TECHNISCHE UNIVERSITÄT MÜNCHEN
Fakultät für Maschinenwesen

One-pot multi-enzyme syntheses: New strategies beyond whole cells and isolated enzymes

Schriftliche Habilitationsleistung bestehend aus einer Kumulation von 17 Fachpublikationen von

Kathrin G. Castiglione, geb. Hölsch
geboren am 13. Mai 1982 in Offenburg

zur Erlangung der Lehrbefähigung für das Fachgebiet Technische Biokatalyse an der Fakultät für Maschinenwesen der Technischen Universität München, eingereicht am 17. Juni 2019

Fachmentorat:

1. Univ.-Prof. Dr.-Ing. Dirk Weuster-Botz (Vorsitz)
   Lehrstuhl für Bioverfahrenstechnik, Fakultät für Maschinenwesen, Technische Universität München

2. Univ.-Prof. Dr. rer. nat., Dr. rer. nat. habil. Johannes Buchner
   Lehrstuhl Biotechnologie, Fakultät für Chemie, Technische Universität München

3. O. Univ.-Prof. Dr. phil. Kurt Faber
   Institut für Chemie, Karl-Franzenz-Universität Graz

Preface

Biochemical engineering is a discipline that deals, amongst others, with the design of processes utilizing biocatalysts for the sustainable manufacturing of value-added products such as bulk or fine chemicals, pharmaceuticals, and food or feed additives. Traditionally, biochemical engineers treated the biocatalyst as a black box and the focus was put on the matching of the equipment to the demanding requirements of the employed enzymes or cells. However, times have changed and the modern methods of protein and cell engineering facilitate the tailored design of biocatalysts and their adaption to process requirements.

The integrated development of bioprocesses with concurrent optimizations of biocatalyst characteristics and reaction engineering concepts is only possible in a highly interdisciplinary working atmosphere. Such a stimulating environment can be found at the Institute of Biochemical Engineering of the Technical University of Munich (TUM), where (molecular) biotechnologists, (bio)chemists, chemical engineers, and bioprocess engineers work hand in hand on the development of economically and ecologically attractive bioprocesses. The work presented here was conducted during my time as leader of the biocatalysis group at this institute.

This cumulative habilitation thesis is a summary of 17 articles published in peer-reviewed journals. The original papers are not included in the published version of this thesis due to copyright reasons. A list of the papers with links to the publishers' websites can be found at the end of this thesis.

The successful completion of the research projects connected to these publications was only possible due to the (co-)supervision of Bachelor's, Master's and doctoral theses. This co-supervision was kindly supported by the head of the Institute of Biochemical Engineering at TUM, Prof. Dirk Weuster-Botz. In 2014, TUM bestowed the title "TUM Junior Fellow" on me since I had started my own independent research group funded by the German Federal Ministry of Education and Research (BMBF).

Since TUM Junior Fellows can serve as primary reviewers for their own graduate students in doctoral procedures, it was possible for me to directly supervise some of the doctoral candidates. I am grateful for this extraordinary opportunity and hope that similar concepts will be installed at other universities to promote the careers of ambitious young scientists.

At this point, I would like to give my sincere thanks to all of the (former) PhD students of the biocatalysis group, which are also acknowledged in the respective sections of the research summary, for the excellent cooperation over the last years: Yilei Fu, Boquiao Sun, Ilka Sührer, Sarah Poschenrieder, Ludwig Klermund, Tom Schwarzer, Christoph Mähler, Ingmar Polte, as well as Florian Golombek and Michael Mertz, who started their doctoral studies at TUM and accompanied me on my way to the Friedrich-Alexander-Universität Erlangen-Nürnberg. I also owe thanks to the laboratory technicians Florian Sedlmaier and Markus Amann as well as to the workshop staff members Norbert Werth and Georg Kojro for their great support.

My special gratitude goes also to my scientific collaborators for their support and the fruitful discussions. Especially, I would like to thank Dr. Marianne Hanzlik (Department of Chemistry, TUM) for excellent transmission electron microscopy (TEM) images, Dr. Martin Haslbeck (Department of Chemistry, TUM) for his support in diverse protein-related questions, and Prof. Friedrich Simmel (Department of Physics E14, TUM) for the collaboration in the field of membrane protein characterization. Moreover, I owe gratitude to Prof. Ulrich Schwaneberg (Institute of Biotechnology, RWTH Aachen), Prof. Wolf-Dieter Fessner (Department of Chemistry, TU Darmstadt), and Prof. Werner Lubitz (Bird-C, Vienna, Austria) for the provision of a bacterial strain and plasmids, respectively.

The financial support of the research projects by the BMBF (grant numbers: 031A178 and 031B0221), the German Research Foundation (DFG, grant numbers: WE 2715/12-1 and WE 2715/12-2) and the Bavarian State Ministry of the Environment and Consumer Protection (grant number: TLK01U-69036) is gratefully acknowledged.

I also would like to thank the members of my “Fachmentorat”, Prof. Kurt Faber and Prof. Johannes Buchner, for their time, professional guidance, and helpful suggestions throughout my habilitation period.

The last paragraph of this preface is dedicated to the person that has been most important for my scientific development: Throughout my habilitation period, Prof. Weuster-Botz provided me with all degrees of freedom that are necessary to pursue my own scientific interests. Having access to the cutting-edge equipment at his Institute of Biochemical Engineering in Garching was of vital importance to the success of my challenging research projects. Prof. Weuster-Botz inspired me with his structured way of thinking about puzzling research questions and found always time to intensively discuss my ideas. I am incredibly thankful for all of these opportunities and experiences, as they have undoubtedly shaped my scientific career and will accompany me on all my future scientific endeavors.

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1. State of the art

1.1 Multi-enzyme syntheses

The steadily growing demand for sustainable production processes of complex enantiopure chemicals drives the trend toward biocatalysis and, in particular, toward multi-enzymatic cascade reactions. Biocatalytic cascades have been proven to be highly attractive tools to rapidly build up molecular complexity from cheap starting materials in one-pot syntheses.[1-2] Multi-step one-pot reactions can enhance the performance compared to step-by-step syntheses by pushing reaction equilibria toward the desired products while at the same time reducing the number of unit operations. This saves time, chemicals and ultimately costs required for intermediate purification. Moreover, reactions with unstable or deleterious intermediates can be performed by immediate scavenging of the respective substance by a subsequent reaction.[3-5]

Biocatalytic cascade reactions can be classified into four different categories: linear, parallel, orthogonal, and cyclic as summarized in Figure 1.[3]

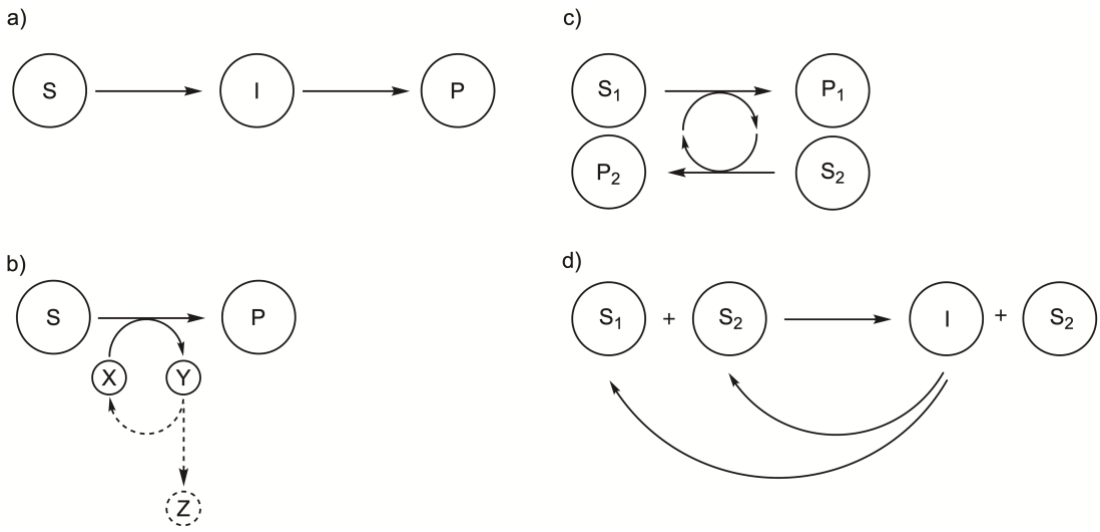


Figure 1: Classification of cascade reactions. a) linear, b) orthogonal, c) parallel, and d) cyclic. S: substrate, P: product, I: intermediate, X, Y, Z: molecules involved in auxiliary reactions. Adapted figure according to Ricca *et al.*[3]

Linear cascades involve one or more enzymes that convert substrates into products via one or more intermediates (Figure 1 a). In orthogonal cascades, the conversion of the substrate into the product is connected to auxiliary reactions that are necessary, e.g., to regenerate cofactors or to remove by-products (Figure 1b). A typical example is the regeneration of oxidized or reduced nicotinamide cofactors in oxidoreductase reactions. Parallel cascades have a similar concept, since two enzymatic reactions are coupled via a shared pool of cofactors or cosubstrates (Figure 1 c). The decisive difference between orthogonal and parallel reactions is the economic value of the products. If both substances are of commercial interest and therefore purified, the cascade is categorized as parallel. Cyclic cascades (Figure 1 d) start with a mixture of substrates, from which only one serves as substrate for a selective catalyst. Subsequently, the formed intermediate is converted back to the starting materials. Iteration of this cycle results in an enrichment of the non-reacted substrate. This reaction scheme has been applied very successfully, e.g., in the deracemization of amino acids.[6] Complex cascade reactions typically comprise reaction modules that can be assigned to different basic cascade types, such as a linear cascade with several auxiliary orthogonal reactions for cofactor supply.[3]

1.2 Design of biotransformations

The design of a biotransformation has substantial impact on the efficiency and economy of the resulting production process. The most important design options, i.e. the choice of the biocatalytic preparation, the reaction medium, and the orchestration of multi-enzyme syntheses, will be discussed in the following paragraphs.

1.2.1 Biocatalytic preparation

One of the first decisions that have to be made when implementing a new biocatalytic process is whether whole cells or isolated enzymes should be employed. Both approaches have their specific advantages and challenges. Whole cells with overexpressed enzymes are cheap to produce and often the preferred option for cofactor-dependent biotransformations due to the cell internal cofactor supply. However, enzymes of the host cell metabolism might catalyze undesired side reactions and the cell wall might cause a diffusion limitation for substrates lowering the activity of the whole cell catalyst. Both drawbacks can be circumvented by using

isolated enzymes instead, but – depending on the degree of purification – they are expensive and it is sometimes necessary to immobilize them to lower the catalyst cost by facilitating their re-use.[7]

Apart from the cost reduction, the immobilization of enzymes can provide further benefits such as a simplified product separation and a stabilization of the biocatalyst. Moreover, the implementation of continuous processes is possible due to the uncoupling from the hydraulic residence time.[8] Negative effects on the specific enzymatic activity or the occurrence of diffusion limitations are strongly dependent on the chosen immobilization method, which range from the (non)-covalent attachment to porous carriers, over cross-linking methods – such as the generation of cross-linked enzyme aggregates – to the entrapment of the catalyst within a matrix or a membrane.[7, 9]

In recent years, there has been growing interest in developing *in situ* immobilization techniques, which are also referred to as *in vivo* immobilization methods.[10-12] By the concurrent production of enzymes and their instantaneous immobilization, the number of unit operations required to obtain the final biocatalytic preparation is reduced, thereby maximizing the cost efficiency. Typically, the *in situ* immobilization of the target enzymes involves protein engineering to facilitate their self-assembly into large supramolecular structures. The most important strategies that have been developed so far are the production of enzyme-coated polyhydroxyalkanoate (PHA) granules[10-11, 13-18], the (triggered) formation of active inclusion bodies[19-24] and the display of enzymes on the surface of microorganisms or spores[25-30].

In most studies dealing with the immobilization of enzymes on PHA granules, the enzyme of interest was genetically fused to the PHA synthase (PhaC) leading to PHA beads decorated with the target enzyme as depicted in Figure 2.[10-11, 13-16] The oriented display of the target enzyme resulting from this strategy can be highly beneficial for the accessibility of the catalytic sites. This is important since the limited accessibility of active centers is a well-known drawback of the uncontrolled immobilization of enzymes on solid carriers.

