or three proteins.[95] In a variation of this approach, combined cross linked enzyme aggregates were prepared from GOx and HRP, and revealed an improved overall reactivity and no susceptibility against perturbation by catalase.[96] A highly sophisticated directional assembly of multidimensional (1D, 2D, or 3D) homooligomeric protein agglomerates was reported by Brodin et al. by exploiting the directionality and strength of metal coordination interactions.[97] The crystalline assemblies, with dimensions that span nearly the entire nano and micrometer scale, could be predictably tuned by external stimuli, such as metal concentration and pH value. Hence, the rational construction of high density enzyme arrangements is progressing rapidly and such agglomerates might indeed be powerful tools for biocatalysis when they are implemented into, for example, the compartmentalized microfluidic reactors or bead based approaches discussed above.

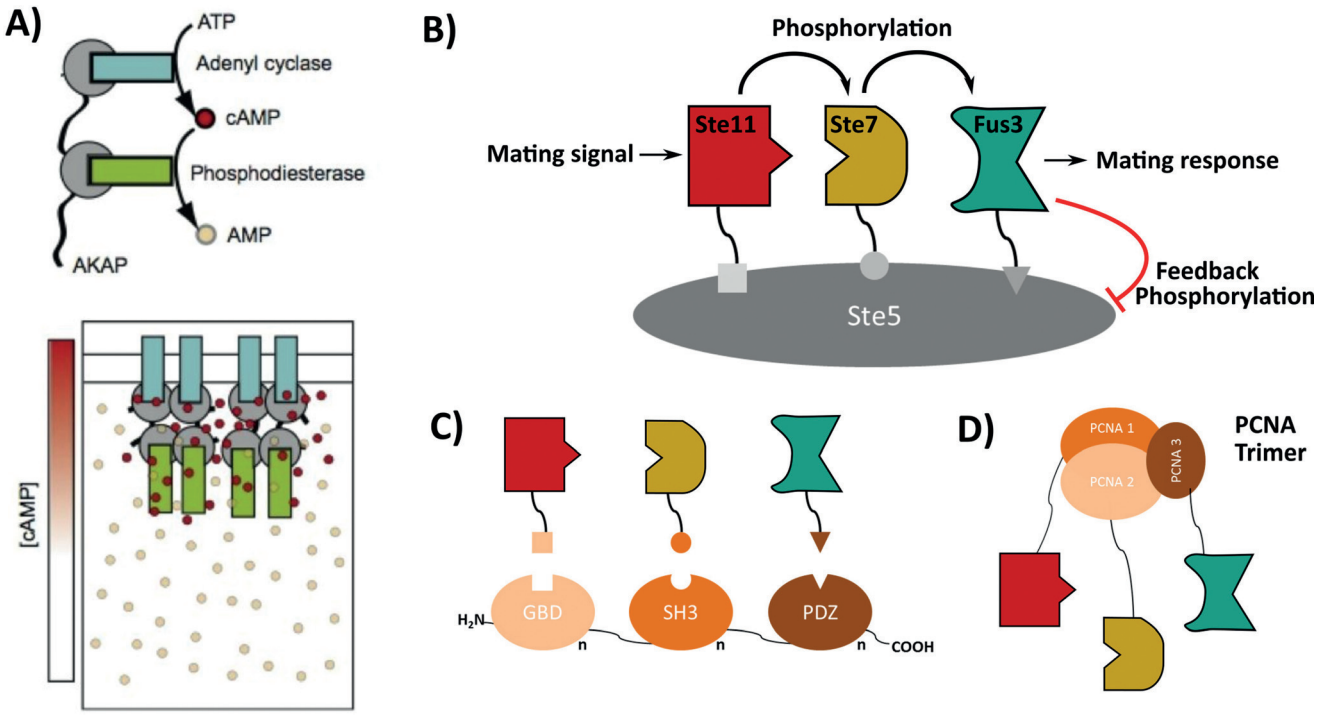

Figure 8. Intracellular scaffold anchored enzyme cascades. A) Membrane associated clusters ("microdomains") of adenyl cyclases (blue) and phosphodiesterases (green) are assumed to produce high concentrations of cAMP in secondary messenger signaling. Reproduced from Ref. [65]. B) Ste5 tethers multiple enzymes to control signaling in the mating pathway of yeast. C) Variable repeats of protein protein interaction domains (GBD, SH3, PDZ) serve as modular "LEGO izable" scaffolds to assemble enzymes in an optimal stoichiometry to enhance pathway flux. D) Fusion proteins of enzymes with domains of the self assembling trimeric PCNA ring structure spontaneously form multienzyme complexes.

