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Mini-Review

Conscious coupling: The challenges and opportunities of cascading enzymatic microreactors

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The continuous production of high value or difficult to synthesize products is of increasing interest to the pharmaceutical industry. Cascading reaction systems have already been employed for chemical synthesis with great success, allowing a quick change in reaction conditions and addition of new reactants as well as removal of side products. A cascading system can remove the need for isolating unstable intermediates, increasing the yield of a synthetic pathway. Based on the success for chemical synthesis, the question arises how cascading systems could be beneficial to chemo-enzymatic or biocatalytic synthesis. Microreactors, with their rapid mass and heat transfer, small reaction volumes and short diffusion pathways, are promising tools for the development of such processes. In this mini-review, the authors provide an overview of recent examples of cascaded microreactors. Special attention will be paid to how microreactors are combined and the challenges as well as opportunities that arise from such combinations. Selected chemical reaction cascades will be used to illustrate this concept, before the discussion is widened to include chemo-enzymatic and multi-enzyme cascades. The authors also present the state of the art of online and at-line monitoring for enzymatic microreactor cascades. Finally, the authors review work-up and purification steps and their integration with microreactor cascades, highlighting the potential and the challenges of integrated cascades.

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1 Introduction

Continuous processes in chemistry have gathered a lot of interest due to their specific advantages over batch processes, such as a constant quality of the product output, the potential to suppress side reactions and to operate in wider temperature ranges, enhanced safety, reduction of solvents and time when cleaning the reactors, and the opportunity to execute processes which would be instable in batch mode [1–3]. Microreactors, i.e. reactors with characteristic dimensions from sub-millimeter to sub-micrometer [4], have found applications in photochemistry, exothermic reactions, radiochemistry, manufacture of nanoparticles, for solid-liquid, liquid-liquid, and gas-liquid reactions [2, 5–9]. Their advantages have been reported in numerous reviews and especially for processes that involve highly toxic, explosive or hazardous compounds, miniaturization can lead to further benefits, for example to further enhance safety of operation and for point-of-use generation of reagents [10].

Microreactors have been widely applied in organic synthesis [8, 11, 12], both for single and multiple reactions in sequence [13, 14]. Traditional syntheses of complex organic molecules using "round-bottomed flask" technol-

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Abbreviations: **AchE**, acetylcholine esterase; **CALB**, *Candida antarctica* lipase B; **ee**, enantiomeric excess; **ChOX**, choline oxidase; **ERY**, L-erythrulose; **GA**, glycolaldehyde; **GOx**, glucose oxidase; **HPA**, hydroxypyruvate; **HRP**, horseradish peroxidase; **IEMR**, immobilized enzyme microreactor; **ISPR**, in-situ product removal; **ISSS**, in-situ substrate supply; **Ni-NTA**, nickel nitrilotriacetic acid; **PFA**, perfluoroalkoxy alkane; **PTFE**, polytetrafluoroethylene; **SBP**, soybean peroxidase; **TAm**, transaminase; **TK**, transketolase; **THN**, 1,3,6,8-tetrahydroxynaphthalene; **UDP-galactose**, uridine diphosphate galactose; **UMP-galactose**, uridine monophosphate galactose

ogy usually involve several steps: not just the key reaction steps, but protection and deprotection, work-up, separation and purification steps, at each of which intermediates and products can be lost, reducing overall yield. By optimization of individual reaction steps in flow reactors, and linking them in series and in parallel, large synthetic yields were obtained: Bogdan et al. reported a three-step synthesis of ibuprofen yielding 9 mg min^{-1} of crude product using a simple setup of perfluoroalkoxy alkane (PFA) tubing and syringe pumps [15]. Note that spatial control and fast heat transfer properties allow the individual reactors to be run at different temperatures (50, 65 and 150°C respectively). Subsequent refinements by Snead and Jamison [16] allowed ibuprofen to be generated at a rate of 8 g h^{-1} with a 90% or higher yield in each step. Chemical reaction cascades also led to higher product qualities compared with their batch equivalent for the continuous synthesis of well-defined poly(2-oxazoline)-diblock and triblock copolymers [17], they increased the purity of 2-(trifluoromethyl)-2H-[1,3]-oxazino-[2,3-a]isoquinolines due to the elimination of byproducts [18] and the purity in the enantioselective synthesis of tetrahydroquinolines [19].

The fact that continuous flow reactors are enclosed systems with good heat transfer properties allows hazardous intermediates to be generated safely in situ for their direct use in subsequent synthetic steps [3]. Materials such as diazomethane [20], isocyanides [21], organic azides [22], iodine monochloride and ozone [23, 24], which are challenging to handle in batch reactions because of toxicity, aggressive oxidation properties or explosion risks, are more easily handled/generated and react safely in flow systems. The cascading of reactions facilitated the in situ generation of toxic reagents followed by their immediate use in the subsequent step, e.g. the continuous flow synthesis of hazardous and toxic ethyl diazoacetate and its utilization in a capillary microreactor with a yield of 99% in 2 min [25].

The advantages for microreactors and for continuous synthesis potentially also apply to enzymatic microreactors [4, 26], though not all have been demonstrated yet. In this review, we are focusing on enzymatic microreactors and in particular on cascades of reactions, either as chemo-enzymatic or as enzyme-enzyme reaction sequences. By this, we mean sequences of spatially separated reactions, where each reaction is performed in a separate reactor, and where the product of one reaction becomes the educt (or substrate) of the subsequent reaction. While there are challenges to be found in these setups, the spatial confinement in microreactors can significantly decrease the complexity of cross-reactivity. Despite this spatial confinement of reactions (or compartmentalization), the product of one reaction step may still potentially act as an inhibitor for the subsequent reaction, be unstable or even cause unwanted side-reactions [27]. Therefore, this review also deals with work-up and purification steps, as they are required to perform reactions in sequence, and build complete cascading systems. With a spatial separation it is also possible to monitor the progress of each reaction individually through the implementation of online or at line monitoring tools, which will also be discussed.