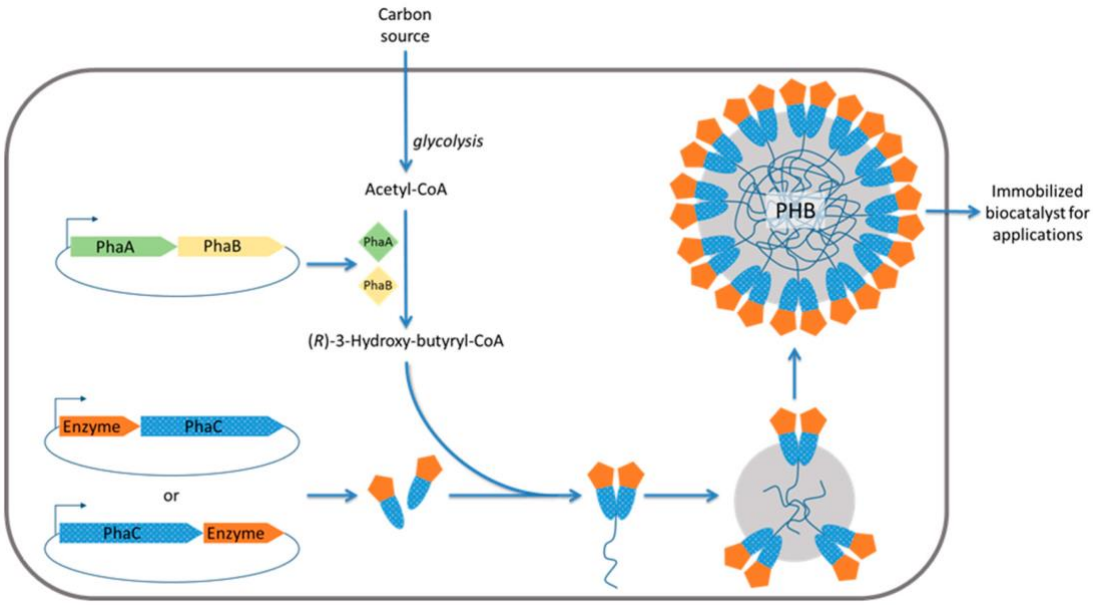


Figure 2: Schematic overview of the *in situ* production of polyhydroxyalkanoate (PHA) beads with immobilized enzymes. PhaA, β-ketothiolase; PhaB, acetoacetyl-CoA reductase; PhaC, PHA synthase; PHB, polyhydroxy-butyrate. Figure from Rehm *et al.*[11] (Creative Commons license type BY).

The generation of active inclusion bodies has the significant advantage over the PHA granule-based approach that the cells do not have to spend resources on the production of a non-catalytic scaffolding material. It has been shown for quite a few enzymes that their overexpression in *Escherichia coli* leads to the formation of inclusion bodies with catalytic activity.[12] This formation can be induced effectively by fusing polypeptide or protein tags to the enzyme of interest. Available tags for this purpose comprise cellulose binding domains[31-32], coiled-coiled domains[19], different kinds of artificial peptides[20-22], or whole proteins such as the pyruvate oxidase from *Paenibacillus polymyxa*[23] or the viral capsid protein VP1 of the foot-and-mouth disease virus[33]. Unfortunately, the triggered formation of inclusion bodies is usually associated with a severe loss of enzymatic activity. However, first attempts to tailor the properties of active inclusion bodies by adapting the fusion design with respect to linker region, C- versus N-terminal fusions or type of aggregation-inducing tag gave promising results and might lay the foundation for a rational design of inclusion bodies with higher specific activities in the future.[19]

For the display of enzymes on the surface of Gram-negative microorganisms they are either fused to so-called autotransporters (Figure 3 a) or to outer membrane proteins, such as the ice nucleation protein from *Pseudomonas* species (Figure 3 b) or the Outer Membrane Protein W (OmpW) from *E. coli* (Figure 3 c).

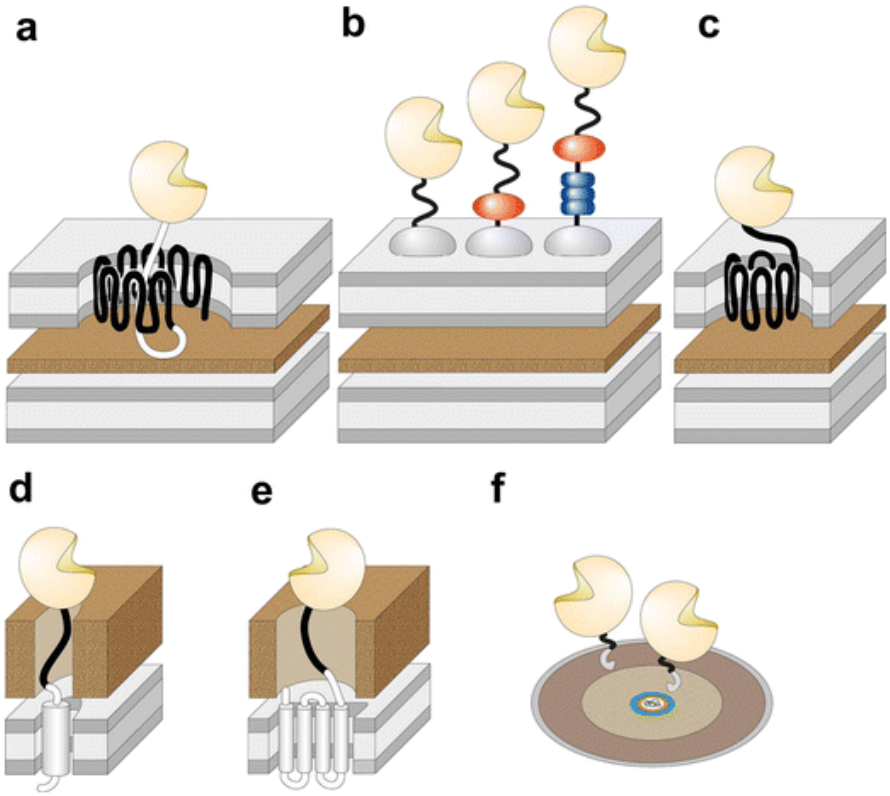


Figure 3: Schematic drawings of the membrane anchoring of enzymes on the surface of Gram-negative (a–c) and Gram-positive (d, e) microorganisms or spores (f). a) autotransporter; b) full length ice nucleation protein with its C-terminal hydrophilic region (red) and the internal repetitive domain (blue). Truncated versions of this protein can also be used for the display of passenger proteins. c) OmpW; d) PgsA; e) NCgl1221; f) spore display via fusion to proteins from the inner and outer spore coat. Figure from Schüürmann *et al.*[26] (reproduced with permission from John Wiley & Sons).

Autotransporters are composed of a signal peptide initializing the translocation through the inner membrane, a passenger domain, and a translocator domain facilitating the surface exposure of the passenger domain. The latter can either be replaced by the enzyme of interest or serve as its fusion partner.[30] Examples of autotransporters that have been used frequently for the display of enzymes are the esterase EstA from *Pseudomonas aeruginosa* and the adhesin involved in diffuse adherence (AIDA-I) from *E. coli*.[26]

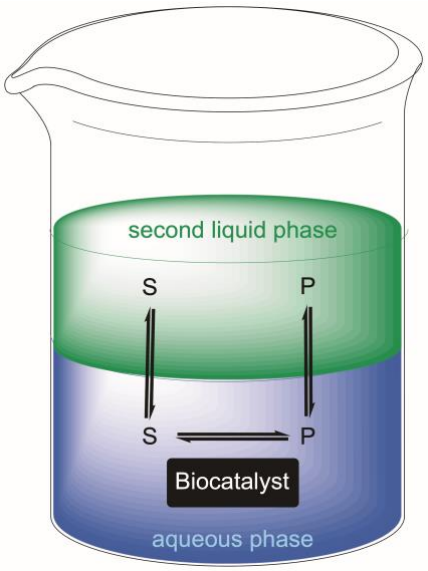
In Gram-positive organisms, other transport proteins have to be used due to the different structure of the cell envelope that lacks the outer membrane and has a much thicker peptidoglycan layer. Here, PgsA (Figure 3 d), an anchoring domain derived from the poly-γ-glutamate synthetase complex PgsBCA from *Bacillus subtilis*, and NCgl1221 (Figure 3 e), a channel protein from *Corynebacterium glutamicum*, have been applied most frequently in the context of biocatalysis.[26]

The specific activity of a whole cell biocatalysts displaying enzymes on its surface is hard to predict, which can be attributed to two facts: Firstly, the number of proteins on the cell surface varies greatly between individual fusion proteins. The reported numbers of immobilized molecules using surface display range between 15,000 and 180,000.[25, 34] Secondly, some enzymes show a significant loss of activity after immobilization on the cell surface.[26]

Whereas the surface display strategies avoid mass transfer limitations caused by the cell membrane, another major disadvantage of whole cell catalysts cannot be circumvented: undesired side-reactions catalyzed by the host cell metabolism. This problem can be overcome by using dormant endospores that are formed by *Bacillus* species and have (almost) no metabolic activity.[27-28] In this case, surface display is achieved by fusing the enzyme of interest to proteins belonging to the inner or outer coat of the spore (Figure 3 f) or to the surrounding crust.[26, 35] The spore surface display has the additional advantage that the fusion proteins do not have to cross a membrane, since the efficiency of this translocation is sometimes very poor and strongly dependent on the characteristics of the passenger domain – most importantly on the size and structure of the protein and its folding characteristics.[36] However, the low display efficiency on the spore surface, which has also been shown to be extremely challenging to improve, is a major limitation of this interesting approach.[28]

1.2.2 Reaction medium

Another important design option for biotransformations is the choice of the reaction medium. Most biotransformations are carried out in simple aqueous solutions. However, if substrates and products are poorly soluble or even unstable in water or display inhibitory or toxic effects on the biocatalyst, a biphasic reaction mode is often highly beneficial. The second phase, either a water-immiscible liquid or a solid phase, thereby serves as substrate reservoir and *in situ* product extractant. Thus, the concentrations of negatively interfering substances in the aqueous phase are kept to a minimum. Figure 4 shows a simplified representation of a liquid-liquid reaction system.



$$D = \frac{c_i^{second\ phase}}{c_i^{aqueous\ phase}} \qquad \text{(equation 1)}$$

$$STR = \beta_i \cdot a_d \cdot \left(c_{i,c}^{*} - c_{i,c}\right) \qquad \text{(equation 2)}$$

STR	substrate transfer rate, mol L^{-1} s^{-1}
a_d	interfacial area, m^{-1}
β_i	mass transfer coefficient of i, m s^{-1}
$c_{i,c}^{*}$	equilibrium concentration of i at the interface, mol L^{-1}
$c_{i,c}$	concentration of i in the bulk of the aqueous (continuous) phase, mol L^{-1}

Figure 4: Schematic illustration of a liquid-liquid biphasic reaction system. The distribution coefficients D for substrate (S) and product (P) can be calculated from the respective concentrations in the aqueous phase and the second liquid phase according to equation 1. The substrate transfer rate (STR) into the aqueous phase containing the biocatalyst can be calculated according to equation 2.

In biphasic systems, the distribution coefficients D of substrates and products, which can be calculated using equation 1, are of utmost importance and values between 100 and 1,000 are ideal for the avoidance of toxic effects on the biocatalysts and a facile product recovery.[37] To ensure a non-limiting substrate transfer rate (STR) from a second liquid phase into the aqueous medium containing the biocatalyst, a high interfacial area should be generated in the reaction system by appropriate stirring, because the STR is linearly dependent on this parameter, as can be inferred from equation 2.

Biotransformations in liquid–liquid systems either use organic or non-conventional solvents, such as supercritical fluids[38] or ionic liquids[37], as the second phase, whereas solid-liquid systems typically employ polymeric adsorbent resins[39]. Although all of these so-called substrate feeding and product removal (SFPR) strategies have been applied in biotransformations with great success[37-45], every strategy has its own advantages and disadvantages. For example, organic solvents can adversely affect the membranes of whole cell catalysts and their cellular toxicity has been shown to be negatively proportional to the octanol-water partition coefficient.[46-47] Toxic effects exerted by the second phase can be kept to a minimum by using either adsorbent resins[39] or ionic liquids in volume fractions up to 40 %[37, 48]. Room temperature ionic liquids have drawn enormous attention of biotechnologists during the last two decades because they have many favorable properties, such as non-volatility, nonflammability, along with, in many cases, high biocompatibility and high chemical as well as thermal stability.[37] Moreover, their characteristics can be fine-tuned by choosing the right combination of ions. This adaptability of the solvent properties, which is hardly accessible with any other class of solvents, is also the reason why ionic liquids have been frequently designated as “designer solvents”.[49] On the contrary, ionic liquids are usually considered to be much more expensive than adsorbent resins and organic solvents. However, the costs for ionic liquids strongly depend on the employed ions and the production amount.[50] In addition, the recyclability of ionic liquids employed in biotransformations has been demonstrated, which significantly reduces their share of expenses in a process.[48]

All in all, the selection of the optimum second phase is a critical factor for the efficiency and economy of a biphasic biotransformation and depends on different factors such as toxic or inhibitory effects on the biocatalysts, distribution coefficients of substrates and products, and costs of the employed resin or solvent.