5. Cascades Arranged on Nanoscaffolds

In addition to the enzyme agglomerates discussed above (depicted schematically in Figure 1E), the clustering of enzymes also occurs on molecular or supramolecular scaf folds. For example, random assemblies of scaffold supported enzyme complexes (shown schematically in Figure 1G) are formed at the cell membrane. They can function as "metab olite microdomains", wherein elevated concentrations of signaling molecules, such as Ca^{2+} or cAMP, are generated by the concerted action of producing and consuming enzymes, such as adenyl cyclases and phosphodiesterases for cAMP, which are held together by A kinase anchoring proteins (AKAPs, Figure 8A).[98] Such microdomains, estimated to be less than 1 µm in size, also contain high concentrations of metabolite sinks (e.g. phosphodiesterases), which are located at the boarders to ensure that metabolites can not escape. An overview of this topic is given by Lee et al.[65] Another example concerns assemblies of transmembrane receptors, whose spatial arrangement and organization is increasingly recognized to play a key role in cell communication.[99,100] These submicrometer signaling clusters, which contain tens to thousands of molecules, have been observed for the epider mal growth factor receptor, ephrin receptors, and "immuno logical synapses" in B and T cells. The size, composition, as well as spatial and temporal organisation of the cluster are thought to influence the final cellular outcome of the signaling events. In addition to these relatively large supra molecular assemblies, more defined clusters, containing only a few enzymes arranged on protein scaffolds in a defined stoichiometry, are abundant in nature. In the following, we briefly describe these systems and discuss attempts to mimic them by arrangement of biocatalytically relevant enzymes on artificial scaffolds made of proteins and nucleic acids.

5.1. Protein Scaffolds

Nature uses protein scaffolds for the directional arrange ment (Figure 1H) of enzymes to promote signaling networks inside a cell. To illustrate the principle, we briefly refer to Ste5, which is one of the best understood protein scaffolding systems (Figure 8B). Ste5 was discovered as the first scaffold protein more than two decades ago.[101] It tethers multiple enzymes that participate in the mating pathway of yeast. The rational engineering of the structure of Ste5 enabled the design of customized cell signaling circuits.[102] Numerous other scaffold proteins exist in biological systems to regulate phosphorylation cascades, protein folding, ubiquitinylation, pathogen activity, and other fundamental processes. Excellent overviews on these systems have been published which also discuss their implications for engineering the cellular path way[1] and the mechanisms of such reaction diffusion sys tems.[89]

In addition to using fusion proteins to investigate prox imity effects[86] and, more recently, to direct pathway flux in living cells to improve biotechnological production process es,[103,104] sophisticated approaches for cascading enzymes in vivo have been developed through modular scaffold systems (Figure 8C,D). For example, the Dueber research

group developed a "LEGO izable" scaffold system in which the eukaryotic protein interaction domains PDZ, SH3, and GBD can be fused in a variable sequence to assemble enzymes of interest, which are then fused with cognate peptides. They heuristically determined optimal stoichiome tries in recombinant *E. coli* cells to overcome the low turnover of bottleneck enzymes and to obtain enhanced product titers for glucaric acid, mevalonate, and resveratrol, compared to cells expressing free enzymes or direct fusion proteins.[105] This system was also adopted for the assembly of an electron transfer system to enable a synthetic hydrogen production pathway.[106] Conceptually similar approaches, used for the in vitro assembly of multienzymes, have har nessed self assembling protein multimers, such as the ternary proliferating cell nuclear antigen (PCNA), which forms a posttranslational ring structure (Figure 8D),[107,108] or a "mini scaffoldin" fusion protein, which was used to assemble three dockerin containing hydrolytic enzymes.[109] Elaborate surveys on this topic have been published by the groups of Silver,[110] Dueber,[65] and Wheeldon.[111]

