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Biocatalysis in Continuous-Flow Microfluidic Reactors

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Contents

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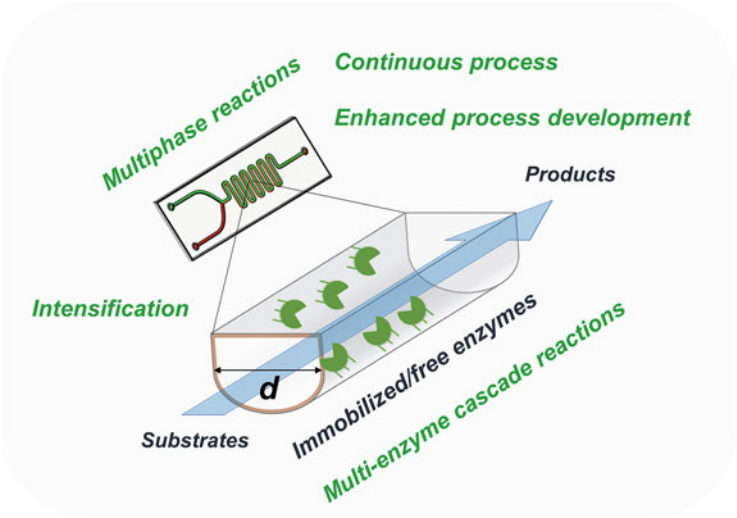
Abstract The implementation of continuous-flow transformations in biocatalysis has received remarkable attention in the last few years. Flow microfluidic reactors represent a crucial technological tool that has catalyzed this trend by promising tremendous improvement in biocatalytic processes across a host of different levels, including bioprocess development, intensification of reactions, implementation of new methods of reaction screening, and enhanced reaction scale-up. However, the full realization of this promise requires a synergy between these biocatalytic reaction features and the design and operation of microfluidic reactors. Here an overview on the different applications of flow biocatalysis is provided according to the format of the enzyme used: free vs immobilized form. Until now, flow biocatalysis has been implemented on a case-by-case approach but challenges and limitations are discussed in order to be overcome, and making continuous-flow microfluidic reactors as universal tool a reality.

Graphical Abstract



Keywords Continuous production, Enzyme immobilization, Flow biocatalysis, Microfluidic reactors, Miniaturization, Reaction intensification

1 Biocatalysis and Continuous-Flow Microreactors

1.1 Biocatalysis Goes with the Flow

Recent years have seen the emergence of a plenitude of new biocatalytic applications, mainly due to advances in protein engineering. These developments have, in

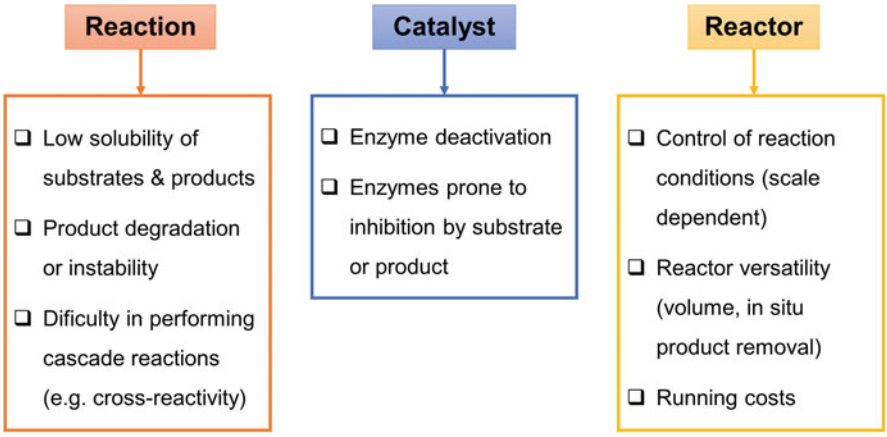


Fig. 1 Commonly encountered challenges to implement biocatalytic reactions in traditional reactors