1.2.3 Orchestration of multi-enzyme syntheses

The optimum interplay between individual biocatalysts in a multi-enzymatic synthesis is strongly influenced by its mode of operation. Especially linear cascades (Figure 1 a) offer the choice between sequential and concurrent reactions. The latter are often desirable since the concurrent operation can exert positive effects on unfavorable thermodynamic equilibria and the atom economy of a process. Moreover, it can help to implement stereo-convergent syntheses and to avoid the degradation of unstable intermediates by their immediate transformation.[51-52]

In comparison to chemical catalysts, biocatalysts are regarded as easily combinable since they generally work under similar mild reaction conditions – usually in the range of 20 to 40°C, around physiological pH and at atmospheric pressure.[53-54] On the downside, many enzymes have quite narrow operational windows and small variations in the reaction conditions can result in a significant reduction of the enzymatic activity. Thus, the implementation of efficient multi-enzyme syntheses can be challenging if there are incompatibilities between individual reaction steps. Aside from the need for different reaction conditions (e.g. in terms of pH, solvent, or temperature) incompatibilities can also arise from reversible or irreversible cross-inhibitions by components of the reaction system (e.g. substrates, intermediates, products, or cofactors).[2-4, 55] Such incompatibilities are not only occurring in artificial reaction systems. Quite the contrary, they are widespread in cells and nature has developed two fundamental organizational principles to deal with this issue: compartmentalization and selective mass transport. The spatial separation of incompatible reactions and the tight control over the microenvironment of the involved catalysts allow the establishment of natural metabolic pathways with outstanding efficiency and stunning elegance. Moreover, these fundamental metabolic concepts do not only exist in eukaryotic cells with their complex systems of specialized organelles, but also in bacterial cells with cytoplasm, periplasm, and microcompartments.[56]

In recent years it has been realized that compartmentalization and selective mass transport are very promising approaches to overcome incompatibility issues also in artificial cascade reactions by the efficient spatial separation of simultaneous reactions.[52] There are quite a few examples for the successful realization of compartmentalization approaches in chemo-enzymatic reactions, e.g. by compartmented continuous flow settings[57-59] or the employment of polydimethylsiloxane (PDMS) membranes or thimbles[60-64]. An example for the latter approach is the one-pot synthesis of enantiomerically pure amines via a combination of Wacker-oxidation and enzymatic transamination as shown in Figure 5. In this reaction setup, without entrapment within the PDMS thimbles the copper-catalyst component of the Wacker-oxidation would deactivate the transaminase.[60]

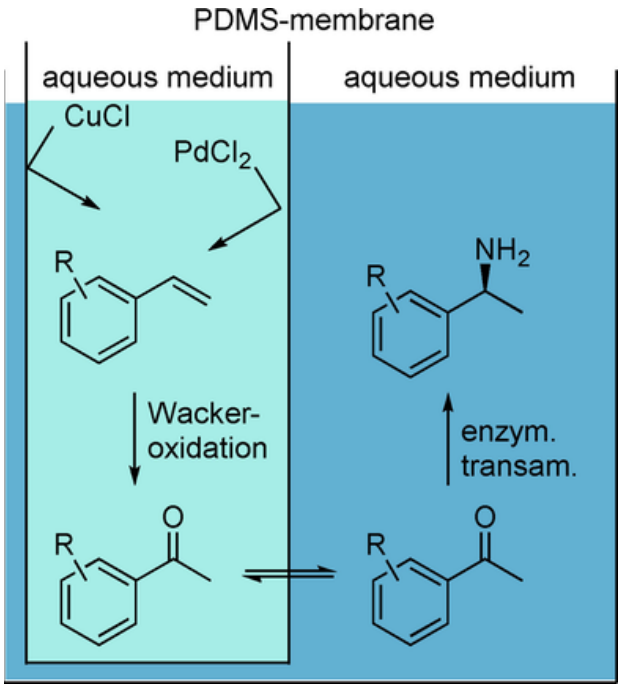


Figure 5: Compartmentalized reaction system for the production of chiral amines. The metal catalysts needed for the Wacker-oxidation cannot cross the membrane of the polydimethylsiloxane (PDMS) thimble. By this means, a deactivation of the transaminase is avoided. Figure from Uthoff *et al.*[60] (reproduced with permission from John Wiley & Sons).

For the spatial separation of incompatible enzymatic reactions some comparable approaches have been described. Most of the concepts rely on the entrapment of enzymes in micro- or nanostructures, such as vesicles, capsids, or polymer matrices.[65-69] However, for most concepts it has only been shown that the encapsulation of different – predominantly compatible – enzymes is possible and positive effects on individual enzyme characteristics, such as an improvement of the operational stability, have been described. In most cases, the proof of principle that incompatibility issues can be solved this way is still missing.

An interesting compartmentalization approach that has already been demonstrated to solve incompatibility issues is the preparation of polyurethane-based polymer matrices with dispersed aqueous inclusions.[69] These matrices are formed by UV-light curing of an emulsion of polyurethane precursors and the respective enzyme solution of interest. Since the reaction conditions that were prevalent in the aqueous solution during the matrix formation are also maintained in the resulting compartments, optimum reaction conditions for individual enzymes can be ensured. By combining several types of artificial compartments, it is thus possible to implement cascade reactions that employ enzymes with incompatible reaction conditions. This was demonstrated on the example of a two-enzyme system consisting of an alcohol dehydrogenase and a hydroxynitrile lyase with incompatible reaction requirements regarding the pH (Figure 6).

A drawback of the polyurethane matrices is the limited transferability of this approach to reaction systems involving compounds with completely different physicochemical properties since the membrane can only be crossed by small polar substances at reasonable rates.[69] This disadvantage can be overcome by employing artificial compartments that allow for a tailor-made mass transport over the compartment boundaries. In this context, vesicles composed of self-assembling amphiphilic block copolymers, so-called polymersomes[70], are of special interest because of their tunable membrane characteristics.[71]

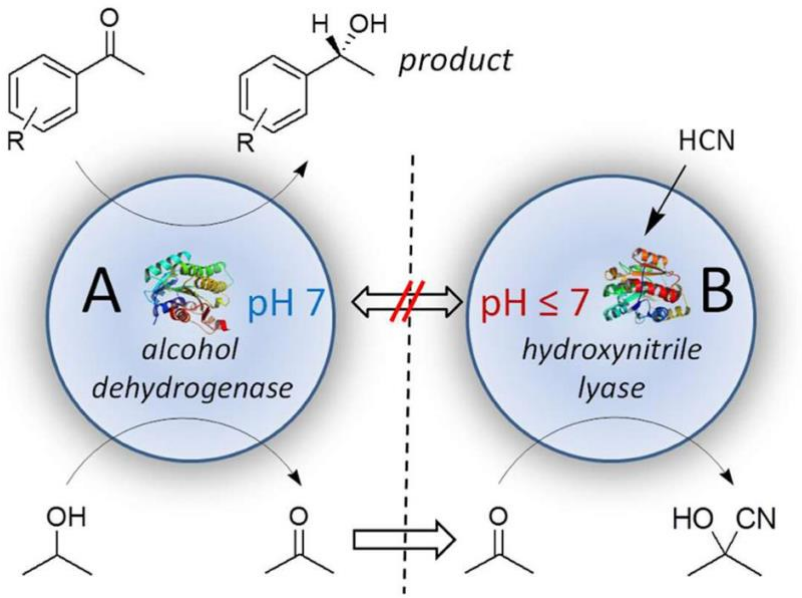


Figure 6: Compartmentalized cascade reaction of an alcohol dehydrogenase and a hydroxynitrile lyase with incompatible pH requirements. The entrapment of the alcohol dehydrogenase was performed at neutral pH, while the hydroxynitrile lyase has to be kept in an acidic environment. All reactants enter and leave the compartments via diffusion through the external solvent. Figure from Uhrich and von Langermann[69] (Creative Commons license type BY).

Polymersomes have been studied for their application as nanoreactors for enzymatic cascade reactions in recent years.[72-74] Typically, polymersomal membranes have a much higher mechanical stability and significantly lower permeability compared to natural phospholipid membranes, which is mainly due to the difference in thickness[75] (up to 40 nm[76] versus 3 – 5 nm[77]). The high diffusional barrier of polymersomal membranes is of critical importance to the implementation of a selective mass transport since it limits the uncontrolled diffusion of compounds over the compartment boundaries. Three different strategies have been described so far to tailor the mass transport of molecules into and out of polymersomes: (1) the integration of channels into the membrane (natural[78-81] or engineered[82-84] channel proteins or even DNA channels[85]), (2) the use of porous membranes[72, 86-88] and (3) the application of stimulus-responsive membranes that swell or shrink in the presence or absence of an external stimulus[74, 89-90].

The permeability of porous and stimulus-responsive membranes is an intrinsic property of the employed polymer and the resulting selectivity is based on size-exclusion with a hardly adjustable molecular mass cut-off. Although exact size exclusion limits have not been determined so far, porous and stimulus-responsive membranes have been shown to facilitate the diffusion of molecules with molecular masses between 515 – 740 Da, at the least.[74, 91] As reactants involved in enzymatic reactions are often significantly smaller, these types of polymersomes have been predominantly used for (model) cascade reactions without incompatibilities[74, 88, 91-92] or with incompatibilities mediated by macromolecules, such as the protection of enzymes from proteolytic degradation[93]. In contrast, with the ability to embed natural or engineered membrane protein channels or DNA nanopores into polymer membranes, a highly selective mass transport across the compartment boundaries can be introduced on different selectivity measures. Thus, this technique has the potential for a spatial segregation on a low molecular mass level. However, also in this case the proof of principle is still missing.

2. Scope and objectives

The central aim of the research conducted during this habilitation was the development of platform technologies that help to overcome common drawbacks of the application of whole cells and isolated enzymes in one-pot multi-enzyme syntheses.

Although many exciting new techniques have been added to the toolbox of chemists and biotechnologists in recent years, such as methods for the *in situ* immobilization of enzymes and the compartmentalization of reactions, the current approaches are far from being optimal. For example, the available methods for the *in situ* immobilization of enzymes are either associated with the synthesis of a large amount of enzymatically non-active scaffold structures, as in the case of enzyme-coated PHA granules, or with a strong activity loss that is typically observed with catalytically active inclusion bodies.[12] Significantly reduced enzymatic activities have also been reported for bacterial surface display systems, which have the additional disadvantages that side reactions can be mediated by the host cell metabolism.[26] As an alternative, metabolically inactive spores with surface-exposed enzymes can be created, but they show very low display efficiencies.[28] Thus, **one objective of this habilitation thesis was the development of a new *in situ* immobilization strategy for enzymes that avoids these disadvantages.** This innovative strategy should be based on the immobilization of enzymes in cellular envelopes that can be generated by the expression of the lysis gene E from the phage PhiX 174.[94] Chapter 3 deals with the development of a suitable production process for biocatalytically active cellular envelopes and their thorough characterization.

The following chapter 4 describes the development of another platform technology that is applicable in the context of multi-enzyme syntheses. Since the incompatibility between individual reaction steps is one of the most critical hurdles that can be encountered during the implementation of concurrent cascade reactions, efficient methods for the spatial separation of enzymatic reactions on a low molecular mass level are urgently needed. Since effective compartmentalization cannot be achieved by the cellular envelope technology, a completely different technological basis was needed for this purpose.

A second objective of this habilitation thesis was the development of nano-scale enzyme membrane reactors (nano-EMRs) that mimic the two main organizational principles of living cells: compartmentalization and selective mass transport. Figure 7 shows a schematic drawing of the envisioned nanoreactors with encapsulated enzymes, embedded channel proteins for a selective mass transport and immobilized enzymes on the vesicle surface.

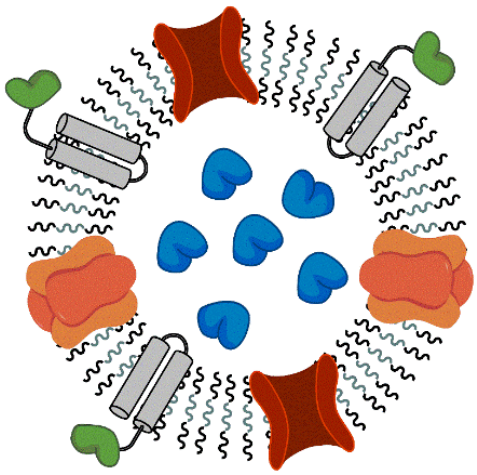


Figure 7: Schematic representation of a nano-scale enzyme membrane reactor with encapsulated enzymes (blue), embedded transport proteins (red and orange) and surface-displayed enzymes (green).