2 Coupled chemo-enzymatic reactions

Given the wide range of multi-step chemical syntheses that have been achieved in continuous flow microreactors [8], it is surprising how few report coupled chemo-enzymatic reactions. These kind of reactions, which consist of a chemical reaction followed by an enzymatic one (or vice versa), are of particular interest for developing new routes to chiral compounds, and to attain an overall reduction of the number of synthesis steps.

One of the challenges in a chemo-enzymatic system lies in matching the reaction media, i.e. often incompatible organic and aqueous phases, in such a way that optimum enzyme activity can be achieved and no inhibition or deactivation of the enzyme occurs. Additionally, enzymes need to have long-term operation stability especially when non-aqueous solvents are required for the chemical reaction. The use of multiphasic reactors for biocatalytic reactions has recently been reviewed by Karande et al. [28]. One of the few examples of a chemo-enzymatic reaction sequence is a multistep synthesis of 2-aminophenoxazin-3-one (APO) using three separate microfluidic devices which was achieved with a yield of 18.9% [29] (Table 1). The devices contained metallic zinc, silica-immobilized hydroxyaminobenzene mutase, and silica-immobilized peroxidase, respectively, within their microchannel. These devices were prepared and connected sequentially and used for the combinatorial synthesis of 2-aminophenoxazin-3-ones. Delville et al. [30] used an (R)-selective hydroxynitrile lyase to generate the required stereoisomer of an unstable cyanohydrin from an aldehyde. To prevent racemization, the cyanohydrin had a protecting group added in a second reaction. The compartmentalization of the two reactions facilitated the combination of incompatible reaction conditions (using an inline separator module). Using this approach, acetylated cyanohydrins were synthesized directly with a yield of 68 and 97% ee, i.e. similar to those of batch reaction steps. However, the continuous microreactor approach saved one work-up and extraction step. The application of directed enzyme evolution or protein design may in the future facilitate new chemo-enzymatic reactions in flow [31]. Some of the most important parameters for successful chemo-enzymatic syntheses are related to enzyme properties such as activity, stability and how these properties are influenced by organic solvents, catalysts, substrates, by-products, pH and temperature [32]. Enzyme engineering methodologies such as directed evolution have been especially successful to tailor the enzyme prop-

erties with respect to these parameters for the achievement of a successful process, for example in enhancing activity towards non-natural substrates [33, 34].

3 Coupled enzyme-enzyme reactions

Some of the challenges for chemo-enzyme reactions do not apply to multi-enzyme cascade reactions, since reaction conditions are generally less harsh. Significant challenges to establish multi-enzyme cascade reactions, include the matching of reaction conditions and overcoming the inhibition of the enzymes in the cascade, such as caused by reactants (temperature, pH, flow rates and substrate concentrations) and products [35]. Compartmentalization of the reactions helps to achieve tailoring the individual reaction conditions to the biocatalyst, but the prevention of cross-inhibition is more difficult. Fig. 1A illustrates all potential sources of inhibition that can arise in a two enzyme cascade. Here, intermediate steps which remove inhibiting reactants or products might be necessary to achieve a fully functioning system. To prevent enzymes from performing side reactions with other substrates and intermediates in subsequent reactors, the removal or retention of enzyme might be necessary. Enzymes can for example be removed with an in-line filtration step [36], which would in principle also allow recirculation and re-use of the enzyme (Fig. 1B).

To retain enzymes, they are typically attached to different surface geometries and chemistries, including immobilization on beads, enzyme entrapment in porous matrices, and immobilization on the walls of a microchannel [37–45]. Preserving activity and stability of the enzyme may dictate the preference of enzyme immobilization versus the use of free enzymes, although the overall performance comparison needs to be considered case by case and is usually enzyme specific. With enzymes, immobilization can increase catalytic stability, though inactivation or a rapid loss of activity can also occur. In cases in which immobilization is suitable to the enzyme and present operational benefits, immobilization can be of advantage due to enzyme compartmentalization into a defined space.

A key opportunity of multi-enzyme reactions in microreactors is the ability to perform in vitro biosynthetic reactions [46]. These offer modular approaches for coupling enzyme reactions and may utilize metabolic reaction cascades performed naturally by cells and organs or create de novo pathways in order to achieve the production of new molecules. The ability to rapidly evaluate the effects of reaction conditions and different enzyme variants in microreactors provides a new paradigm for performing multi-step biosynthetic reactions. The number of two- and three-step coupled enzyme reactions reported in literature is growing, though there are still only a limited number of them used for synthess of organic compounds (Table 1). Ku et al. [47] constructed a synthetic metabolic pathway consisting of a type III polyketide synthase, 1,3,6,8-tetrahydroxynaphthalene (THN) synthase, and soybean peroxidase, both immobilized in an enzymatic microreactor. The THN synthase was immobilized to Ni–NTA agarose beads prepacked into a microfluidic