turn, facilitated the improvement of catalytic properties of enzymes to better match the needs of industry (e.g., by facilitating the synthesis of chiral alcohols and amines). Furthermore, since enzymes are highly selective, renewable, and operate under mild conditions in aqueous media, these processes are also generally regarded as environmentally sustainable since they present a good atom economy, a reduced E-factor ($kg_{waste} \cdot kg_{product}^{-1}$), and reduced downstream costs associated [1]. Nonetheless, in order to realize all the benefits of the industrial use of enzymes, new routes to target molecules and feedstocks must be found by using biocatalytic retrosynthesis [2] and biocatalytic reactions must be operated close to industrial conditions (e.g., by matching sustainable process metrics, such as $g_{product} \cdot L_{reactor}^{-1} \cdot h^{-1}$, $g_{product} \cdot g_{biocatalyst}^{-1}$, and $g_{product} \cdot g_{substrate}^{-1}$). As a result, there is a subtle but important interplay between biocatalysts and process properties for process optimization [3], whereby enzyme activity and stability can be fine-tuned. While traditionally biocatalytic applications are carried out in classic stainless-steel batch reactors, novel reactor designs are increasingly being sought out by researchers interested in intensifying processes and overcoming common issues that have historically plagued these applications (Fig. 1). Continuous processing presents itself as a suitable alternative to these reactors, allowing researchers to obtain a constant product output quality while reducing the overall footprint of the process. Perhaps not surprisingly recent years have seen a strong trend towards continuous operation models [4–12].

Continuous-flow reactors offer an improved control over reaction conditions, with benefits in yield and productivity levels. This increase in efficiency and the concomitant minimization of waste result not only in cleaner processes, but also in lower overall production costs. Furthermore, continuous processes enable a reduction in process lines and facility footprints, which in turn results in less up-front capital cost. To exploit the full benefits of continuous processing, however, rigorous

kinetic analyses are necessary – as well as the characterization of reactor performance at all scales. Implementation of sensor technology to control the quality profile of the products and a product stability assessment are also requirements for a successful continuous process – and, of course, both process scalability and cost-effectiveness must be established [6, 7, 9, 13, 14].

Miniaturized continuous-flow reactors (with volumes ranging from μL to mL) are systems used to evaluate the suitability of biocatalytic reactions or, in particular cases, for production [7, 9, 13, 14]. The small dimensions of these reactors allow experiments to be performed with much smaller volumes compared to traditional batch systems, thereby offering significant cost reduction when using expensive substrates or enzymes. But the benefits do not stop there: these reactors also offer the ability to closely manage the parameters of an experiment; in-line purification with recovery of products can be more easily performed [6, 13]; and no mechanical mixing is typically required. In addition, reactions can be potentially accelerated due to enhanced mass transfer with a decrease in reaction time and significantly improved space-time yield.

1.2 New Demands of Biocatalysis for Reactor Engineering

Increasing demand for enzyme-catalyzed reactions by industry presents new opportunities for reaction engineering [15–23]. These fall into the framework of process intensification, whereby reaction intensification is manifested in terms of decrease in reaction time, reactor volumes, energy demands, and overall costs (Fig. 2).

In response to this demand, there has been a general shift away from batch production towards continuous production for biocatalytic reactions – and microfluidic approaches have become increasingly important as a result [4, 5, 7, 8,