To establish such nano-EMRs as platform technology in biocatalysis, several prerequisites have to be met. Firstly, a fast, reproducible, and scalable production process for the nano-EMRs is needed. Therefore, a new method for the generation of polymer vesicles in stirred-tank reactors should be implemented and the produced vesicles should be characterized in detail. Secondly, a toolbox of membrane proteins for the tailored mass transport of compounds with diverse physicochemical properties across the polymer membrane must be available. Thus, a set of different membrane proteins should be recombinantly produced and characterized regarding their transport characteristics in the artificial membrane. Thirdly, a facile method for the immobilization of enzymes on the vesicle surface must be available. By this means, the bulk volume surrounding the vesicles can be used as additional reaction space. Compared to adding soluble enzymes to the outer reaction space, their immobilization on the vesicle surface results in a single biocatalytic entity, which can be easily

recovered and reused. Lastly, the acquired knowledge should be used for the implementation of a compartmentalized reaction system for the synthesis of CMP-*N*-acetylneuraminic acid (CMP-NANA) in nano-EMRs. This linear three-step cascade reaction suffers from a strong cross-inhibition between the first and the third reaction step caused by the small molecule cytidine triphosphate (CTP, molecular mass 483 g mol^{-1}), which should be abolished by the nano-EMR technology.

3. Research summary: *In situ* immobilization of enzymes in cellular envelopes

Cellular envelopes, which are also referred to as “bacterial ghosts”, can be obtained by the expression of the lysis gene E from the phage PhiX174 in proliferating Gram-negative cells. Although the lysis gene E has been identified more than 50 years ago[95], the molecular basis of its action is still not completely understood and several theories have been proposed to explain the ability of the encoded protein E to effect lysis in the absence of muralytic activity.[96] As summarized in a recent review by Chamakura and Young[96], these hypotheses range from an interference with the peptidoglycan biosynthesis, which is currently the model with the strongest experimental evidence, over an activation of unspecified autolytic functions to the formation of a multimeric transmembrane tunnel. The latter mechanism has been proposed by researchers around Werner Lubitz, who pioneered in the field of biotechnological application of protein E for the generation of vaccines.[97] According to this transmembrane tunnel model, protein E inserts into the cytoplasmic membrane, where a conformational change is induced that leads to the fusion of the inner and outer membranes, the multimerization of protein E molecules and, finally, to the formation of a single lysis pore with 40 to 200 nm in diameter.[98]

Independent of the concrete mode of action of protein E, the cytoplasm is released upon lysis due to the difference in osmotic pressure between the cell interior and the surrounding medium and an empty cellular envelope is retained. The remaining shell is ideally suited to serve as carrier for immobilized enzymes, because proteins that are attached to the phospholipid membranes prior to lysis remain in the cellular envelopes.[94, 99]

In this chapter, a summary of the conducted research in the field of *in situ* enzyme immobilization in cellular envelopes is given. This summary is based on the following peer-reviewed publications, in which more detailed results and comprehensive information about the employed materials, methods, and devices can be found:

[KC1] Hölsch K, Sührer I, Heusel M, Weuster-Botz D (2013): Engineering of formate dehydrogenase: Synergistic effect of mutations affecting cofactor specificity and chemical stability. Appl Microbiol Biotechnol 97: 2473-2481.

[KC2] Fu Y, Castiglione K, Weuster-Botz D (2013): Comparative characterization of novel ene-reductases from cyanobacteria. Biotechnol Bioeng 110: 1293-1301.

[KC3] Sührer I, Haslbeck M, Castiglione K (2014): Asymmetric synthesis of a fluoxetine precursor with an artificial fusion protein of a ketoreductase and a formate dehydrogenase. Proc Biochem 49: 1527-1532.

[KC4] Sührer I, Langemann T, Lubitz W, Weuster-Botz D, Castiglione K (2015): A novel one-step expression and immobilization method for the production of biocatalytic preparations. Microb Cell Fact, 14: 180-189.

[KC5] Castiglione K, Fu Y, Polte I, Leupold S, Meo A, Weuster-Botz D (2017): Asymmetric whole-cell bioreduction of (*R*)-carvone by recombinant *Escherichia coli* with *in situ* substrate supply and product removal. Biochem Eng J 117: 102-111.

3.1 Process development and *in situ* immobilization of a β-galactosidase[a]

So far, most studies investigating the formation of cellular envelopes have been dealing with the production of vaccines for the immunization against pathogenic Gram-negative bacteria since the cellular envelopes have the same morphology (including all cell surface structures) as whole cells and can be additionally decorated with recombinantly expressed antigens.[99]

[a] The results presented in sections 3.1 and 3.2 are part of the dissertation of Ilka Sührer. I conceived and co-supervised the project that was funded by the German Research Foundation (DFG, grant no. WE 2715/12-1).

To make this technology applicable in biotransformations, several challenges had to be addressed. First of all, the enzymes had to be immobilized in active form. This was achieved by genetic fusion of the target enzymes to (artificial) membrane anchors tethering them to the cytoplasmic membrane. As an example, the β-galactosidase from *E. coli* K12 was fused to the membrane anchoring domain of cytochrome b_5 from rabbit liver. Thus, the enzymes were not expelled with the soluble components of the cytoplasm and cellular envelopes with immobilized biocatalysts could be produced as illustrated in Figure 8.

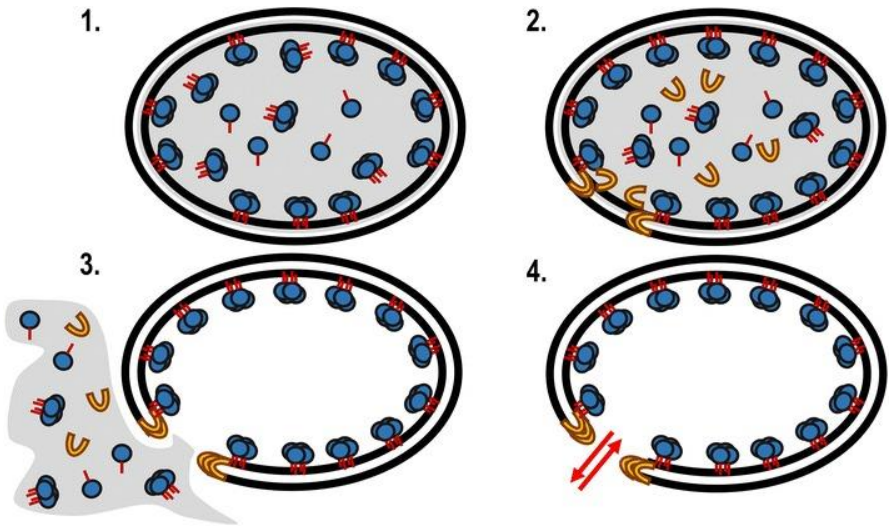


Figure 8: Schematic depiction of the *in situ* enzyme immobilization in cellular envelopes. The graphical representation of the lysis procedure is based on the transmembrane tunnel model.[98] 1. Expression of the enzymes of interest fused to artificial membrane anchors and spontaneous insertion into the cytoplasmic membrane. Multimerization, as in the case of the tetrameric β-galactosidase, is not hampered by the anchoring domain. 2. Expression of the gene encoding the lytic phage protein E, which inserts into the cell membranes. 3. Pore formation by protein E and lysis with release of the cytoplasm. 4. Cellular envelope with immobilized enzymes and a single lysis pore. An improved mass transfer into cellular envelopes (compared to whole cells) is indicated by the red arrows. Figure from Sührer *et al.*[KC4] (Creative Commons license type BY).

Secondly, the vitality of the cells had to be preserved during the protein production since only dividing cells can be lysed efficiently.[99] Therefore, all cultivations were performed in stirred-tank reactors to ensure a sufficient oxygen supply throughout the process. Whereas recombinantly expressed antigens in cellular envelopes reach their full immunogenic potential already in tiny amounts, the production of cellular envelopes with a high number of immobilized enzymes, which is a prerequisite to reach high catalytic activities, is challenging due to the negative effect of the metabolic burden onto the cell vitality. Therefore, two vectors with differing copy numbers as well as batch and fed-batch processes were compared to find the best expression conditions for the β-galactosidase. Figure 9 gives an overview of the employed four-step production processes.

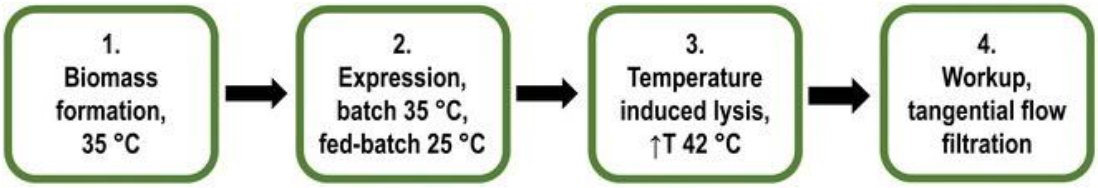


Figure 9: Overview of the production of cellular envelopes in batch or fed-batch processes. Figure from Sührer *et al.*[KC4] (Creative Commons license type BY).

The biomass formation was always performed in batch mode, whereas expression of the target protein took place with or without feeding of glucose. The expression of the β-galactosidase was induced by the addition of the chemical inducer isopropyl-β-D-1-thiogalactopyranoside (IPTG). In contrast, the expression of the lysis gene E was tightly controlled by the rightward phage λ pR promoter and the corresponding temperature-sensitive repressor cI857, which gets inactivated at temperatures above 37°C.[100] Thus, the lysis was induced not earlier than after a temperature shift to 42°C.

It was possible to obtain successful overexpression of the β-galactosidase and lysis via protein E (> 99.0 % lysed cells) in all experimental setups with low concentrations (≤ 0.1 mM) of IPTG. The highest specific activity was detected in cellular envelopes that were produced in batch mode with a high copy number plasmid. In this case, 27,200 ± 10,460 enzyme molecules were immobilized per cellular envelope with an activity of 753 ± 190 U/$g_{dry\ weight}$. The specific activity of the membrane bound β-galactosidase was 57 % higher than of the purified β-galactosidase with N-terminal

hexahistidine-tag, which might be due to a stabilization of the catalytically active enzyme tetramer via the membrane attachment. A comparison to whole cells showed that the mass transfer limitation for the β-galactosidase substrate ortho-nitrophenyl-β-galactoside was significantly reduced by the lysis pore resulting in a 3-fold higher activity of the cellular envelopes.[KC4]

3.2 Co-immobilization of a two-enzyme system

The limited available space within the cytoplasmic membrane could be a critical factor for the production of cellular envelopes with high enzymatic activities – especially if several different enzymes should be co-immobilized. The immobilization of fusion proteins would be space-saving since more than one catalytic entity could be anchored within the smallest possible membrane area. To test this hypothesis, a fusion protein of a ketoreductase (KR) from *Synechococcus* PCC 7942 and a formate dehydrogenase (FDH) from *Mycobacterium vaccae* N10 was constructed. Because the KR has an absolute requirement for NADPH as cofactor, a $NADP^+$-accepting mutant of the FDH was used that had been designed in an earlier study.[KC1] This fusion protein performed equal or even better than the free enzymes in the asymmetric reduction of the prochiral ketone ethylbenzoyl acetate (EBA) (Figure 10), due to a lowered half-saturation constant ($K_{m,EBA}$) of the KR subunit.[KC3]

Unfortunately, it was not possible to immobilize the fusion protein in functional form in the cytoplasmic membrane of *E. coli* cells because the anchoring in both possible orientations resulted in a complete loss of activity of one of the enzymatic domains. Thus, other approaches for the maximization of the number of immobilized enzymes within cellular envelopes are currently under investigation.[b] However, both enzymes could be successfully co-immobilized within cellular envelopes as separate moieties demonstrating the general applicability of the cellular envelope technology for the co-immobilization of enzymes involved in cascade reactions.

[b] This is subject of the ongoing doctoral studies of Ingmar Polte, which are funded by the DFG (grant no. WE 2715/12-2).

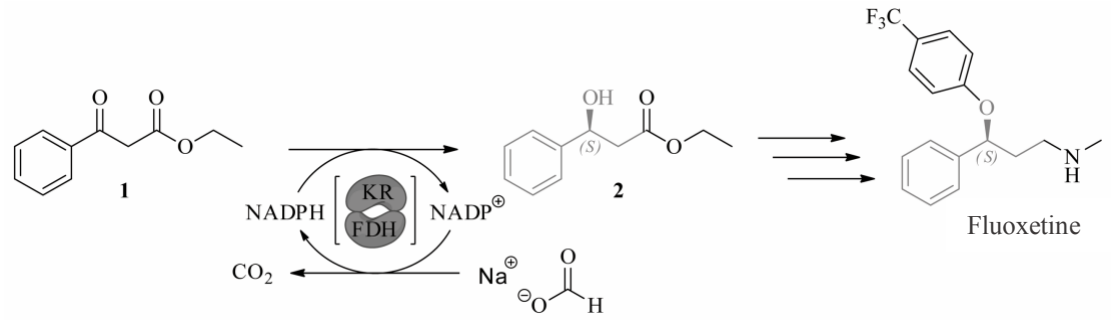


Figure 10: Reaction scheme of the asymmetric reduction of ethylbenzoyl acetate **1** to the chiral alcohol ethyl-(*S*)-3-hydroxy-3-phenylpropanoate **2** by a fusion protein composed of a ketoreductase (KR) and a cofactor-regenerating formate dehydrogenase (FDH). The formed alcohol is a precursor for the synthesis of the antidepressant fluoxetine. The final product moiety originating from the chiral alcohol is highlighted in gray. Modified figure according to Sührer *et al.*[KC3]

3.3 Avoidance of undesired side-reactions[c]

In a next step, the capability of the cellular envelope technology to reduce undesired side-reactions catalyzed by soluble host cell enzymes was investigated. As an exemplary reaction system, the production of the chiral building block (2*R*,5*R*)-dihydrocarvone by asymmetric reduction of the C,C-double bond of (*R*)-carvone was studied. The orthogonal cascade reaction involved an ene reductase from *Nostoc* PCC 7120 (NostocER1) and the above mentioned $NADP^+$-accepting FDH mutant (see section 3.2). The reaction scheme is depicted in Figure 11.