5.2. Nucleic Acid Scaffolds

In a similar fashion to the protein based scaffolds described above, nucleic acid templates have been used for the colocalization of enzymes in live cells (Figure 9). For example, plasmid DNA has been used as a stable and configurable scaffold to arrange biosynthetic enzymes in the cytoplasm of *E. coli*. To this end, the individual enzymes were genetically modified with zinc finger domains that specifically bind unique DNA sequences of the plasmid (Figure 9A). Indeed, the production of several metabolic products, such as

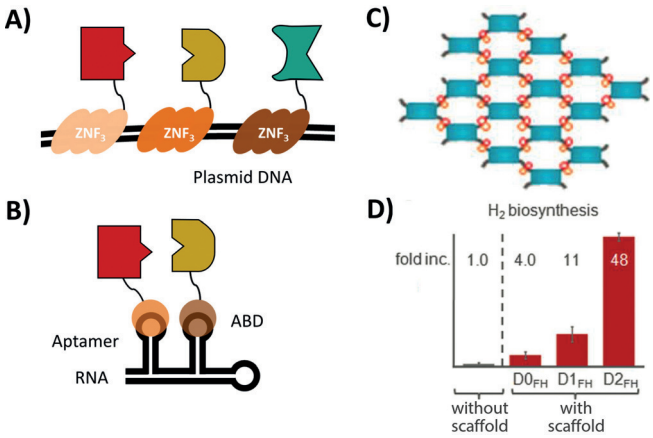

Figure 9. In vivo systems of spatially arranged enzyme cascades on nanostructured nucleic acid templates. A) Plasmid bound enzymatic cascade as reported by Conrado.[112] B) RNA aptamer motifs as scaffolds for recombinant enzymes bearing aptamer binding domains (ABDs). C) Synthetic self assembled 2D RNA scaffolds D2 bearing aptamers (red and orange circles) to facilitate binding and assembly of ABDs fused to hydrogen producing enzymes in live *E. coli* cells. D) Hydrogen production increases with the dimensionality of the RNA scaffold that assembles the ferredoxin (F) and hydrogenase (H) enzyme cascades ($D0_{FH}$ discrete hairpin, as in (B), $D1_{FH}$ 1D RNA scaffold, $D2_{FH}$ 2D RNA scaffold as in (C)). Data taken from Ref. [113], with permission.

resveratrol, 1,2 propanediol, and mevalonate was increased as a function of the scaffold architecture.[112] To mimic microdomain like assemblies, recombinant *E. coli* cells have been engineered to produce synthetic RNA scaffolds equipped with aptamer binding sites (Figure 9B). These binding sites induce the formation of clusters of the cooper ating enzymes [FeFe] hydrogenase and ferredoxin, and increase the production of hydrogen (Figure 9C,D).[113] More recently, in vivo colocalization of enzymes on RNA scaffolds has been used to increase the metabolic production of pentadecane and succinate in a geometrically dependent manner. This illustrated the importance of the relative orientation of the enzyme active sites.[114] These demonstra tions of rationally designed nucleic acid/protein assemblies suggests that functional multimeric architectures can indeed be constructed in vivo. As a consequence of the rapid advancement of RNA nanotechnology,[115] it seems likely that this concept could be further exploited for production processes in biotechnology.