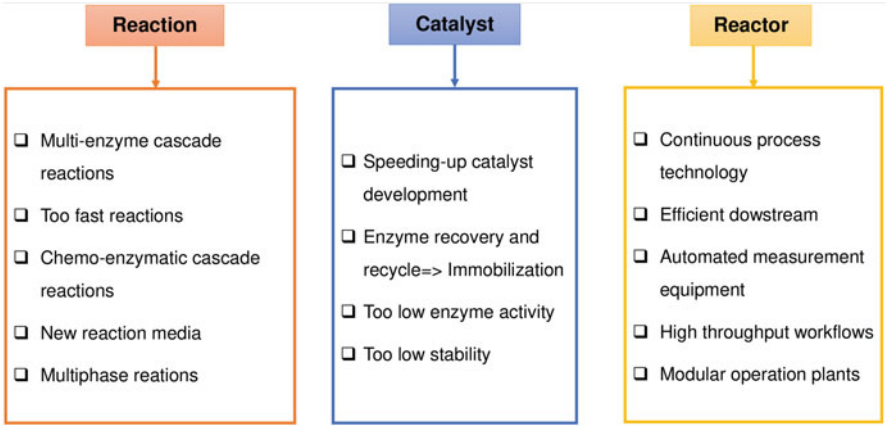


Fig. 2 Demands for reactor engineering in the context of new biocatalytic trends

24, 25]. The microfluidic approaches rely on the miniaturization of continuous-flow reactors, usually in the form of tubular reactors. This is a deviation from the common assumption that continuous reactors for biochemical engineering applications are stirred tank bioreactors, in which the reaction medium is kept at a maximum internal homogeneity by the means of mixing, or packed/fluidized bed macroreactors are used. To clarify this approach, important definitions are here introduced which are based on similar explanations previously applied in microprocess engineering and chemical flow microreactors [5, 6, 26, 27].

- The characteristic channel dimensions of the miniaturized continuous-flow reactors (with volumes ranging from μL to mL) range from the micrometer to the millimeter scale. Nonetheless, the dimension of the reactor channel is relative since the key aspect is whether at the selected channel dimension there is a specific enhancement of, for example, transport intensification with the absence of mass transfer limitations.
- Miniaturized continuous-flow reactors operate under continuous-flow conditions and flow regime is laminar.
- Integrated approaches are necessary where catalyst characteristics, kinetic data, transport phenomena, and reactor engineering are combined to develop flow system. The use of dimensionless numbers is particularly important to identify rate limiting steps and offer opportunities to enhance the overall reaction performance, in particular in solid−liquid biocatalytic reactions [28, 29].
- Transport phenomena that are beneficial for chemical synthesis (e.g., enhanced mass and heat transfer) usually take place below a certain channel diameter where regular laminar flow or surface-tension dominated droplet/bubble flow regimes are encountered [28, 29].

In this chapter, the analysis is limited to microfluidic reactors in continuous-flow tubular configuration and on applications where the miniaturized dimensions have a well-defined influence or advantage.

Miniaturized continuous-flow reactors can be manufactured using a variety of fabrication methods, depending on the reactor materials and feature sizes [5, 6, 14, 30–33]. Direct writing methods – such as CO_2 laser writing – offer rapid fabrication, but are limited to polymeric devices and only permit sizing down to approximately 100 μm. Devices with low aspect ratio features can also be produced using soft lithography techniques; but this requires access to a clean room, and this process is both time consuming and comparatively costly. CNC (computer numerical control) micromachining can be used to fabricate molds (i.e., cast and mold techniques to fabricate poly(dimethylsiloxane), PDMS, devices) or devices themselves in any microfluidic geometry. More recently, additive manufacturing (3D-printing) has been used to create whole devices [14, 32, 34] – although the resolution of printing must be such that fluid leakage is avoided. Flow reactors can also be realized using tubing (e.g., coil microreactors made of polytetrafluoroethylene, PTFE). In all cases, however, the hydraulic diameter and the length of the reactors will dictate the residence time of the fluid within the system.