[c] The results presented in this chapter are part of the dissertation of Yilei Fu, where I had the role as co-supervisor, and the Master's thesis of Ingmar Polte, where I acted as project designer and co-supervisor.

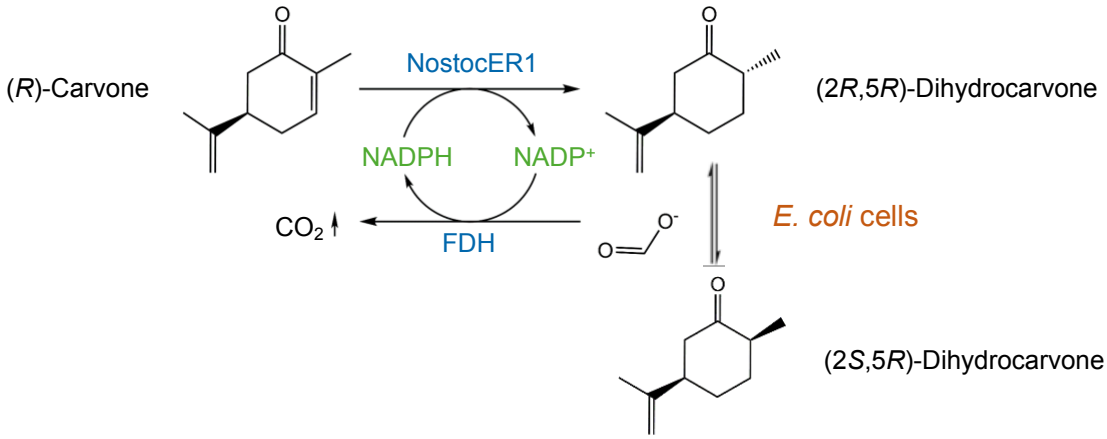


Figure 11: Enzymatic synthesis of (2*R*,5*R*)-dihydrocarvone using an ene reductase from *Nostoc* PCC 7120 (NostocER1) for the reduction of the C,C-double bond and a formate dehydrogenase (FDH) for cofactor regeneration. The undesired isomerization of the product to the diastereomer (2*S*,5*R*)-dihydrocarvone by the *E. coli* cells is also shown.

If recombinant whole *E. coli* cells overexpressing NostocER1 and FDH were employed for the biotransformation of 50 mM (*R*)-carvone, a host cell-mediated product isomerization was observed resulting in a low diastereomeric excess (*de*) of 81.7 % after 5 h reaction time. In contrast, the isolated NostocER1 synthesized the product with high optical purity (*de* > 97 %).[KC2] In addition, the substrate was toxic for the cells leading to a low conversion of 27.2 %. Further investigations of the toxic effect of (*R*)-carvone revealed that the substrate concentration in the aqueous phase should be kept below 2 mM during the biotransformations to avoid negative effects on the biocatalyst. Thus, the biotransformation was greatly improved by *in situ* substrate feeding and product removal (SFPR) using polymeric adsorbent resins of the XAD series or water-immiscible ionic liquids. The concentrations of substrate and product in the aqueous phase were most effectively lowered by using the adsorbent resin XAD4 at a resin to substrate mass ratio of 5 or 20 % (v/v) of the ionic liquid 1-hexyl-1-methylpyrrolidiniumbis(trifluoromethylsulfonyl)imide ([HMPL][NTF]). The distribution coefficients of (*R*)-carvone and dihydrocarvone between [HMPL][NTF] and sodium phosphate buffer (100 mM, pH 7.0) were determined as 437 and 933, respectively. Thus, the substrate loading in the liquid-liquid system was restricted to

~ 200 mM[d] to keep the (*R*)-carvone concentration in the aqueous phase below the toxicity limit of 2 mM. A significant increase of the substrate loading would have only been possible with a concomitant change of the volume fraction of [HMPL][NTF], which might exert negative effects on the membrane integrity of the biocatalyst and will be evaluated in future studies. Due to the higher substrate loadings, higher space time yields were achieved with the solid-liquid system. Under optimized conditions (300 mM (*R*)-carvone, 400 mM formate, 36 g L^{-1} biocatalyst, XAD4 at a resin to substrate mass ratio of 5, 300 mM phosphate buffer, pH 6.3), (2*R*,5*R*)-dihydrocarvone was obtained with 96.5% *de* and 96.8% conversion within 9 h. From these values it can be inferred that the yield and the stereoselectivity of the biotransformation were dramatically enhanced by the SFPR technique, but the optical purity of the product was still not perfect.[KC5]

Since there was experimental evidence that the undesired isomerization of (2*R*,5*R*)-dihydrocarvone to (2*S*,5*R*)-dihydrocarvone was catalyzed by soluble components of the cytoplasm, cellular envelopes with immobilized NostocER1 and FDH were produced. The cellular envelopes displayed both NostocER1 and FDH activity and the (2*R*,5*R*)-dihydrocarvone isomerization rates were reduced up to 77 % in comparison to whole cells. Detailed investigations of the effectiveness of the cell lysis and the work-up procedure are currently ongoing to identify the root-cause of the remaining isomerization activity.[e] Nevertheless, these first results demonstrated clearly that the cellular envelope technology is suitable to reduce undesired side-reactions mediated by the host cell metabolism by the removal of cytoplasmic enzymes.

[d] This value refers to the volume of the aqueous phase.

[e] This is subject of the ongoing doctoral studies of Ingmar Polte, which are funded by the DFG (grant no. WE 2715/12-2).

4. Research summary: Development of nano-scale enzyme membrane reactors[f]

Notwithstanding the advantages of the cellular envelope technology, the bacterial shells are not suitable for the construction of compartmentalized reaction systems. Since the implementation of artificial reaction compartments with tunable mass transfer across the membrane is of high interest for biocatalytic cascade reactions with incompatible steps, nano-scale enzyme membrane reactors (nano-EMRs) were developed using polymersomes as chassis. This development was structured in four parts: vesicle production and characterization, membrane functionalization, surface functionalization, and, finally, application to an exemplary cascade reaction.

The amphiphilic polymer that was used for the vesicle formation in all sub-projects was the ABA-type triblock copolymer poly(2-methyloxazoline)$_{15}$-poly(dimethyl-siloxane)$_{68}$-poly(2-methyloxazoline)$_{15}$ (PMOXA$_{15}$-PDMS$_{68}$-PMOXA$_{15}$, shown in Figure 12).

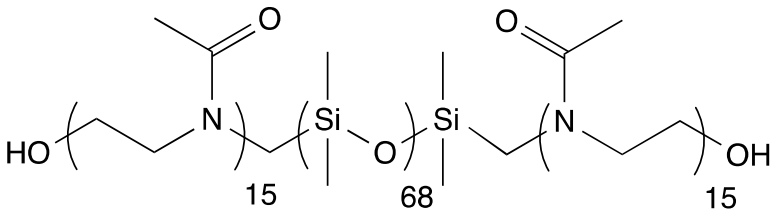


Figure 12: Chemical structure of the poly(2-methyloxazoline)$_{15}$-poly(dimethyl-siloxane)$_{68}$-poly(2-methyloxazoline)$_{15}$ (PMOXA$_{15}$-PDMS$_{68}$-PMOXA$_{15}$) polymer that was used for the production of nano-scale enzyme membrane reactors.

[f] The results presented in this chapter are part of the dissertations of Sarah Poschenrieder, Tom Schwarzer and Ludwig Klermund. They were members of my junior research group working on the development of "Synthetic reaction compartments for multi-enzyme syntheses" (BMBF grant no. 031A178). For all three doctoral studies, I designed and supervised the research projects.

This polymer was chosen since the functional integration of membrane proteins into PDMS-based membranes is favored by the high flexibility and fluidity of the polymers[101] and there have been several reports on the successful integration of proteins into membranes of this type.[78-83]

This research summary is based on the following peer-reviewed publications, in which more detailed results and comprehensive information about the employed materials, methods, and devices can be found:

[KC6] Klermund L, Groher A, Castiglione K (2013): New *N*-acyl-D-glucosamine 2-epimerases from cyanobacteria with high activity in the absence of ATP and low inhibition by pyruvate. J Biotechnol 168: 256-263.

[KC7] Klermund L, Riederer A, Groher A, Castiglione K (2015): High-level soluble expression of a bacterial *N*-acyl-D-glucosamine 2-epimerase in recombinant *Escherichia coli*. Protein Expr Purif 111: 36-41.

[KC8] Klermund L, Riederer A, Hunger A, Castiglione K (2016): Protein engineering of a bacterial *N*-acyl-D-glucosamine 2-epimerase for improved stability under process conditions. Enzyme Microb Technol 87-88: 70-78.

[KC9] Poschenrieder ST, Wagner S, Castiglione K (2016): Efficient production of uniform nanometer-sized polymer vesicles in stirred-tank reactors, J Appl Polym Sci 133: 43274.

[KC10] Klermund L, Poschenrieder ST, Castiglione K (2016): Simple surface functionalization of polymersomes using non-antibacterial peptide anchors. J Nanobiotechnol 14:48.

[KC11] Poschenrieder ST, Schiebel SK, Castiglione K (2017): Polymersomes for biotechnological applications: large-scale production of nano-scale vesicles. Eng Life Sci 17: 58-70.

[KC12] Poschenrieder ST, Klermund L, Langer B, Castiglione K (2017): Determination of permeability coefficients of polymersomal membranes for hydrophilic molecules. Langmuir 33: 6011-6020.

[KC13] Schwarzer TS, Hermann M, Krishnan S, Simmel FC, Castiglione K (2017): Preparative refolding of small monomeric outer membrane proteins. Prot Expres Purif 132: 171-181.

[KC14] Klermund L, Poschenrieder TS, Castiglione K (2017): Biocatalysis in polymersomes: Improving multienzyme cascades with incompatible reaction steps by compartmentalization. ACS Catal 7: 3900-3904.

[KC15] Poschenrieder ST, Hanzlik M, Castiglione K (2018): Polymersome formation mechanism and formation rate in stirred-tank reactors. J Appl Polym Sci 135: 46077.

[KC16] Poschenrieder ST, Schiebel SK, Castiglione K (2018): Stability of polymersomes with focus on their use as nanoreactors. Eng Life Sci 18: 101-113.

[KC17] Schwarzer TS, Klermund L, Wang G, Castiglione K (2018): Membrane functionalization of polymersomes: alleviating mass transport limitations by integrating multiple selective membrane transporters for the diffusion of chemically diverse molecules. Nanotechnology 29:44LT01

4.1 Vesicle production and characterization

Firstly, an efficient and scalable production process for the polymersomes was established in miniaturized stirred-tank reactors. Driving an S-shaped stirrer[102] at its maximum rotational speed (4,000 min^{-1}) in unbaffled reactors led to a monomodal particle size distribution with a low polydispersity index (PDI < 0.2). Vesicles with a number-based mean diameter of ca. 100 nm, as determined by dynamic light scattering measurements, were generated in less than 1 h in a single production step. The developed process was robust with respect to changes in temperature (8 – 40°C), pH (5 – 8) and buffer molarity (0 – 100 mM). Cryo-transmission electron microscopy (cryo-TEM) images revealed that the polymersomal membranes had a thickness of 14 nm. Moreover, the aggregation number, which denotes the mean number of polymer chains per vesicle, was determined by static light scattering as 43,000.[KC9]

The developed production process was successfully transferred from the milliliter-scale (12 mL) to the liter-scale (1.5 L). Standard propeller stirrers with a dimensionless diameter (d D^{-1})[g] larger than 0.65 led to the desired narrow particle size distribution if they were driven in an unbaffled stirred-tank reactor when providing a Froude number[h] of Fr= 6.52 at the same time.[KC11]

In further investigations, the self-assembly mechanism of the amphiphilic co-polymers in stirred-tank reactors was elucidated on the basis of TEM images. The results revealed two simultaneously occurring pathways as illustrated in Figure 13.