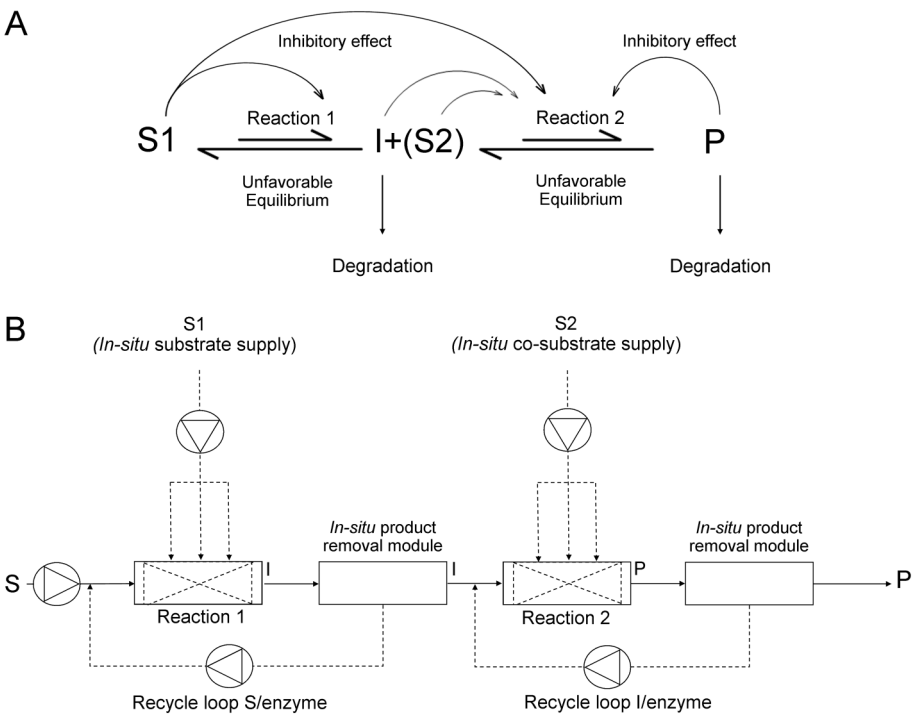


Figure 1. (**A**) Cascade reaction schematic in a linear two reactor mechanism. Substrates (S1, S2), intermediate (I) and product (P) are the chemical species involved in the reaction. The substrate, S, as well as the intermediate I and product P, can present inhibitory effects. I and P can be prone to degradation. This imposes challenges to the process which can be overcome by in situ product removal (ISPR). (**B**) Cascade reaction process with in-situ substrate supply (ISSS), and ISPR strategies in place. The dashed lines annotate possible implementation routes of ISSS, ISPR, enzyme recovery and recycle and the use of immobilized enzymes (packed bed reactor).

Table 1. List of published examples of multi-step chemo-enzymatic and enzymatic cascade reactions in microreactors. Reactions are carried out in continuous flow mode unless otherwise stated

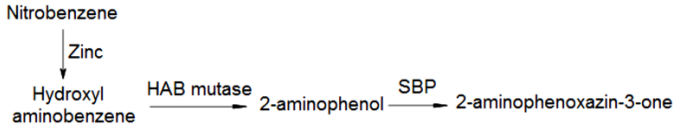
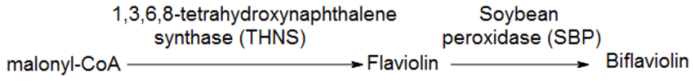
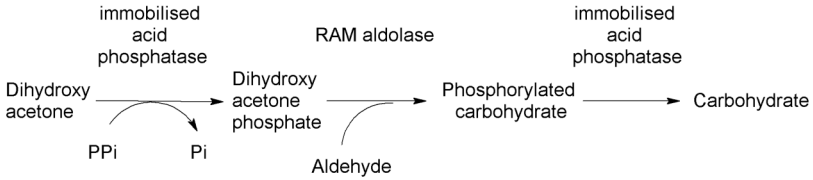
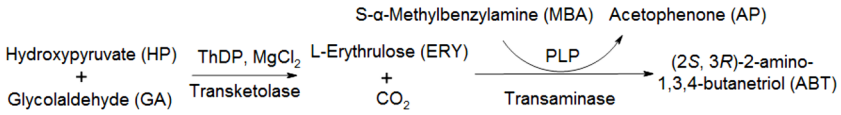
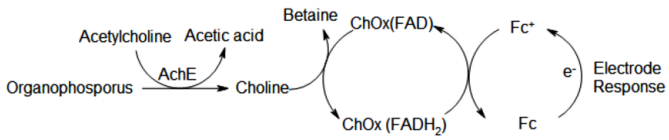
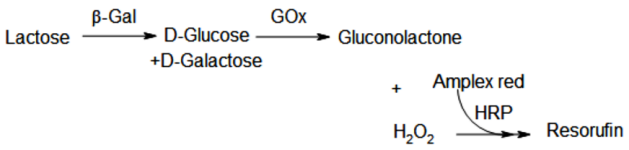
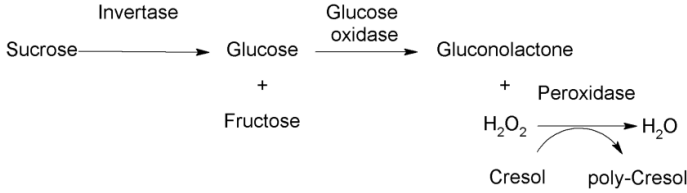
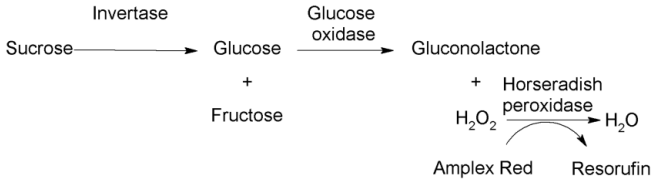
System	Enzymes	Immobilization Method	Product(s)/Reaction	Reaction	Reference
Chemo-enzymatic	(1) HAB-mutase (2) Soybean peroxidase (SBP)	Silica-immobilization in microchannel	Chemo-enzymatic synthesis of 2-aminophenoxazin-3-one from nitrobenzene		[29]
Enzyme-enzyme	(1) 1,3,6,8-tetrahydroxy naphthalene synthase (THNS) (2) Soybean peroxidase (SBP)	His-tag affinity on Ni-NTA agarose beads and covalent immobilization on microchannel wall	(1) Flaviolin; (2) Biflaviolin		[47]
	(1) Acid phosphatase (2) Fructose-1,6-diphosphatase aldolase or rhamnulose-1-phosphate aldolase (3) Acid phosphatase	Immobilized on beads-details	Complex chiral carbohydrate analogues e.g. D-fagomine precursor		[48]
	(1) Transketolase (2) Transaminase	His-tag affinity on Ni-NTA derivatized silica microchannel wall	Chiral amino alcohols e.g. 2-amino-1,3,4-butanetriol		[50]
	(1) Transketolase (2) Transaminase	His-tag affinity on Ni-NTA agarose beads	Chiral amino alcohols e.g. 2-amino-1,3,4-butanetriol		[51]