Based on our seminal work on the construction of DNA based supramolecular multiprotein arrangements,[116] we suggested early on that this approach should be ideally suited to the generation of stoichiometrically and spatially well defined artificial multienzyme complexes in vitro.[117] Indeed, by arranging two enzymes on surface bound oligo nucleotides, we could demonstrate this principle for the coupled reduction of NADH and the oxidation of flavinmo nonucleotide (FMN) to generate light (Figure 10A).[118] Similar experiments were later conducted with GOx/HRP cascades by using either surface immobilized linear scaf folds[93] or oligomeric 1D or 2D nanostructures in homoge neous solution.[119,120] The advent of the so called "scaffolded DNA origami" technique,[121] which allows an unlimited variety of nanoscaffolds of arbitrary shape to be readily generated,[122,123] makes it possible nowadays to arrange proteins on molecular pegboards with a single "pixel" resolution of about 6 nm,[124] This approach has been exploited by the Yan research group to advance the field of DNA based multienzyme cascades in the broader context of spatially interactive biomolecular networks.[125,126] Recent examples concern, for example, the optimization of geometric arrangements of a three enzyme pathway to facilitate effi cient substrate transfer (Figure 10B)[127] or the directional regulation of enzyme pathways by the physical control of substrate channeling (Figure 10C).[128] Since the latter exam ple nicely illustrates that DNA scaffolded multienzyme complexes can be steered by synthetic switches and are not limited in terms of the incorporated biocatalytic entities, such concepts might be useful for the design of regulatory circuits for synthetic biology.

The hypothesis discussed in Section 4 that spatial prox imity leads to an enhancement of activity is one of the major drivers for the strong and growing interest in DNA based multienzyme catalysis. So far, more than 20 different DNA templated enzyme cascades have been reported and a detailed assessment of these systems is given in the recent survey by Morii and co workers.[129] Therein, a quantitative comparison is made of the literature data on the "*n* fold increase in efficiency by cascading" as a function of the distance between