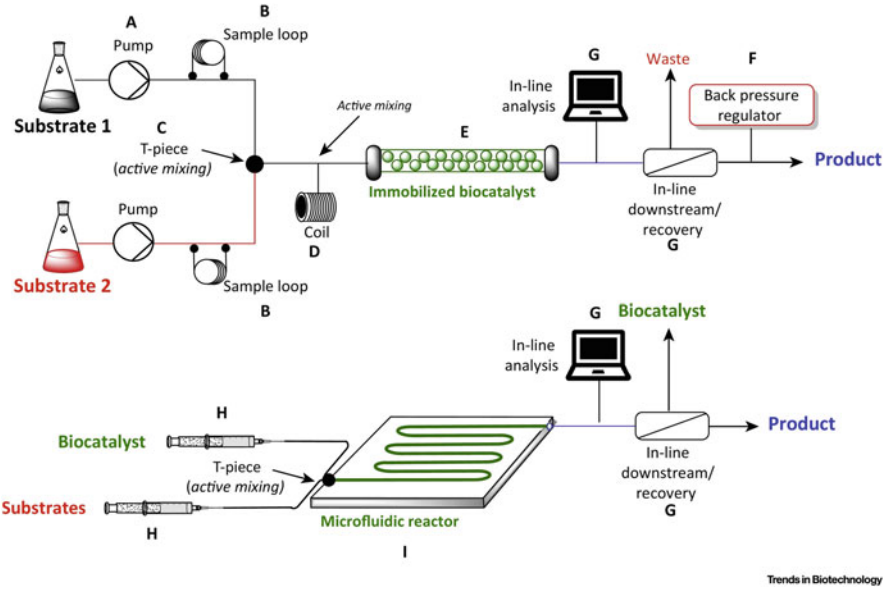


Fig. 3 Main type of miniaturized continuous-flow reactors: fixed-bed reactor (top panel) and tubular reactor (bottom panel). The reactors are complemented with several peripheral equipment comprising of pumps (delivery of substrates or enzyme in the case of tubular reactors), in-line purification or recovery units (e.g., modular microfluidic reactor and inline filtration system for the biocatalytic synthesis of chiral metabolites [30]) and *at-line* reaction analytics. The figure was reproduced with permission from [4]

These devices all require several pieces of peripheral equipment, such as pumps and actuators, to be operated. With recent advances in analytical methods, there now exist several ways to monitor reaction conditions (e.g., pH, temperature, and oxygen), reaction parameters (e.g., substrate and products concentrations), and operational conditions (e.g., flow rates and pressure). The reactors must therefore be fabricated using materials and configurations that permit interrogation with sensors and other analytical methods for the online monitoring of chemical and physical variables (pH, temperature, oxygen, and CO_2) [35], and for *at-line* reaction analytics (GC- and LC-MS). Online monitoring is crucial to ensure robust process control strategies and, ultimately, guarantee a stable process with precise synthesis of products (APIs and value-added chemicals). This robust analytical data will allow building high-quality models for process design and optimization and will ultimately enable feedback control strategies (e.g., controlled addition in multi-inlet reactors of acid and base [36] or oxygen rich fluid in case of oxygen-dependent reactions) [36–38].

There are two main categories of miniaturized continuous-flow reactors: tubular reactors (Fig. 3 bottom panel) and fixed-bed reactors Fig. 3 top panel). Combined with the form of enzymes used (i.e., free or in immobilized form), this fundamental delineation allows us to categorize these applications (Fig. 3).

1.3 Scope of this Book Chapter

Continuous-flow microreactors are promising tools to expand the applicability of biocatalysis in industry. This reactor type not only addresses some of the current limitations of conventional enzymatic, but also helps researchers to meet increasing industry demand for modern enzyme catalyzed transformations [3, 15–23]. They will ultimately facilitate the establishment of complex multi- or chemo-enzymatic reaction cascades, due to the modular nature of the microreactors system [39] and intensifying reactions and processes while generating new operating windows. Advances in continuous-flow microreactors will also help effectively further the development of continuous bioprocesses, and, ultimately, contribute to the establishment of modular, small efficient production plants. In this chapter, recent advances of biocatalysis in continuous-flow microreactors will be discussed based on the form of biocatalyst used (i.e., free or immobilized enzymes).

2 Biocatalytic Microfluidic Reactors with Free Enzymes

2.1 Modern Biocatalysis with Free Enzymes and Emerging Demands: The Context of Microfluidic Technology

Although continuous processing is frequently associated with the retention of enzymes inside the reactor by immobilization, there are also cases where the enzyme is used in a soluble form in the reaction medium. Since the dimension of the characteristic magnitude is volumetric (amount/activity of enzyme suspended per unit of volume), in principle there is no specific advantageous feature of reaction intensification due to reactor miniaturization (e.g., due to the increase of the specific surface area) [9, 28, 29]. The interest of using free enzymes in microfluidic reactors must be found, therefore, either in practical reasons or due to the presence of several fluid phases in contact. Practical reasons include the use of microfluidics as an enabling technology – for example, due to their ability to allow for the precise manipulation of small amounts of fluids, and control of reaction times. Additionally, these systems would allow the implementation of advanced scale-up strategies (including numbering up and scaling out) and the incorporation of advanced reactor instrumentation which enables the establishment of Process Analytical Technologies (PAT) and Quality by Design (QbD) approaches. Plug and play configurations of miniaturized continuous-flow reactors and miniaturized downstream unit operation allow the assembly of complex synthetic cascades, and ultimately the creation of automatized systems for reaction screening and the study of whole bioprocess sequences [36, 40]. Different system configurations can be operated as miniaturized continuous-flow reactors depending on device architecture and biocatalytic reaction conditions (Fig. 4). To maximize productivities and yields it may be necessary to implement in situ substrate supply (ISSS) [41] and in situ product removal (ISPR)