Table 1. List of published examples of multi-step chemo-enzymatic and enzymatic cascade reactions in microreactors. Reactions are carried out in continuous flow mode unless otherwise stated (continue)

System	Enzymes	Immobilization Method	Product(s)/Reaction	Reaction	Reference
Enzyme-Enzyme (Monitoring)	(1) Acetylcholine esterase (AchE) (2) Choline oxidase (ChOx)	Covalent attachment to channel walls and microparticles	Bioelectrocatalytic reaction to determine AchE-inhibiting organophosphorus		[72]
	(1) β-Galactosidase (β-Gal) (2) Glucose oxidase (GOx) (3) Horseradish peroxidase (HRP)	de-PG2/avidin mediated immobilization of enzymes inside glass tubes	Synthesis of fluorescent compound resorufin which is detected with inverted fluorescence microscope		[67]
	(1) Candida antarctica lipase B (CALB) (2) Glucose oxidase (3) Horseradish peroxidase	Non-covalent immobilization, DNA-directed immobilization (DDI) technique	Hybridizationof 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)		[68]
	(1) Invertase (2) Glucose oxidase (3) Soybean peroxidase	Covalent attachment to PMA-coated glass microchip	Poly(*p*-cresol)		[46]
	(1) Invertase (2) Glucose oxidase (3) Horseradish peroxidase	Photopatterning on porous polymer monoliths	Oxidation of Amplex Red by HRP from invertase-hydrolyzed sucrose		[74]

channel, whereas the second enzyme was covalently attached to the walls of a second microfluidic channel pre-coated with a reactive poly(maleic anhydride) derivative. Novel polyketide derivatives such as flaviolin and biflaviolin were synthesized with a yield of 40%. Lee et al. [46] immobilized enzymes onto the modified glass surface of the microfluidic channels to form bi- and tri-enzymatic systems. The glass channels were modified using a highly reactive poly(maleic-anhydride-alt-1-olefin) (PMA)-based coating that reacts with the free amino groups of the enzymes to generate an immobilization support. Soybean peroxidase was coupled with a lipase in a bienzymatic system, or with an invertase and glucose oxidase to form a three-enzyme system. In the sequential two-enzyme cascade, the lipase was used to catalyze the hydrolysis of *p*-tolyl acetate to yield *p*-cresol, which was the substrate of the polymerization reaction catalyzed by peroxidase, whereas the three-enzyme system was aimed at generating the cosubstrate of the peroxidase, H_2O_2, by the invertase-catalyzed formation of glucose molecules from sucrose and their subsequent glucose oxidase-catalyzed oxidation with oxygen to yield H_2O_2. Coupled enzymatic reactions in continuous-flow systems can be considered as a powerful tool for multistep organic synthesis as shown by the work of Babich et al. [48]. They demonstrated the synthesis of a complex chiral carbohydrate analogue from achiral inexpensive building blocks in a three-step cascade reaction consisting of immobilized acid phosphatase, fructose-1,6-diphosphate aldolase and acid phosphatase in a packed bed reactor system. This continuous-flow microreactor system enabled the synthesis of 0.6 g of D-fagomine. A seven step continuous flow enzymatic reaction was established by Liu et al. [49] to produce uridine diphosphate galactose (UDP-galactose). All of the enzymes were immobilized individually on Ni-NTA agarose beads, which were packed into a column. The authors achieved 50% conversion of UMP into UDP-galactose within 48 h and the stability of the immobilized enzymes was also improved compared to the enzyme in solution.

In addition to synthetic applications microfluidic reactors have also been used for the screening of multi-step biotransformation. An immobilized enzyme microreactor (IEMR) was developed and applied towards the characterization of a de novo transketolase-ω-transaminase cascade to synthesize chiral amino alcohols [50]. This prototype dual microreactor consisted of two surface-derivatized fused silica capillaries containing the His_6-tagged enzymes immobilized by reversible attachment via Ni-NTA linkage. Transketolase was employed in the first reaction to catalyze the production of a chiral ketone from an achiral aldehyde substrate. In a second reaction, an ω-transaminase was used for the biocatalytic amination of a ketone to generate chiral amino alcohols, such as 2-amino-1,3,4-butanetriol. To take the in vitro study of multistep enzyme kinetics further, His_6-tagged TK and TAm enzymes were immobilized onto Ni-NTA agarose beads and packed into the tubes of the IEMR [51]. This 'packed bed' configuration enabled higher enzyme loadings and the coupled reaction reached higher conversion yields (83%) [51] compared to the enzyme immobilized on the capillary walls (5%) [50]. Synthesis of a chiral amino alcohol via the dual enzyme reaction demonstrated the utility of these IEMR systems to enable in vitro multi-step pathway evaluation. The development of de novo pathways represents one of the attractive opportunities of microsystems for broadening the windows of operation in enzyme cascades. Using microreactors to screen for different enzyme variants to find the best possible combination of enzymes and substrates has the advantage of being quick and efficient due to the small amount of reagents and enzymes needed. Applications in process development represent another attractive feature of microreactors as an inexpensive platform for the optimization of reaction conditions [4].