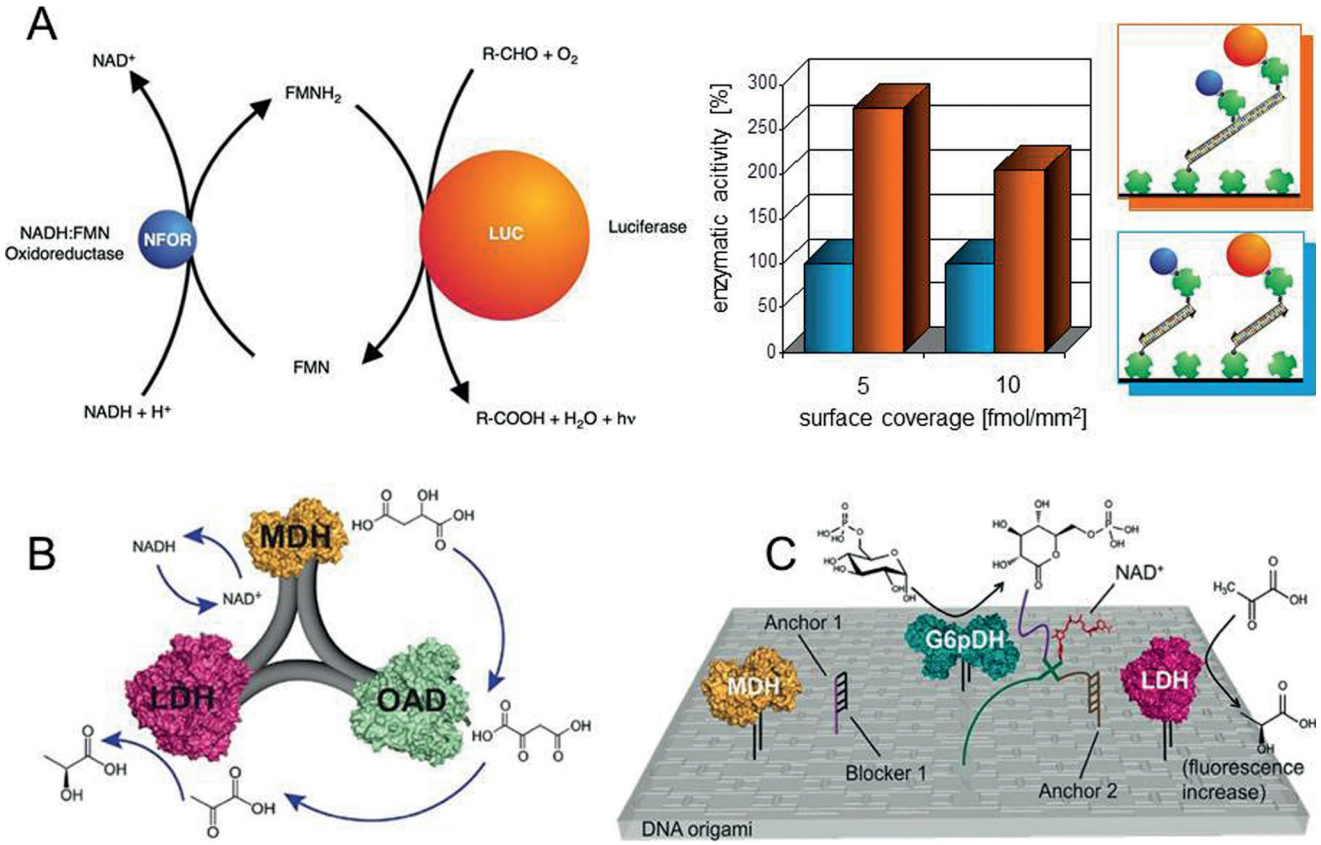

Figure 10. In vitro systems of spatially arranged enzyme cascades on DNA nanstructures. A) Surface bound bienzymatic cascade comprised of NADH/FMN oxidoreductase (NFOR) and luciferase (LUC).[118] NADH is oxidized by NFOR to produce $FMNH_2$, which is consumed by LUC for the bioluminescent oxidation of an aldehyde. The diagram shows that the overall enzymatic activity is higher when the two enzymes are located in proximity on the same oligonucleotide (orange) compared to random immobilization on two different surface bound oligomers (blue). As expected, the relative increase depends on the surface coverage. B) Three enzyme pathway comprised of malate dehydrogenase (MDH), oxaloacetate decarboxylase (OAD), and lactate dehydrogenase (LDH) arranged on a triangular three point star DNA nanoscaffolds. This geometric arrangement of the enzymes showed a fivefold higher activity than the unassembled free enzymes and almost no NADH was detectable in the bulk solution, thus suggesting that almost all the NADH was coupled in the enzyme pathway without leakage.[127] C) Three enzyme pathway comprised of MDH, glucose 6 phosphate dehydrogenase (G6pDH), and LDH arranged on a rectangular DNA origami platform. Through oxidation of glucose, G6pDH can reduce the NAD^+ cofactor, whose position on the origami can be switched to channel the reduction equivalents to either MDH or LDH.[128]

the individual enzymes. Two major conclusions can be derived from this overview: Firstly, about half of the studies have focused on the GOx/HRP model cascade system and, secondly, the *n* fold factors vary to a large extent, with a majority of the studies showing moderate enhancements of two to fivefold and a few exceptions up to several hundred fold, with no apparent correlation to the distance between the two interacting enzymes. One may argue that that different enzyme pairs and/or nucleic acid scaffold systems would lead to different degrees of enhancement, however, one would expect quantitatively comparable results for the same type of enzyme cascade at least, such as the GOx/HRP system. One possible explanation is that the differences in the reported activity enhancements might stem from technical impreci sions, insufficient characterization, or purification of the derivatized enzymes and the assembled complexes. The (bio)chemical modification of the engaged enzymes often leads to changed activities and stabilities, and the remaining free enzymes can distort activity measurements from the assembled complexes. It is, therefore, of utmost importance for both the DNA based and all other cascading approaches to precisely characterize the kinetic parameters of free and assembled enzymes and also to strictly implement controls that are mandatory to really pin down proximity effects and further develop this approach.

By demonstrating that proximity does not contribute to activity enhancement in the GOx/HRP cascade, Hess and co workers have recently challenged the notion of a proximity effect in multienzyme systems immobilized on a DNA scaf fold.[91] Combined experimental and theoretical studies showed no proximity effect, which is in agreement with the models predicting that the rapidly diffusing hydrogen perox ide intermediate enters from the bulk solution (Figure 7C). Hence, when both enzymes have reached their steady state, the overall activity of the cascade is limited by the one exhibiting the lowest turnover. Critical to support the claim that proximity causes activity enhancement are thus questions such as whether there is a steady state and if it has been reached as well as which enzyme is the rate limiting one and did the individual activity of this enzyme change upon its

integration into the multienzyme complex. The last aspect was identified as the possible reason for the activity enhancement of DNA arranged GOx/HRP cascades: The pH value near the surface of the negatively charged DNA nanostructures is lower than that in the bulk solution, thereby creating a better suited pH environment for the anchored enzymes.[91]