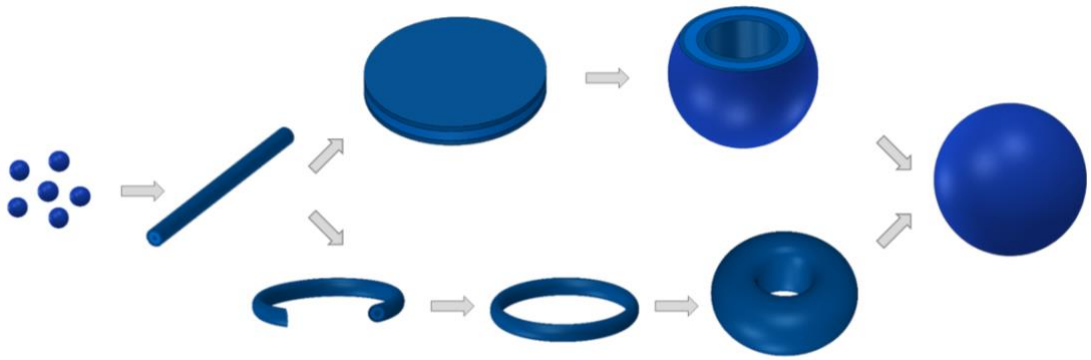


Figure 13: Polymersome formation pathways in stirred-tank reactors. At the beginning of the process, spherical micelles were formed, which then fused to worm-like micelles. Subsequently, two simultaneously occurring pathways were found, which involved either basket- or donut-like structures as intermediates. Figure from Poschenrieder *et al.*[KC15] (reproduced with permission from John Wiley & Sons).

Since a low intrinsic permeability of the polymer membrane is of vital importance to the establishment of a selective mass transfer over the compartment boundaries via incorporated transport proteins, the permeability coefficients for diverse molecules were determined. For this purpose, a new assay was developed that is based on the diffusion of compounds into empty vesicles, which is illustrated in Figure 14.

[g] d: stirrer diameter (m), D: reactor diameter (m)

[h] Fr = $d*n^2/g$ with d: stirrer diameter (m), n: rotational speed of the stirrer (s^{-1}), g: gravitational force (m s^{-2})

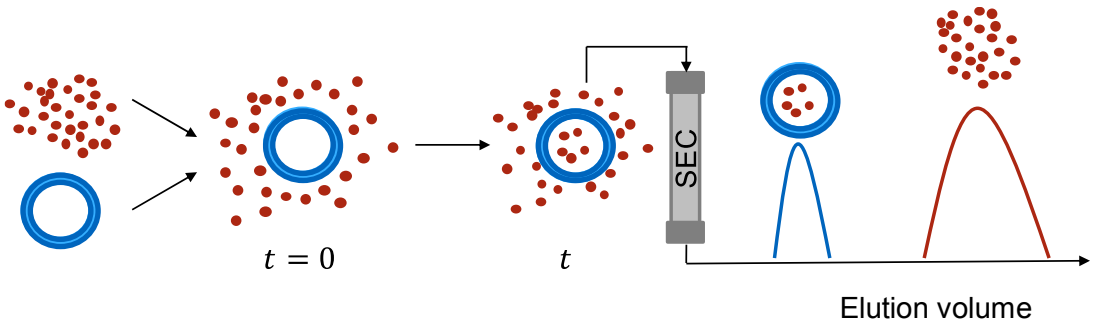


Figure 14: Schematic overview of the developed influx assay. Empty vesicles are mixed with the compound of interest at t = 0. During the incubation period, the molecules diffuse across the membrane. After certain time intervals (t), the polymersomes are separated from the surrounding molecules by fast size exclusion chromatography (SEC) employing 2.5 mL columns. Subsequently, the vesicles are lysed and the concentration of the compound of interest is determined using suitable analytical techniques (not shown).

The influx assay is applicable to hydrophilic molecules without significant membrane retention such as carboxy acids, nucleotides or sugars. Using this method, permeability coefficients as low as 1.9×10^{-14} cm s^{-1} could be measured. For all investigated compounds, the determined permeability coefficients of $PMOXA_{15}$−$PDMS_{68}$− $PMOXA_{15}$ membranes were at least 2.5 orders of magnitude lower than the corresponding permeability coefficients reported for artificial liposomal membranes. These results demonstrate that polymersomal membranes are ideally suited for the implementation of nano-EMRs with selective permeability, since there is only minimal passive diffusion of compounds into and out of the vesicles.[KC12]

A detailed investigation of the stability of the formed polymersomes revealed that the vesicles are resistant towards the mechanical stress typically prevailing in stirred tank reactors. A maximum local energy dissipation of up to 1.23 W kg^{-1} (corresponding to a stirrer speed of 1,000 min^{-1} in the employed miniaturized stirred tank reactors) did not lead to a detectable disintegration of the vesicles. Nevertheless, almost 7 % release of the encapsulated model dye calcein was determined at 25°C within 48 h. The occurrence of local membrane defects is the most likely explanation for this observation. [KC16]

Moreover, the stability of the polymersomes in biphasic liquid-liquid reaction systems was investigated. The encapsulant retention in the presence of organic solvents and ionic liquids was low, indicating that the polymersomes in their current form are not well suited for the application in biphasic reaction systems.[KC16] A possible approach to improve the vesicle stability in the presence of solvents is the generation of polymersomes with cross-linkable membrane, which is currently under investigation.[i]

4.2 Membrane functionalization

For the establishment of a toolbox of selective transport proteins, eight different membrane proteins were characterized regarding their functional integration into the polymer vesicles and their transport characteristics in this artificial environment. These transporters comprise the unspecific pore OmpF from *E. coli* and its G119D mutant, which has a 50 % lower molecular mass cut-off than the wildtype (~600 Da vs. ~300 Da).[103] Since many substrates and products involved in biotransformations are hydrophobic, four transporters mediating the improved mass transfer of hydrophobic compounds were characterized: the transporters AlkL, OprG and TodX from *Pseudomonas* species as well as OmpW from *E. coli*. These membrane proteins were selected because they had already been recombinantly expressed and purified. Nevertheless, the spectra of compounds that are transported by these proteins were still largely unexplored. In addition, two anion-selective channels originating from *E. coli* were integrated into the polymer membranes: the moderately selective protein PhoE and the specific formate transporter FocA.

A fundamental prerequisite for the functionalization of the polymer membranes with selective transporters is the preparative production of the involved membrane proteins. The unspecific and anion-selective porins, i.e. OmpF, OmpF G119D, PhoE, and FocA, which are oligomeric, were recombinantly produced in *E. coli* in their native form. In contrast, the structurally less complex small monomeric outer membrane proteins AlkL, OprG, TodX, and OmpW were produced as inclusion bodies and then refolded into their native conformation. For each of these four monomeric proteins, the refolding conditions were optimized by studying the effect of different detergents,

[i] This is subject of the ongoing doctoral studies of Florian Golombek, which are funded by the BMBF (grant no. 031B0221).

detergent concentrations and folding additives on the folding efficiency. The preparative folding was performed at a final protein concentration of 0.5 g L^{-1} in miniaturized stirred-tank reactors equipped with a liquid handling system. Under these conditions, an almost complete folding of OprG (96 %) was observed. Very good results were also obtained with AlkL (84 % folding) and OmpW (71 % folding), whereas only a moderate folding efficiency of 52 % was determined for TodX.[KC13]

The purified membrane proteins were used to alleviate mass transport limitations of molecules into and out of the nanoreactors. The above-mentioned fusion protein composed of a ketoreductase (KR) and a formate dehydrogenase (FDH) (see section 3.2) was encapsulated in the vesicle lumen. This ensured that the enzymatic activities of FDH and KR were balanced in each vesicle. Moreover, the cofactor $NADP^+$ was also encapsulated in high concentration. With a molecular mass of 744 g mol^{-1}, $NADP^+$ cannot diffuse through any of the studied porins and was therefore successfully retained within the polymersome lumen. As a model reaction, the asymmetric reduction of pentafluoroacetophenone (PFAP) to (*S*)-pentafluoro-phenyl ethanol ((S)-PFE) was studied (Figure 15).

For the transport of the hydrophobic substrate and product of the KR, the membrane proteins AlkL, OmpW, OprG or TodX were employed. Formate was transported by OmpF, PhoE or FocA. Among these seven transporters, AlkL showed the highest integration efficiency. On average, 39 % of the added AlkL molecules integrated into the membrane. Under the conditions tested, a maximum of 120 AlkL transporters per polymersome was detected. The highest channel-specific effects on the mass transfer were achieved using TodX and PhoE, respectively. The combination of both proteins led to an improvement of the space-time yield of the product (*S*)-PFE by 2.32-fold compared to nanoreactors without transport proteins. This moderate improvement of the space-time yield can be attributed to the fact that the encapsulated activity of the fusion protein was low. Therefore, the reaction was usually allowed to proceed for 112 h at 25 °C to generate a detectable signal with the system. Since also very slowly permeating substances will reach the vesicle lumen if the considered time frame is long enough, the passive diffusion of PFAP and formate, for which a permeability coefficient of 6.3 x 10^{-12} cm s^{-1} was determined, led to some background activity.[KC17]

Thus, the augmentation of the encapsulated enzyme activity is of utmost importance to improve the effectiveness of the nano-EMRs.[j]

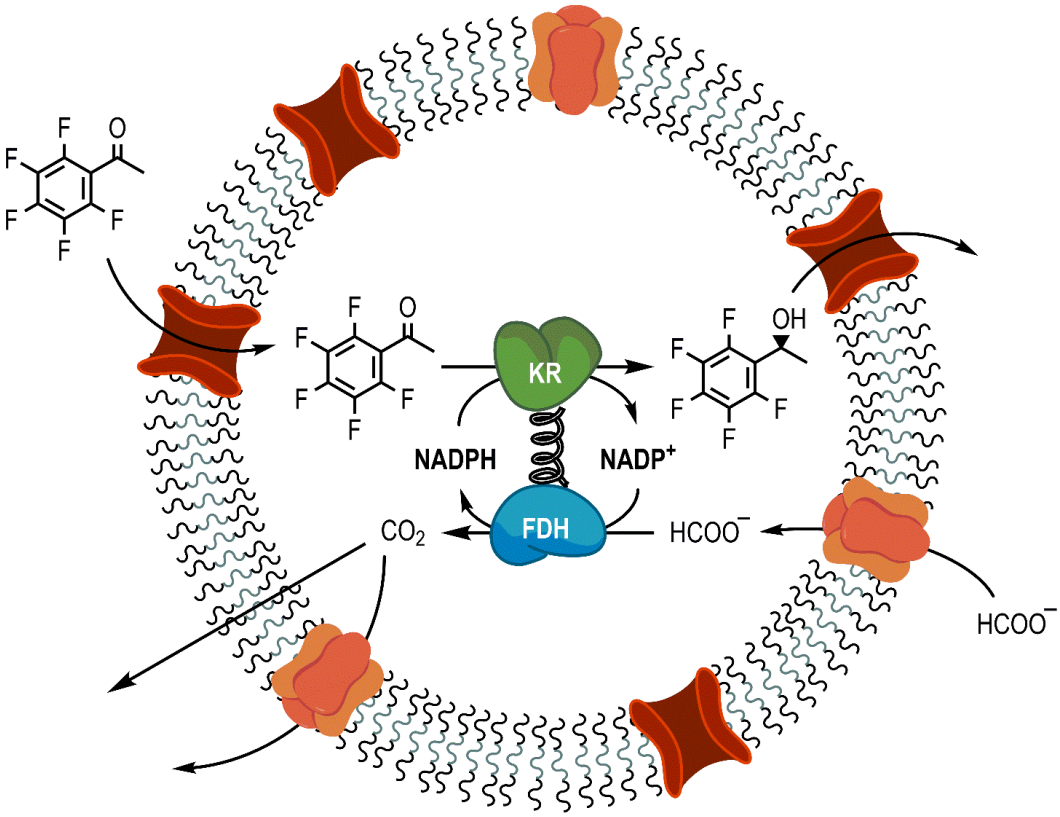


Figure 15: Scheme of the studied orthogonal cascade reaction based on a ketoreductase (KR) and a formate dehydrogenase (FDH) which are encapsulated as a fusion enzyme. The cofactor $NADP^+$ was also encapsulated in the vesicles and cannot diffuse through the employed membrane proteins. The compounds pentafluoroacetophenone, (*S*)-pentafluorophenyl ethanol and formate require different transport proteins to increase their mass transfer into and out of the vesicle lumen, respectively. Modified figure according to Schwarzer *et al.*[KC17]

[j] This is subject of the ongoing doctoral studies of Michael Mertz, which are funded by the BMBF (grant no. 031B0221).