4 Online monitoring

Monitoring the reaction progress of cascading reactions and individual reaction rates at different positions in the cascade can significantly reduce process development times and underpin quality by design approaches. A large number of optical [52, 53], spectroscopic, optofluidic [54, 55], and electrochemical [56] detection methods are available (Fig. 2) and allow on-line and real-time measurements of a broad set of reaction variables (oxygen, pH, glucose, carbon dioxide, or specific reactants/products). Although a few individual enzymatic reactions have been monitored, these detection methods have not been applied to enzymatic cascades [57–61]. As a number of detection methods depend on suitable chemical, optical or spectroscopic properties that enable easy measurement, generalized detection methods are of much interest. NMR opens the door for a variety of advanced reaction monitoring techniques with high information content in real-time under flow conditions as well as for the self-optimization of processes employing single or multiple NMR methodologies [62]. The integration of microreactors with chiral analysis and detection of the enantiomers without the need of labeling is an important step for investigating stereoselective biocatalytic transformations [63]. However, most methods to detect substrate and product concentrations can currently not be performed on-line.

For this reason, cascading reactions can be a useful monitoring tool if the cascade can transform an otherwise not quantifiable analyte into a chemical species that can be measured with existing and easily implementable sensor technology e.g. oxygen sensor. To achieve such an indirect measurement, one microreactor is used as a last step in a linear cascade or branches out from a linear cas-

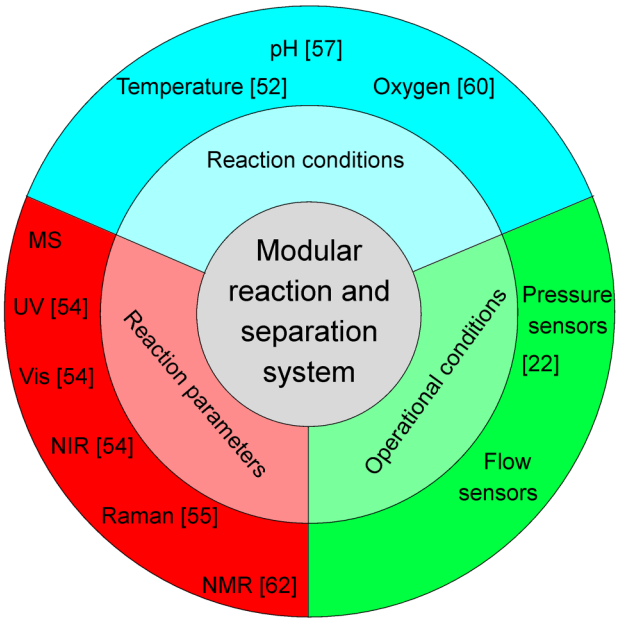


Figure 2. Diagram highlighting options for monitoring modular reaction or separation systems to improve control over reactions. The monitoring has to address reaction parameters (e.g. substrates and products), reaction conditions (e.g. pH and temperature) and operational conditions (e.g. flow rates and pressure). A number of on-line spectroscopic methods and sensors have been developed and implemented in microfluidic systems which can now address this.

cade. The analyte is taken from the main reaction path into a separate cascade which quantitatively yields an easily-measured product [64]. An example for such a cascade model system is coupling glucose oxidase (GOx) with horseradish peroxidase (HRP) to monitor oxygen by a proportionate color-change caused by the production of hydrogen peroxide in the presence of the oxygen and glucose [65]. This has been used by Wei et al. [66] to detect cocaine in urine samples, providing a point-of-care and stand-alone device based on capillary action only. Similarly, Fornera et al. [67] added β-galactosidase to the GOx-HRP sequence to measure lactose. Another three-enzyme cascade was achieved by Vong et al. [68] who coupled *Candida antarctica* lipase B (CALB) to the GOx-HRP couple. CALB and GOx were immobilized using a novel ssDNA-ssDNA interaction method to immobilize the enzymes in separate areas within the same capillary. Atalay et al. [69] presented enzyme cascades to differentiate and quantify sucrose, D-glucose and D-fructose in one assay using an array of enzymes (β-fructosidase, hexokinase, glucose-6-phosphate dehydrogenase, phosphoglucose isomerase). Mao et al. [70] used a cascade of GOx and HRP in order to measure glucose in a microfluidic total analysis system to determine kinetic parameters of the individual enzymes.

Ferrer et al. [71] developed a "paper microfluidic-based enzyme-catalyzed double microreactor assay" using the enzymes lactate dehydrogenase and diaphorase to create a fluorescent complex that could be detected through optical readout. A cascade of acetylcholine esterase (AchE) and choline oxidase has been used by Han et al. [72] to detect organophosphorus compounds. Lin et al. [73] presented a multi-enzyme system consisting of AchE and choline oxidase for the selective monitoring of acetylcholine using electrochemical detection.

Invertase, horseradish peroxidase, and glucose oxidase were patterned on porous polymer monoliths in separate regions of a single channel by Logan et al. [74]. All possible arrangements of the three enzymes were tested, whereby product formation was only observed when the enzymes were placed in the correct sequential order. According to these results, the photo-patterning of enzymes on polymer monoliths can be applied as a simple technique for preparing spatially located multi-enzyme microreactors for directional synthesis [74]. A summary of cascade reactions used for monitoring can be found in Table 1. Further miniaturization of these indirect measurement techniques would facilitate their integration with microreactor cascades for organic synthesis, and could lead to real-time detection of reactant or product compounds as the reaction progresses.