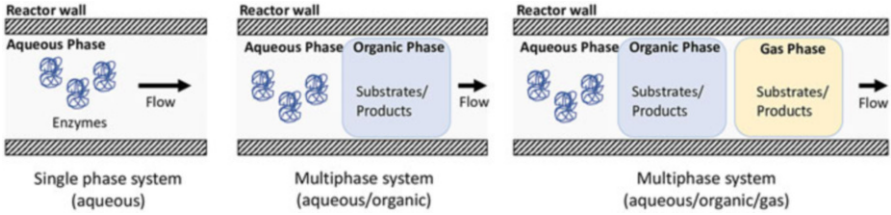


Fig. 4 Example of reactor configurations for free enzyme systems. The multiphase systems can have different flow characteristics rather than a droplet system (train of droplets of different phases) depending on the microfluidic device architecture. The enzymes are contained in the aqueous phase while substrates and products can be present in the organic and gas phase depending on their characteristics

strategies [42]. These strategies are commonplace in batch reactors and at larger scales, and different methodologies have been established according to the different physicochemical properties of both reactants and products. ISSS and ISPR can be applied in miniaturized continuous-flow reactors by the use of organic solvents and/or gases, in multiphasic systems (e.g., aqueous-organic, aqueous-gas, or aqueous-organic-gas system). However, one challenge associated with multiphase systems is the inactivation of enzyme at the phases interface which can be circumvented by enzyme optimization via enzyme engineering or reduce solvent polarity difference.

2.2 Biocatalysis in Monophasic Aqueous Medium

2.2.1 Compartmentalization of Complex Reactions in Microfluidic Devices

The continuous production of high value, or difficult to synthesize, products is of increasing interest to the pharmaceutical industry. These reactions typically rely on the implementation of complex, multistep reaction sequences that resemble biological processes seen in living systems. Cascading reaction systems have already been employed for chemical synthesis with great success, allowing a quick change in reaction conditions and the easy addition of new reactants, as well as the expedient removal of unwanted 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 chemoenzymatic or biocatalytic synthesis. Microreactors are promising tools for the development of such processes [40].

Multistep or cascading continuous-flow reactors are essentially several different reactors connected into a single flow sequence to carry out complex cascade reactions (Fig. 5). In this reactor cascade several biotransformations can be carried out at different conditions. For example, the fluid can be rapidly heated or cooled in

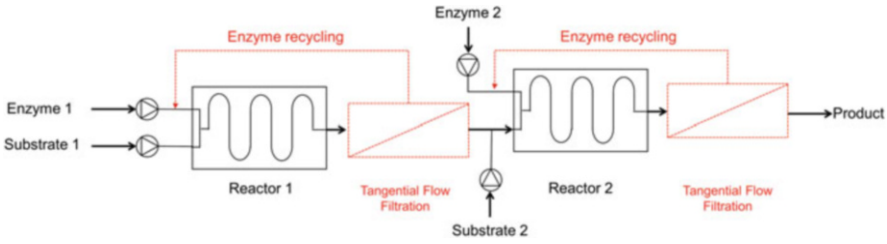


Fig. 5 Conceptual setup of a cascading reaction system using free enzymes. The two enzymes are compartmentalized and the enzymes are recovered through tangential flow filtration units [30]

different reactors to mediate an effective (bio)transformation. The reactor dimensions, e.g., hydraulic diameter and length, dictates the residence time inside the reactor and particular features, e.g., heat transfer [40]. Nonetheless, there can be issues when coupling these reactions in terms of incompatibility of reaction conditions, the balancing of suitable catalyst amounts, and the need to overcome inhibition issues. Guidelines and considerations how to overcome these key issues have been provided to set a framework to couple cascade reactions [40].