4.3 Surface functionalization

The immobilization of proteins on the surface of polymersomes was investigated to make the bulk volume surrounding the vesicles available as additional reaction space. Compared to adding enzymes to the outer reaction space in solution, their immobilization on the vesicle surface results in a single biocatalytic unit and facilitates an easy biocatalyst recovery and reuse. For this purpose, the proteins of interest were fused to hydrophobic, non-antibacterial peptide anchors such as the transmembrane domain of cytochrome b_5 from rabbit liver, which had also been employed in the production of biocatalytically active cellular envelopes (see section 3.1).

For the initial characterization of this immobilization strategy, enhanced green fluorescent protein (eGFP) was used. Several eGFP-fusion proteins equipped with different anchoring domains were capable of spontaneously inserting into the preformed polymeric membrane. A detailed characterization of the surface functionalization revealed that the peptide insertion was linearly dependent on the applied protein concentration and possible at a broad temperature range of 4 – 42 °C. Up to 2,320 ± 280 eGFP molecules were immobilized on a single vesicle, which agrees well with the calculated maximum loading capacity of the polymersomes of 2,254 eGFP molecules. This value was estimated based on the highest-density hexagonal packing arrangement of spheres on the vesicle surface. The peptide insertion had no negative effect on the membrane integrity as demonstrated by calcein leakage experiments and the surface-functionalized polymersomes remained stable for at least six weeks.[KC10]

4.4 Application of the nano-EMR technology to an exemplary cascade reaction

As an exemplary cascade reaction suffering from an incompatibility, the three-step enzymatic synthesis of cytidine monophosphate-*N*-acetylneuraminic acid (CMP-NANA) was investigated. CMP-NANA is, *inter alia*, an important building block for human milk oligosaccharides.[104]

In the first reaction step, the amino sugar *N*-acetylglucosamine (GlcNAc) is converted to *N*-acetylmannosamine (ManNAc) by an *N*-acyl-D-glucosamine 2-epimerase (AGE). The majority of the known AGEs are of eukaryotic origin, display only moderate enzymatic activities, and tend to form inclusion bodies in heterologous expression hosts such as *E. coli*. In contrast, we identified a highly active enzyme in the cyanobacterium *Anabaena variabils*[KC6] that can be efficiently produced in *E. coli* via co-expression of the chaperonin system GroEL/GroES. [KC6] For the production of ManNAc in the cascade reaction, a mutant with enhanced operational stability was constructed.[KC8] The formed ManNAc then reacts with pyruvate to *N*-acetylneuraminate (NANA) in an aldol condensation reaction catalyzed by the *N*-acetylneuraminate lyase (NAL) from *E. coli* K12. In the third reaction step, NANA is activated with cytidine triphosphate (CTP) by a CMP-sialic acid synthetase (CSS) from *Neisseria meningitidis*.

The multi-enzyme synthesis in one pot is favored over sequential reaction steps because both the AGE and the NAL reaction exhibit unfavorable reaction equilibria ($K_{eq,AGE}$ = 0.26; $K_{eq,NAL}$ = 2.1 L mol^{-1}). However, the quasi-irreversible CSS reaction drives the cascade reaction to completion. The incompatibility of the implemented system mainly arises from a strong inhibition of the AGE by CTP with an inhibition constant of 1 mM. This incompatibility between reaction steps 1 and 3 essentially requires the spatial separation of the AGE from CTP. Therefore, the AGE was encapsulated together with its allosteric activator adenosine triphosphate (ATP) in the lumen of polymersomes, whereas the NAL and the CSS were immobilized on the surface using hydrophobic peptide anchors. The compartmentalized reaction system is shown in Figure 16.

When the enzymatic synthesis of CMP-NANA was performed with free enzymes, no product formation could be measured. This has two reasons: (1) the mentioned strong inhibition of the AGE by CTP and (2) the low stability of the CSS with a half-life of 1.5 h at 30°C. The stability issue was solved by the immobilization of the CSS on the polymersome surface because the immobilized enzyme had a 24-fold longer half-life. The key element for the spatial separation of the first and the third reaction step was the engineered membrane channel OmpF G119D, which facilitates the passive transport of compounds with molecular masses < 300 g mol^{-1}.[103, 105] Thus, the substrate and product of the AGE reaction (each 220 g mol^{-1}) can penetrate the porin,

while CTP (483 g mol^{-1}) is selectively excluded from the vesicle lumen. As a consequence, the compartmentalized system reached a 2.2-fold higher product concentration than the system with immobilized CSS, but without spatial separation of the incompatible reactions.[KC14]

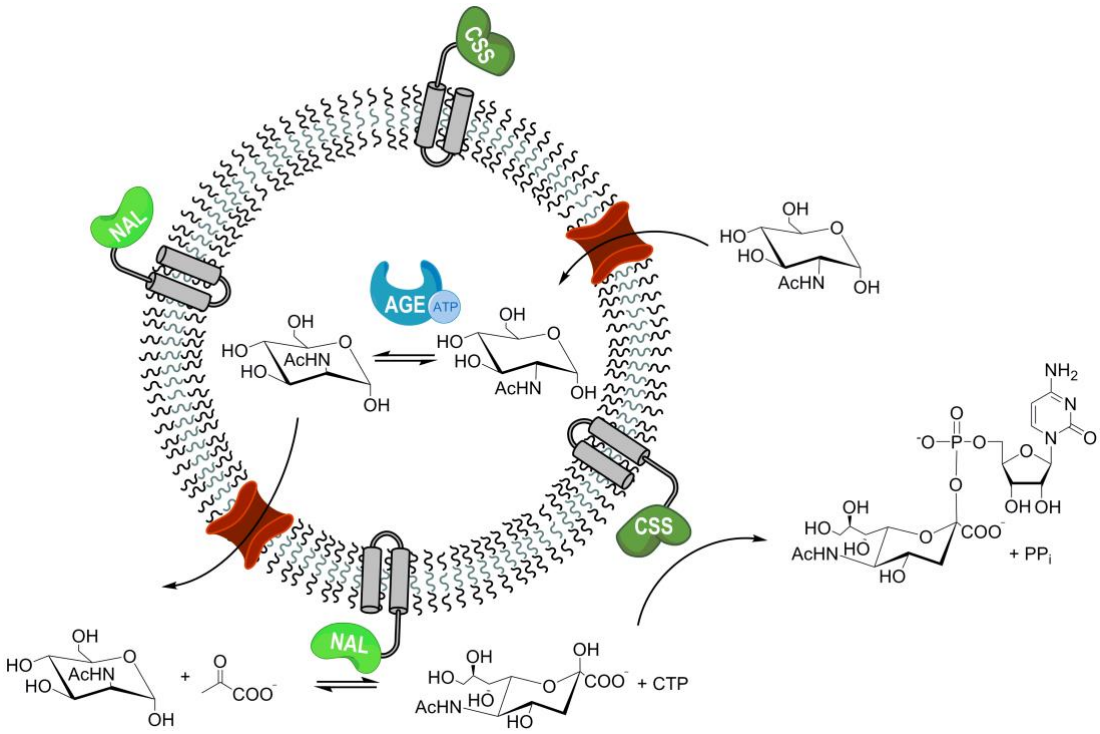


Figure 16: Three-step synthesis of CMP-*N*-acetylneuraminic acid in polymersomes using *N*-acyl-D-glucosamine 2-epimerase (AGE, depicted with its allosteric activator adenosine triphosphate (ATP)), *N*-acetylneuraminate lyase (NAL) and CMP-sialic acid synthetase (CSS). The G119D mutant of the Outer Membrane Protein F (OmpF) from *E. coli* permits selective mass transport (molecular mass cut-off ~ 300 g mol^{-1}).[103, 105] The substrate and product of the AGE reaction (220 g mol^{-1}) diffuse readily, while CTP (483 g mol^{-1}) is excluded from the vesicle lumen. Figure from Klermund *et al*.[KC14] (Reprinted with permission from the American Chemical Society).

5. Discussion

According to the state of the art, currently either whole cell biocatalysts or isolated enzymes are applied in biotransformations. However, depending on the specific characteristics of a given enzyme-catalyzed reaction either choice might represent a bad compromise between cost and efficiency of the bioprocess. Whole cells can pose two main difficulties on biotransformations: mass transfer limitations caused by the cell wall and undesired side-reactions mediated by enzymes of the host cell. Within this work it was demonstrated that the cellular envelope technology is suitable to significantly reduce both of these unwanted effects. Thus, cellular envelopes with immobilized enzymes combine the advantages of cost-intensive isolated enzymes, which do not suffer from mass-transfer limitations and undesired side-reactions, with the low production cost of whole cell biocatalysts.

For an assessment of the performance of this novel *in situ* immobilization strategy, a comparison with alternative methods is necessary. The anchoring of more than 27,000 β-galactosidase molecules within the cytoplasmic membrane of a single cellular envelope lies within the broad reported range of surface-displayed molecules on bacterial cells, which is between 15,000 and 180,000.[34] These numbers are two to three orders of magnitude higher than the values reached with typical spore display systems. For example, the immobilization of only 104 molecules per *Bacillus subtilis* spore of a xylose reductase has been reported.[106] Although the spores have typical dimensions of 1 x 0.5 µm (length x width) and are thus significantly smaller than a bacterial cell, the low number of displayed molecules underpins the identification of the display efficiency as bottleneck of the spore display technology.[28] Taken together, the *in situ* immobilization of enzymes is already competitive when using the newly developed methodology in its current state, but there is still some room for improvement of the molecule number per cellular envelope, which will be addressed in further studies.

A very positive result obtained during the in-depth characterization of the β-galactosidase-containing cellular envelopes was the 57 % higher specific enzyme activity that was observed upon immobilization. A similar effect had been described by George *et al.*, who also determined an increased activity of the membrane-bound β-galactosidase that was fused to the cytochrome b_5 anchoring domain.[107] However,

the enzymatic activity of the β-galactosidase can also be negatively affected by its *in situ* immobilization. For example, in one of the first reports on catalytically active inclusion bodies an activity loss of two thirds of the activity of the soluble enzyme was determined.[108] In further studies it was shown that the activity loss can be avoided by using a proper aggregation-inducing domain for the inclusion body formation. Fusions between the aggregation-prone VP1 capsid protein (VP1LAC) of the foot-and-mouth disease virus and the β-galactosidase resulted in inclusion bodies with 66 % higher activity than the soluble enzyme.[33] These results demonstrate that the ideal *in situ* immobilization approach has to be identified on a case-to-case basis and – if possible – several strategies should be investigated in parallel to find the most suitable one.

The newly developed method for the *in situ* immobilization in cellular envelopes expands the design options for biocatalytic preparations and could also serve as starting point for the simultaneous employment of different immobilization strategies. For example, the concurrent immobilization of enzymes within the cytoplasmic membrane and as catalytically active inclusion bodies in a single cell could lead to powerful biocatalytic preparations with maximized enzymatic activity if these cells are subsequently turned into cellular envelopes. In a recent study by Ehgartner *et al*. it was shown that the formation of inclusion bodies does not hamper the protein E mediated lysis and that the inclusion bodies were effectively retained within the cellular envelopes.[109] The non-interference with the lysis procedure is of vital importance to the success of such hybrid approaches, which will also be subject of further studies.

Whereas *in situ* immobilization strategies represent elegant measures to maximize the cost efficiency of bioprocesses, they are usually not suitable to solve incompatibility issues within cascade reactions. This is the domain of compartmentalization techniques. Within this work, nano-EMRs were created that can be employed as artificial reaction compartments. Polymersomes made from amphiphilic triblock copolymers served as chassis for these nanoreactors.

For the fast, reproducible, and scalable production of the polymersomes a new production process in stirred tank reactors was established. In general, polymersomes can be generated by different methods such as electroformation[70, 110], film rehydration[78, 111], direct dispersion of the polymer powder into water or an

aqueous buffer[112], or by the switch from an organic solvent that is suitable to dissolve the hydrophobic as well as the hydrophilic polymer parts to an aqueous solution[113-114]. Because water is a selective precipitant for the hydrophobic block, the self-assembly of the polymer chains is triggered. Most of these procedures lead to polymersomes with broad particle size distributions and the polydispersity is reduced in additional unit operations, e.g. by repeated extrusion through polycarbonate filters.[115] In contrast, the formation of polymersomes in stirred-tank reactors leads immediately to vesicle dispersions with low polydispersity in a single production step. There are some further examples for one-step production processes, which directly produce uniform polymersomes. All of these procedures involve a solvent switch, typically from ethanol to an aqueous buffer, that is carried out in microfluidic devices[116-119], continuously working micromixers[120-121] or modified inkjet printers[122]. However, solely the newly established production process in stirred tank reactors allows for a vertical scale-up, whereas the other approaches are only (horizontally) scalable by parallelization.