While this study[91] clarifies that proximity in itself does not influence the maximal reaction rate when the enzymes are in the steady state, several questions remain. For example, it is not clear to what extent the motion of the intermediates can be represented in reaction diffusion models as a normal diffusion.[130] In other words, the specific hydrophilicity/lipophilicity properties of the intermediate might either lead to 3D diffusion into the bulk or along the 2D surface of the scaffold. Specifically, diffusion into the bulk solution could be electrostatically restricted, which would direct the intermediate to the second enzyme. Diffusion along DNA origami surfaces and hydration layers of proteins was suggested to explain the 14 fold enhancement of a GOx/HRP cascade reaction.[125] Simulations of the same system carried out by Roberts and Chang using the coarse grained Brownian dynamic package revealed that interenzyme distance and rotational orientation were the primary factors for the efficacy of the system. The diffusive barrier played only a secondary role, but became more important at longer distances.[131] Eun et al. found that the electrostatic potential increased the efficiency of transferring a substrate between enzymes. By numerically solving reaction diffusion models extended by coulomb interactions, the authors provided evidence that the role of attractive electrostatic interactions in confining intermediate substrates in the vicinity of the enzymes can contribute more to net reactive throughput than the directional properties of the electrostatic fields.[132] Altogether, this discussion illustrates that the theoretical treatment and simulation of complex diffusion reaction networks is still in its infancy. However, the significant advances in the rational construction of well defined cascades (Figure 10B,C) will enable a better integration of theory and experiment.

6. Summary and Outlook

Biocatalytic transformations are considered to be a key domain of industrial ("white") biotechnology, wherein biological systems, living organisms, or derivatives thereof, are used to make or modify products or processes. The economic relevance of white biotechnology is enormous, because it is also considered a key domain for the conversion and efficient use of renewable biomass as an alternative to petrochemical synthesis for sustainable production processes and energy supply in the future.[133] It is expected that by 2030, the products of white biotechnology and bioenergy will have an estimated worth of 300 billion €.[134] Biomimetic compartmentalization offers an enormous potential to substantially contribute to these developments. Based on the above summary of the current state of the art of understanding and mimicking compartmentalization, we would like to emphasize the following points.

6.1. Compartmentalization on All Scales

Nature uses compartments at dimensions that span from the meter (e.g. organ systems in higher animals) over millimeter (tissues) and micrometer (cells) down to the lower nanometer scale of biomolecular assemblies. As a consequence of the great success of this strategy, it is plausible that future developments of technical systems will also benefit from a multiscale approach (see Figure 1). Although macroscopic compartmentalized bioreactor systems have been studied for more than a decade for processing milliliters to liters of reactants,[21] miniaturized compartments for the precise handling of micro and nanoliter volumes have only recently become available (see Section 2). Advances in top down engineering and surface patterning have led to the availability of a high number of compartmentalized microstructured reactors, which were developed in the context of lab on a chip, micro total analysis systems (μTAS), and biocompatible micro electronical and mechanical systems (bioMEMS).[135] These systems are used for point of care diagnostics and high throughput screening.[38,136] They have already reached a high level of sophistication, but their use for biocatalytic production processes is only at the beginning (see Figure 3B).[37] To overcome one of the major hurdles, innovative chemical approaches are currently being developed for the efficient and selective immobilization of biocatalysts.[137,138] At the (supra)molecular level, bottom up engineering and self assembly along with genetic engineering and heterologous protein expression provide novel means for the construction of biomolecular architecture almost at will. As discussed in Section 5, two complementary approaches for supramolecular scaffolding, based on either proteins (see Figure 8) or nucleic acids (see Figures 9 and 10), are at the focus of current developments. Whereas the first seems better suited for whole cell applications and large scale production, the second approach offers a greater degree of modularity and spatial control, which is particularly important for fundamental studies of cascade systems.