5 Operational considerations

When implementing cascading reactions, even though intermediate products are not isolated, there is still a need to remove side-products from the upstream reaction and deliver a reasonably pure reactant to the downstream reaction. In purely chemical reaction systems, some ingenious solutions to this challenge have been reported: the removal of water (either as a solvent for ionic or polar species, or a reaction product) is for example a common feature of multi-step organic syntheses; traditional methods relying on adsorption by anhydrous magnesium sulfate are not an option in micro-flow systems. When Noel et al. [75] carried out a two-step Suzuki-Miyaura coupling reaction, the first step was quenched by an aqueous solution of HCl, which had to be removed before the intermediate could be passed through the catalyst bed for the second reaction. A semipermeable Zefluor membrane, selectively wetted by the organic phase, was important for the process to achieve a 95% yield in 4 min. Gas-liquid separation is also possible: in the protocol for the continuous flow synthesis of carbamates, Sahoo et al. [76] reported that nitrogen generated in the acid-catalyzed decomposition of organic azides could be separated with a fluoropolymer membrane (99% yield). Occasionally, the solvent used for early reactions in a multistep synthesis is incompatible with reactions further downstream. Hartman et al. [77] report a continuous-flow distillation, where volatile dichloromethane is replaced by higher-boiling toluene by manipulating the temperature above the boil-

ing point of dichloromethane so that the vapor is retained by a semi-permeable PTFE membrane allowing the recovery of a yield up to 76%. Delville et al. [30] showed a successful synthesis and purification of a chiral cyanohydrin (64% yield and 98% ee) in an integrated chemo-enzymatic synthesis system. Water was removed from the system by connecting the microreactor to a liquid-liquid extraction module, using a PTFE membrane to separate the two different liquid phases.

In addition to the challenges of cascading enzymatic reactions, which have already been discussed (see Sections 2 and 3), the cascade development needs also to consider solubility aspects of the reactants and products involved, as well as unfavorable reaction equilibria. As mentioned, the compromises between reactor operation and reaction conditions are often not enough to achieve high productivities [78]. Furthermore, confinement of the reaction space by implementing a reactor for each reaction is often not sufficient to truly compartmentalize reactions in a continuous-flow setting, as cross-inhibition between successive reactions may occur. It may thus be necessary to implement in situ substrate supply (ISSS) [79] and in situ product removal (ISPR) strategies in order to fully compartmentalize the reactions, in order to maximize productivities and yields [80].

ISSS (or fed-batch), ISPR (or by-product removal) and SFPR (substrate feed and product recovery) [81] are commonplace for batch reactors and at larger scales, and different methodologies for different physicochemical properties of the reactants/products, i.e. their volatility, charge, solubility and hydrophobicity, have been established. For microreactors, such strategies would fit to approaches where reactors, separation and work-up units can be assembled in plug-and-play configurations (Fig. 1B) [82–84].

ISSS can be implemented by creating side entry reactors. For chemical reactors, this has been pioneered by Roberge et al. [85], Barthe et al. [86] and later by Cervera-Padrell et al. [87]. For enzymatic reactors, O'Sullivan et al. reported complete conversion of hydroxypyruvate (HPA) to L-erythrulose (ERY) with transketolase derived from genetically modified *E. coli* [36], and Lawrence et al. improved the output and throughput with a microfluidic side-entry reactor 4.5-fold [88]. A similar side-entry reactor design was used by Gruber et al. [57] to increase ERY production while at the same time monitoring the pH by integrating pH sensors at several locations along the channel of the side entry reactor. A final conversion yield of 95% (250 mM HPA added in total) was obtained with an increase in one pH unit over time. Monitoring of pH at several positions of the channel provides additionally, real-time information on the reaction progress in the reactor [57, 61].

Substrate supply can also be implemented in microfluidic devices by the use of organic solvents, in multi-phasic systems. The use of an additional phase is beneficial when water solubility of the desired substrate is problematic. Marques et al. [89] demonstrated this concept by converting cholesterol ($solubility_{water} \approx 4.66$ µM) into 4-cholesten-3-one using cholesterol oxidase. Cholesterol and oxygen were added to *n*-heptane 0.17 mM and 0.65 mM, respectively, while the enzyme remained in the aqueous phase, reaching a total conversion of 67% after approx. 62 s. Furthermore, due to the low water solubility of the product ($solubility_{water} < 1.30$ mM), 4-cholesten-3-one was continuously removed from the aqueous phase turning this approach into an ISSS and ISPR system simultaneously while allowing the reuse of the enzyme. The challenge in multi-phasic systems for ISSS and ISPR is to avoid inactivation of enzymes at the aqueous-organic interface. A way forward to overcome this is to reduce the difference in polarity between the solvents or to optimize the enzyme via enzyme engineering.

In lipase mediated synthesis of isoamyl acetate, to overcome low reaction yields and enzyme deactivation in an aqueous-organic two-phase system due to the use of acetic acid as acyl donor [90], the aqueous phase (water) was substituted by 1-butyl-1-methylpyrrolidinium dicyanamide ([bmpyr][dca]) [91], enabling a productivity of 48.4 g m^{-3} s^{-1} of isoamyl acetate.