An application of this cascading approach is the synthesis of chiral amino-alcohols [39]. Chiral amino-alcohols are of particular practical interest, since they represent key industrial synthons for the production of complex molecules and optically pure pharmaceuticals. They can be synthesized from simple, non-chiral starting materials, by coupling a transketolase and a transaminase-catalyzed reaction. Low enzyme activities and inhibitory effects have limited their implementation. Usually, the systems are far from full conversion – and long reaction times are also commonly reported, making process modifications and improvement challenging. By implementing microreactor technology, however, full conversion can be achieved. Using the compartmentalization of the reactions afforded by the microreactor cascade, researchers have also successfully overcome inhibitory effects, increased the activity per unit volume, and optimized individual reaction conditions. The transketolase-catalyzed reaction was completed in under 10 min, following optimization of the transaminase-catalyzed reaction, and a volumetric activity was attained which led to full conversion of the coupled reaction in 2 h. This example represents a paradigmatic case of how continuous-flow microreactors can be applied for the design and optimization of biocatalytic processes.

2.2.2 Advanced Monitoring in Continuous Reactors

Controlling and monitoring intensive variables along reactors is more difficult within continuous-flow reactors, which creates difficulties in both understanding and optimizing reactions [43]. Controlling and monitoring pH, in particular, is essential to stabilize reaction conditions and reaction progress for many biocatalytic processes. The suitable design of microfluidic devices integrated with pH sensors can enable

the real-time pH monitoring of the progression of an enzymatic reaction in a microfluidic reactor and is a first step towards achieving pH control [35, 44]. To achieve this, fluidic inputs along the reaction channel can be implemented to adjust the pH of the reaction [37]. This concept was tested with reactions catalyzed by a transketolase and a penicillin G acylase with time-course profiles of pH were recorded within a microfluidic device. Without pH adjustment, the former showed a pH increase of one pH unit, and the latter a pH decrease of about 2.5 pH units. However, with pH adjustment the pH drop of the penicillin G acylase-catalyzed reaction was significantly attenuated and the product yield increased significantly, up to 29%.

2.3 Biocatalysis in Multiphasic Medium

Multiphase microreaction systems are emerging as powerful tools for the development of enzyme-catalyzed transformations involving two or more partly immiscible fluids in continuous flow [28, 29]. Mass transfer intensification due to miniaturization of the reactor dimensions, and the associated enlargement of the interfacial area, presents a powerful approach of effective reaction rate enhancement. Coupling microreactors and biocatalytic reactions in these systems is a highly complex process that requires an integrated approach addressing biocatalyst features, reaction kinetics, mass transfer, and reactor engineering [28, 29]. Multiphase flows are generated when two or more partially immiscible fluids are brought into contact. Such flows can be classified as either gas–liquid or liquid–liquid. Heterogeneous catalytic reactions are often encountered in process biocatalysis, where immobilized enzymes typically constitute the preferred form of catalyst (viz. Sect. 3). In liquid–liquid reactions, biocatalytic reactions take place in the water phase or directly at the fluids' interface. On the other hand, in gas–liquid reactions the gaseous substrate usually requires transport into the aqueous liquid phase, where it reacts upon contact with the soluble form of the enzyme [28, 29].

2.3.1 Biocatalysis with Free Enzymes in Liquid–Liquid Flow

Multiphase conditions can facilitate substrate supply, product removal, or both through in situ extraction between the aqueous and organic phase [28, 29, 45]. The potential benefits to researchers are several but outstanding is the potential to substantially increase productivity, enhance the space-time yield, and intensify transport [28, 29]. The application of free lipases in a biphasic medium (aqueous/organic phase) has received considerable attention for the development of intensified reactions. The interest in the use of this organic system lies in the solubility enhancement of hydrophobic substrates, elimination of side reactions caused by water, and improvement of product recovery. Lipases have been used for the synthesis of isoamylacetate [28, 46, 47]. A two-phase system was used composed

of water and *n*-hexane, either in segmented or parallel flow, where the enzyme dissolved in the aqueous phase or hydrophilic ionic liquid and *n*-heptane containing enzyme adsorbed to the liquid–liquid interface. The microchannel microreactor showed superior performance to the well-mixed conventional batch reactor, in terms of both reaction rate and maximum conversions reached in relation to residence time needed, specifically 2.8 times faster than the batch reactor for the same conversion and 286% more productive. Product removal into the organic phase and continuous phase separation were also successfully accomplished.