6.2. Microfluidics, Machines, and Big Data

The advent of flow chemistry is a clear demonstration of the power of machine assisted synthesis programs. They can take advantage of the advanced connectivity of equipment and devices linked through the internet (so called "internet of things", IoT), extend the scope of chemical transformations, and allow for a more efficient use of human resources.[15,16] Current flow chemistry, however, uses macroscopic vessels and reaction interfaces, where fluid mechanics and mass transport are difficult to control and predict. Microfluidics offers a much higher degree of control over the temperature profiles and diffusion based mixing,[35,139] which is mandatory for the simulation and rational implementation of the improved reaction kinetics observed in spatially organized

biological cascades. Once optimized, microfluidic processes can be scaled up by simply increasing the number of fluidic devices, which are often accessible by low cost manufacturing. Combined with high throughput handling of liquids, robotics, and (in line) analytical methods, based on mass spectrometry, NMR spectroscopy, and various other spectroscopic methods, microfluidic platforms can generate an enormous amount of data, which can be fed back into optimization of the device and process. Such iterative rounds of improvement benefit from methods for rapid prototyping and added manufacturing (e.g. 3D printing), which enable ready access to specific reactor geometries made of tailored materials for integration of the catalyst. Likewise, the development, production, and immobilization of (bio)catalysts dramatically benefits from machine assisted programs. Platforms for (semi)automated cloning and directed evolution of proteins are available,[140] but the testing of immobilization procedures, compatibility with solvents, and characterization of the kinetic properties under distinctive environmental conditions is still done by laborious handwork. Machine assistance should help over come the current limitations and even facilitate the combi natorial production and assessment of cascades confined by molecular templates or nano and microstructured carriers.

6.3. The Role of Theory

With improved microfluidic "hardware" and (bio)catalyt ic "wetware" at hand, systems engineering on the basis of modeling and simulation becomes increasingly important. At present, theoreticians are developing models to describe the kinetic properties of multienzyme clusters (see Sections 4 and 5). The first modeling studies on enzymatic cascades have led to such insights as the proximity in simple fusion proteins not leading to enhanced activity,[91] but coclustering of enzymes enhancing the overall reaction rates.[90] This development even provided a computationally tractable means for design ing de novo engineered clusters for controlling metabolic networks. However, advances in theory are restricted by the current lack of accessible model systems that allow direct comparison of simulated and experimental data.[130,141] In view of applications in biotechnology, it is also not clear what happens in cascades when the concentration of the inter mediates in the bulk is kept low, as is the case in heteroge neous catalysis under flow conditions. Whereas microfluidics is optimal for simulating fluid dynamics, and computer assisted synthetic planning[142] and biomacromolecular struc tures can be simulated by force fields,[143] it is still very difficult to describe the properties of a solid surface in contact with an aqueous solution. So called "enhanced sampling algorithms" are being developed to cover the typical simulation time scales during which events occur at the interface between hard and soft matter.[144] Multiscale simulation, for example, by coarse grained methods, will be needed for the description of specific catalytic turnovers in the context of a mesoscopic cluster confinement and the macroscopic scale of the fluidic system. Researchers have begun with the in silico evaluation of complex multienzymatic systems.[145] The recent reports of computational approaches for the simulation and prediction of three [23] and even ten step[146] enzymatic cascades illustrate that continuous operation of the system accompanied with in line analytics can pave the way to a new era of "in vitro metabolic engineering" with accelerated "design build test" cycles for cell free bioproduction.[147]

In conclusion, we expect that machine assisted biocatal ysis will have a large impact on the future of chemical manufacturing in the context of sustainable production processes and energy supply for an information based econ omy. Fundamental research in chemistry plays a key role in this development. It will refine enzymes to have high stability and well characterized catalytic properties, create novel enzymes for "new chemistries",[148,149] provide access to hybrid systems of enzymes and organo(metallic) cata lysts,[21,150] and establish means for combinatorial and evolu tionary approaches for the generation of integrated cascades comprised of cell free and cell based production systems, which may even include multispecies microbial consortia.[151] We believe that the very successful partnership between chemists and engineers will continue to produce essential and innovative solutions for future challenges.[152]

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Conflict of interest

The authors declare no conflict of interest.

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