The implementation of combined ISSS and ISPR in modular microfluidic devices has been recently demonstrated by Heintz et al. [84] for the asymmetric synthesis of 1-methyl-3-phenylpropylamine (MPPA) from benzylacetone. A two-step liquid–liquid extraction process was employed to continuously extract 1-methyl-3-phenylpropylamine and feed benzylacetone, avoiding product inhibition and overcoming process challenges due to low substrate solubility, respectively. A product concentration of 26.5 g_{MPPA} L^{-1} was achieved in 20 h with high purity (70% g_{MPPA} g_{tot}^{-1}) and recovery yield (80% mol mol^{-1}). The use of modular reaction and separation system enables a higher degree of freedom to assemble easily configurable trains of units of operations, including ISPR modules. Furthermore, the integration of online as well as at-line monitoring is by this way facilitated, allowing real-time monitoring of reaction progress and ultimately, a precise control of the entire process.

Nonetheless, there are still challenges to be addressed: namely the overall gain in process intensification and economic feasibility when, e.g. applying ISPR strategies; long term robustness and stability of the process; recycling of streams including recycling/regeneration of cofactor; enzyme preparation and associated costs; the selectivity of the ISPR strategy employed and matching reaction and recovery times.

6 Outlook

Continuous synthesis has moved from an innovative concept to a practical and versatile high-performance technol-

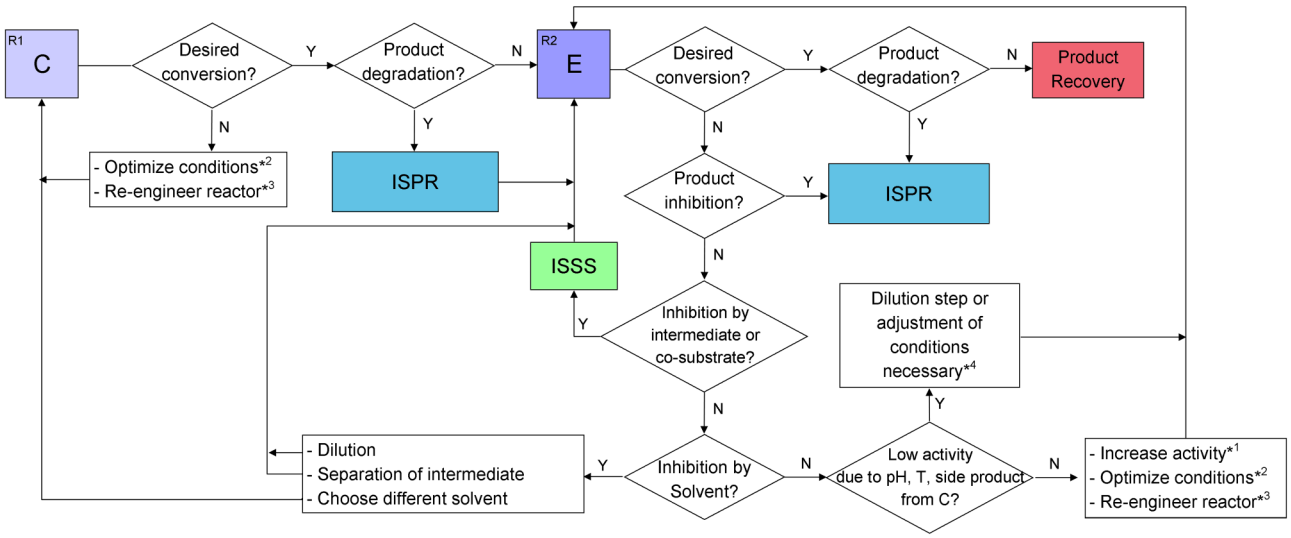


Figure 3. Implementation diagram showing the key decisions required for an optimized coupled chemical (C) and enzymatic (E) reaction cascade. The points in a process where key issues can cause poor conversion (inhibition, product degradation, difference in reaction conditions between the two reactions) are identified, and potential solutions to these are offered. In situ product removal (ISPR) or a substrate supply (ISSS) can help deal with inhibition and product degradation, while choice of solvent, re-engineering the reactor, or even modifying the activity of the enzyme can overcome difficulties with reaction condition differences. The conversion yields can be increased by an increase in the volumetric activity (stability and immobilization of the enzyme and enzyme engineering among others, *1), optimization of reaction conditions (reaction time, pH and temperature, catalyst, among others, *2) and re-engineering of the reactor (mixing, immobilization of the enzyme, among others, *3) and the adjustment of reaction conditions (temperature, pH and dilution of effluent stream of chemical reaction, *4).

ogy, which inspires further innovation along the workflow [92], from process development, product recovery and purification to final product formulation. Reactions which cannot be done in batch reactors are of special interest for flow reactors in chemical and pharmaceutical industries, e.g. reactions requiring high-resolution reaction time control [93] or high safety precautions. Great advances have been made in equipment and workflow for continuous processes [94], from complex synthesis and multi-step reaction sequences [95], continuous-flow manufacturing up to formulation of active pharmaceutical ingredients [83]. For pharmaceutical production, industrial applications of continuous manufacturing have been estimated to increase between 5 and 30% over the next few years [96]. With the quality-by-design process, analytical technologies are also of key importance, but many challenges exist in process analysis that are not specific for continuous processes, but also exist in batch reactors [97]. The principle of function determining form allows many degrees of freedom for coupling reactions in cascades which could be designed as convergent, linear or circular cascades. Conditions for phase contact and mixing of immiscible phases are essential for mass transfer across immiscible phases and their control and improvement at microscale represent important parameters for process design. Active and passive mixing of miscible and immiscible phases can be very efficiently achieved in microsystems [98, 99]. Multiphase microreactors offer therefore an interesting approach for taking advantage of phase boundaries such as gas-liquid, liquid-liquid and liquid-solid interfaces for intensifying biocatalytic reactions and transport in continuous flow processes [28, 100–103].