The detailed characterization of the formed vesicles revealed that the high mechanical stability as well as the relatively low intrinsic permeability of the polymer membrane provide an ideal basis for the establishment of nano-EMRs. The measured permeability coefficients for various substances were generally several orders of magnitude lower than the corresponding values of lipid membranes. The novel influx assay that was developed for the determination of the extremely low permeability coefficients of hydrophilic molecules is ideally suited for their accurate determination since it avoids the dilution of the compound of interest. The strong dilution, typically by a factor of 100 or higher, is a major disadvantage of the so-called release assays, which are commonly applied for the determination of permeability coefficients of artificial membranes.[123] These assays rely on the quantification of the concentration of the compound of interest in the bulk phase surrounding the polymersomes that had been loaded with the respective substance. Therefore, compounds with very low permeability should be encapsulated in high concentrations in the vesicles to generate a detectable signal in reasonable time. However, this is often not possible because high concentrations of certain substances can disturb the vesicle formation. Alternative methods for the determination of permeability coefficients are either restricted to certain permeating molecules or involve specialized pulsed field gradient nuclear magnetic resonance (PFG-NMR) measurements.[124-127] The broad

applicability of the influx assay as well as the fact that it does not involve specialized equipment are significant advantages over these methods. On the contrary, permeability coefficients of fast-permeating compounds or hydrophobic substances with significant membrane retention cannot be accurately determined with the influx assay. PFG-NMR measurements would be the method of choice in this case.

For the immobilization of proteins on the vesicle surface similar membrane anchoring domains were used as for the anchoring of enzymes within the cytoplasmic membrane of cellular envelopes. This immobilization strategy has the advantage that it avoids the chemical pre-conjugation of the polymer with a reactive group or a ligand and the additional functionalization of the protein, which is required in almost all surface functionalization strategies that have been described in the literature so far. Functional end groups for covalent binding can be added to the polymer in the course of its synthesis and range from hydroxyl or amine groups to *N*-hydroxysuccinimidyl esters that can be used for click chemistry.[128-137] With these techniques up to 162 molecules per polymersome (Ø = 100 nm) have been immobilized so far.[137] Ligands that have been used to mediate non-covalent binding of proteins to polymersomal surfaces comprise biotin[138-142], cyclodextrin[143-144] and Ni^{2+}-nitrilotriacetic acid[145-146] to immobilize streptavidin-conjugated, adamantane-conjugated and oligohistidine-tagged proteins, respectively. With these methods a maximum of 24 molecules per polymersome (Ø > 100 nm) has been immobilized so far.[145] In contrast, with the newly developed immobilization method based on membrane anchoring domains up to 2,320 proteins could be immobilized on a single polymersome (Ø = 110 nm), which corresponds to a complete coverage of the available surface area and underpins the high efficiency of this novel technique.

Several years ago, it has already been postulated that polymersomes can be used for the spatial separation of incompatible reaction steps in multi-enzymatic syntheses of chemicals.[91] However, up to now, it has only been demonstrated that polymersomes can protect enzymes from proteolytic degradation by exclusion of macromolecular proteins from a confined reaction space.[93, 147] Yet, in most cases, incompatibilities between individual enzymatic reactions arise from small molecules leading to enzyme inhibition and/or inactivation. Two review papers on vesicular nano-compartments published in 2016 have highlighted this missing piece of evidence.[148-149] Only one year later, we showed for the first time that polymersomes

can be used to improve multi-enzyme reactions with incompatibilities mediated by small molecules. As exemplary reaction, the three-step enzymatic synthesis of CMP-NANA was chosen, which suffers from a strong cross-inhibition between the first and the third reaction step. The reactions were spatially separated by entrapping the first enzyme of the cascade within polymersomes while immobilizing the other two enzymes on the outer surface. The engineered channel protein OmpF G119D was the key element for the effective compartmentalization since it allowed the diffusion of necessary substrates and products while keeping the inhibitor outside of the nanoreactors.

To make the nano-EMR technology applicable to other cascade reactions, a toolbox of suitable transport proteins is needed. In this work, eight different channel proteins were characterized regarding their transport characteristics in the polymer membrane, which represents a significant increase in available tools to tailor the selective permeability of polymer membranes. Since only the wildtype OmpF had been functionally inserted into polymersomes before,[150] the number of channel proteins reconstituted into artificial polymer membranes was augmented by 70 % from 10 to 17. However, due to the immense number of different incompatibilities that could arise in artificial cascade reactions, a much larger number of specialized transport proteins is required for the effective compartmentalization of any imaginable cascade reaction. The further expansion of the toolbox is not a trivial task, because there is only limited knowledge on the transport characteristics of many natural transporters. Therefore, advances in the field of transport protein characterization are a fundamental requirement for the universal applicability of the nano-EMR concept. Scientific progress in the field of membrane protein engineering would additionally speed up this process by facilitating the tailored design of the transport characteristics of membrane channels. Thus, the largely untapped biotechnological potential of membrane proteins, which on average account for 20 – 30 % of all genes in genomes, could be unleashed.[151] Additionally, the integration of DNA channels into polymersomal membranes, which has been demonstrated recently, represents an interesting alternative, especially due to the possibility to easily engineer DNA nanostructures.[85]

6. Conclusions

In recent years, the biomimetic concept of combining multiple enzymes in one pot has drawn immense interest, since the resulting cascade reactions have tremendous potential for cutting short conventional step-by-step syntheses.[4, 52] Particularly, the avoidance of intermediate purification results in a significant reduction of both waste and production costs. However, the implementation of eco-efficient biotransformations is not trivial due to the inherent complexity of cascade reactions. The greater the reaction network gets, the higher is the probability that incompatibilities between individual reaction steps arise and that undesired side reactions are catalyzed by the host cell metabolism, if cost-efficient whole cell biocatalysts should be employed for the biotransformation.

In this habilitation thesis, two novel platform technologies are presented that can help to overcome frequently encountered drawbacks of the application of whole cells and isolated enzymes in one-pot multi-enzyme syntheses. The generation of cellular envelopes is especially interesting for enzyme systems, which are not suitable for whole-cell biocatalysts due to severe mass transfer limitations caused by the cell wall or undesired side reactions mediated by cytoplasmic enzymes. On the other hand, the nano-EMR technique can help to realize multi-enzyme syntheses with incompatible reaction steps, which would otherwise result in significantly less or even no product formation at all.

By this expansion of the design options for biotransformations, the production of a variety of high-value chemicals that cannot be synthesized by the current methods could become feasible. However, there is still a long way to go until one-pot multienzyme syntheses become a routinely used approach in organic synthesis. More interdisciplinary research effort, e.g. in the fields of (bio)chemistry, chemical and bioprocess engineering, and materials science, is needed to develop a next generation of biotechnological processes that employ robust reaction systems which have the potential to reach the levels of elegance and efficiency of self-regulating natural metabolic pathways. By this means, economically and ecologically processes with an efficient utilization of natural resources can be developed. The importance of such processes for the transition to a bio-based industry with the ultimate goal to secure the prosperity of modern societies in the long term cannot be overestimated.

7. Publications

The publications used for this habilitation thesis are listed in inverse chronological order. In the unabridged version of this thesis, off-prints of these publications can be found in the same order in the annex.

- Schwarzer TS, Klermund L, Wang G, Castiglione K (2018) Membrane functionalization of polymersomes: alleviating mass transport limitations by integrating multiple selective membrane transporters for the diffusion of chemically diverse molecules. Nanotechnology 29:44LT01

- Poschenrieder ST, Schiebel SK, Castiglione K (2018): Stability of polymersomes with focus on their use as nanoreactors. Eng Life Sci 18: 101-113

- Poschenrieder ST, Hanzlik M, Castiglione K (2018): Polymersome formation mechanism and formation rate in stirred-tank reactors. J Appl Polym Sci 135: 46077

- Poschenrieder ST, Klermund L, Langer B, Castiglione K (2017): Determination of permeability coefficients of polymersomal membranes for hydrophilic molecules. Langmuir 33: 6011-6020

- Klermund L, Poschenrieder TS, Castiglione K (2017): Biocatalysis in polymersomes: Improving multienzyme cascades with incompatible reaction steps by compartmentalization. ACS Catal 7: 3900-3904

- Schwarzer TS, Hermann M, Krishnan S, Simmel FC, Castiglione K (2017): Preparative refolding of small monomeric outer membrane proteins. Prot Expres Purif 132: 171-181

- Castiglione K, Fu Y, Polte I, Leupold S, Meo A, Weuster-Botz D (2017): Asymmetric whole-cell bioreduction of (*R*)-carvone by recombinant *Escherichia coli* with *in situ* substrate supply and product removal. Biochem Eng J 117: 102-111

- Poschenrieder ST, Schiebel SK, Castiglione K (2017): Polymersomes for biotechnological applications: large-scale production of nano-scale vesicles. Eng Life Sci 17: 58-70.

- Klermund L, Poschenrieder ST, Castiglione K (2016): Simple surface functionalization of polymersomes using non-antibacterial peptide anchors. J Nanobiotechnol 14:48

- Klermund L, Riederer A, Hunger A, Castiglione K (2016): Protein engineering of a bacterial *N*-acyl-D-glucosamine 2-epimerase for improved stability under process conditions. Enzyme Microb Technol 87-88: 70-78

- Poschenrieder ST, Wagner S, Castiglione K (2016): Efficient production of uniform nanometer-sized polymer vesicles in stirred-tank reactors. J Appl Polym Sci 133: 43274

- Sührer I, Langemann T, Lubitz W, Weuster-Botz D, Castiglione K (2015): A novel one-step expression and immobilization method for the production of biocatalytic preparations. Microb Cell Fact 14: 180-189

- Klermund L, Riederer A, Groher A, Castiglione K (2015): High-level soluble expression of a bacterial *N*-acyl-D-glucosamine 2-epimerase in recombinant *Escherichia coli*. Protein Expr Purif 111: 36-41

- Sührer I, Haslbeck M, Castiglione K (2014): Asymmetric synthesis of a fluoxetine precursor with an artificial fusion protein of a ketoreductase and a formate dehydrogenase. Proc Biochem 49: 1527-1532

- Klermund L, Groher A, Castiglione K (2013): New *N*-acyl-D-glucosamine 2-epimerases from cyanobacteria with high activity in the absence of ATP and low inhibition by pyruvate. J Biotechnol 168: 256-263

- Fu Y, Castiglione K, Weuster-Botz D (2013): Comparative characterization of novel ene-reductases from cyanobacteria. Biotechnol Bioeng 110: 1293-1301

- Hölsch K, Sührer I, Heusel M, Weuster-Botz D (2013): Engineering of formate dehydrogenase: Synergistic effect of mutations affecting cofactor specificity and chemical stability. Appl Microbiol Biotechnol 97: 2473-2481

Further publications that were published within the formal habilitation period (2014 – 2019):

a) Original articles

- Mähler C, Kratzl F, Vogel M, Vinnenberg S, Weuster-Botz D, Castiglione K (2019) Loop Swapping as a Potent Approach to Increase Ene Reductase Activity with Nicotinamide Adenine Dinucleotide (NADH) Adv Synth Catal, DOI: 10.1002/adsc.201900073

- Najbauer EE, Movellan KT, Schubeis T, Schwarzer T, Castiglione K, Giller K, Pintacuda G, Becker S, Andreas L (2019) Probing membrane protein insertion into lipid bilayers by solid-state NMR. ChemPhysChem 20: 302–310

- Schmideder A, Schottroff F, Klermund L, Castiglione K, Weuster-Botz D (2017): Studies on the enzymatic synthesis of *N*-acetylneuraminic acid with continuously operated enzyme membrane reactors on a milliliter scale. Biochem Eng J 119: 9 - 19

- Sun B, Hartl F, Castiglione K, Weuster-Botz D (2015): Dynamic mechanistic modeling of the multi-enzymatic two-step one-pot reduction of dehydrocholic acid to 12-keto ursodeoxycholic acid with competing substrates and cofactors. Biotechnol Prog 31: 375-386

b) Review articles

- Wang G., Castiglione K (2019) Light-Driven Biocatalysis in Liposomes and Polymersomes: Where Are We Now? Catalysts 9, DOI: 10.3390/catal9010012

- Klermund L, Castiglione K (2018): Polymersomes as nanoreactors for preparative biocatalytic applications: current challenges and future perspectives. Bioproc Biosys Eng 41:1233–1246

- Schmidt S, Castiglione K, Kourist R (2017): Overcoming the incompatibility challenge in chemoenzymatic and multi-catalytic cascade reactions. Chem Eur J 23: 1-15

c) Book chapters

- Castiglione K (2018): Enzymkinetik. In Chmiel H, Takors R, Weuster-Botz D (Eds): Bioprozesstechnik. 4. Edition, Springer-Verlag GmbH, Berlin: 1-448.

- Castiglione K, Weuster-Botz D (2018): Enzymatische Prozesse. In Chmiel H, Takors R, Weuster-Botz D (Eds): Bioprozesstechnik. 4. Auflage, Springer-Verlag GmbH, Berlin: 403-448.

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9. Annex

Off-prints of the publications used for this cumulative habilitation thesis in inverse chronological order (unabridged version only).