To provide a framework for setting up coupled cascade reactions, some guidelines and consideration on how to overcome key issues are provided. Important hurdles to overcome in both chemo-enzymatic (Fig. 3) and enzyme-enzyme reactions (Fig. 4) comprise product degradation, reduced enzyme activity either by substrate or product inhibition, deactivation of enzymes by solvents, and non-compatible reaction conditions or unmatched reaction rates (between the flow reactors) which lowers process productivity. Enzyme inhibition by either substrate or product can be addressed with the implementation of ISPR strategies, as well as with the isolation of unstable intermediates and products. Non-compatible reaction conditions are a major concern in cascade reactions. A lot of solvents, commonly used in chemical synthesis, can deactivate the enzymes in the subsequent steps of a cascade reaction. In a chemo-enzymatic reactor, the dilution of the chemical reactor output or the isolation of the intermediate product must therefore be considered. In extreme cases, the solvent with which the chemical reaction is performed must be replaced in order to accommodate the subsequent enzymatic steps in the cascade. This implies that the chemical reaction should be re-optimized. A similar issue is present in enzyme-enzyme cascades when the reaction conditions do not match, e.g. when the enzymes perform their different

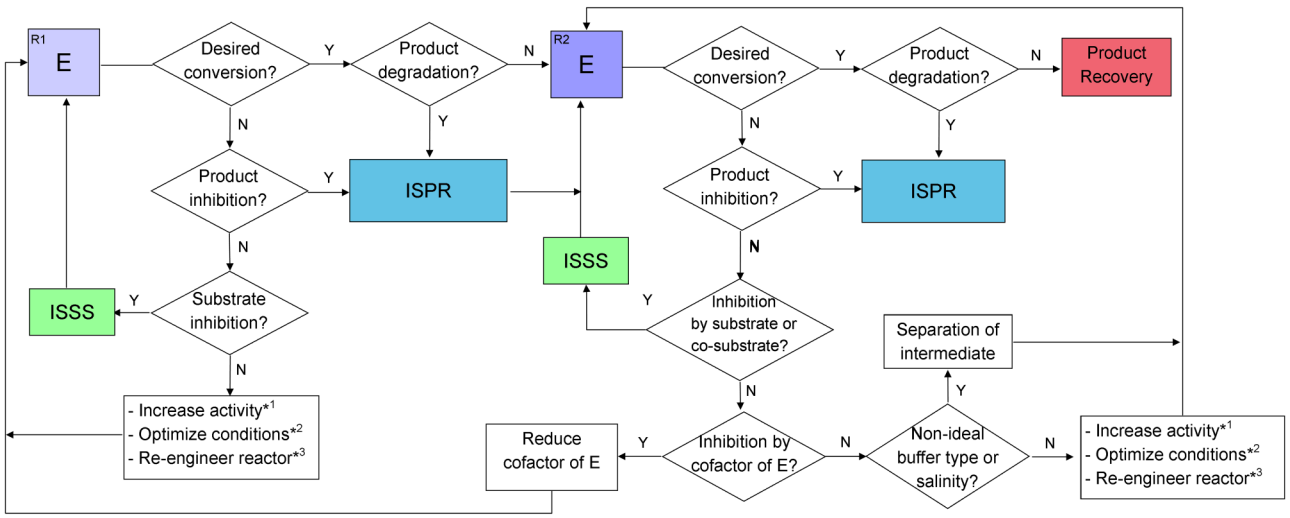


Figure 4. Implementation diagram of a coupled enzyme-enzyme (E-E) cascade. Similar to the diagram for the chemo-enzymatic reaction, the flow chart for the optimization of an enzyme-enzyme cascade deals with the issues caused by the complex interplay of substrate, cofactor and product inhibitions of different enzymes. The implementation of in situ product removal (ISPR) and substrate supply (ISSS) or intermediate separation steps of reactants are dependent on the desired conversion yields obtained as well as the presence of inhibition effects. The conversion yields can be increased by an increase in the volumetric activity (stability and immobilization of the enzyme, higher enzyme load, and enzyme engineering among others, *1), optimization of reaction conditions (reaction time, pH and temperature, catalyst, among others, *2) and re-engineering of the reactor (mixing, immobilization of the enzyme, among others, *3).

catalytic activities best in different buffers. In this specific case, the isolation of the intermediate product or a buffer exchange is mandatory. However, if a compromise between the enzymes activities and the reaction conditions has to be made due to process constraints, the conversion yields can be improved by adapting the reaction times. This can either be achieved by changing the residence time, i.e. by changing the flow rate or by re-engineering the reactor (e.g. changing reactor dimensions to increase or decrease residence time for a given flow rate) or by manipulating the volumetric activity of the enzyme in the respective reactor. Stabilization of the enzymes via immobilization can be considered. However, in the small volumes of microfluidic reactors it can be difficult to introduce immobilization supports (e.g. packed bed reactor), and coating of the inside of reactor walls (such as micro capillaries) limits the surface area upon which enzyme can be immobilized, imposing a limit to the maximum enzyme activity a microfluidic device can sustain. Potentially, this limited density of activity can be overcome by using free enzyme in combination with a recycling step, using a filtration device [38], though this has not been applied yet for cascading systems.

In summary, a large number of enzyme-enzyme microfluidic reaction cascades have been reported which were based on the progress made in microfluidic device development and the understanding of flow reactions in microreactors. Together with the more recent advances made with microfluidic separation units, and the ever-increasing possibilities in online monitoring and control, there is now an arsenal of tools to create well-controlled reaction cascades at the microfluidic scale. This will pave the way towards the engineering of more complex synthetic cascades, such as mimicking multiple enzymatic reactions which take place in living cells, e.g. for sensing, acting on and synthesizing molecules. These are an inspiration for multiplexing along different dimensions, such as space, time or orthogonal EC code.

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