Similar system is used in the oxidation of cholesterol performed by cholesterol oxidase. Reactions were carried out in stirred batch reactors and miniaturized microreactors in a two-phase parallel flow composed of water and n-heptane. In this particular case, both the substrate and the product of the reaction (cholestenone) are poorly soluble in water. Furthermore, the heptane was used to increase the concentration of oxygen (co-substrate) in the reaction mixture. The residence time required to reach target conversion was decreased almost 20-fold in the microchannel reactor when compared with the conventional batch reactor. A normalized residence time concept was used to account for differences in enzyme concentration applied in the different reactor configurations [28, 48, 49].

Most recently, the transfer of the enzyme synthesis of cephalexin from a batch reactor configuration to a continuous-flow microfluidic system was studied. The reactor system also comprised of integrated reaction product separation and enzyme recovery. Production of cephalexin is a paradigmatic example of synthesis in a kinetic regime, which is characterized by the appearance of a concentration maximum during the enzyme reaction. The control of the reaction time and reaction features is critical in order to achieve maximum conversion. The systems consisted of a biphasic reaction medium, with optimum composition of phosphate buffer, polyethylene glycol and water, forming a two-phase slug flow within a microfluidic capillary as the reaction-separation environment. Such a flow arrangement enabled a uniform residence time of the reaction mixture as well as providing in situ extraction of cephalexin and enzyme recycle [50].

Reaction optimization in biphasic systems is accomplished not just by miniaturization itself, but also by the fine-tuning of the microreactor geometry. The performance of the Corning AFR™ Low Flow (LF) fluidic module was shown for the Candida Antarctica lipase B (CALB) catalyzed isoamyl acetate synthesis in an n-heptane–buffer two-liquid phase system. The flow regime consisted in dispersed n-heptane droplets in a continuous buffer phase, which enables in situ extraction of the produced isoamyl acetate to the n-heptane phase. Additionally, it provides a very large interfacial area for the esterification reaction performed by an amphiphilic lipase B, which positions itself on the n-heptane–buffer interface. Productivities obtained (six-fold more per volume and 2.4-fold more in catalyst mass) were the highest reported so far for this reaction and indicate that Corning Advanced-Flow Reactor™ (AFR™) modules are also very efficient for carrying out biotransformations in two-phase systems [51].

2.3.2 Biocatalysis with Free Enzymes in Gas–Liquid Flow

Oxygen-dependent reactions are of great importance in biocatalytic applications [52]. O_2 is usually supplied to the liquid reaction medium containing the free enzyme via contact with a gas phase. The reaction rate is typically limited by the low O_2 transfer rate and low solubility of O_2 in aqueous reaction medium [53–55]. Different approaches based on increasing the oxygen transfer rate by reactor and reaction engineering have been studied [54, 56, 57]. Using continuous-flow microreactors the interfacial surface-to-volume ratio can be maximized while the overall reaction time is minimized [54, 56]. As an example, a continuous falling-film microreactor can be applied for the oxidation of glucose catalyzed by free glucose oxidase [58]. An agitated cell reactor (ACR) has also been applied to enhance the rate of biocatalytic oxidation reactions for the same transformation [59, 60]. Another interesting reactor setup is the tube-*in*-tube configuration where the aqueous and gas phase are physically separated physically by a membrane [29, 61–64]. This allows a continuous supply of gas while avoiding direct interfacial contact between enzyme and gas phase. Oxygen can also be produced in the reaction media itself for a bubble free supply of gas based on the controlled decomposition of hydrogen peroxide [65]. Under the confinement of a porous particle or a flow reactor [66, 67], this feature was exploited to enable the concentration of aqueous O_2 to be increased beyond equilibrium solubility under safe and practical conditions [67, 68].

3 Biocatalytic Microfluidic Reactors with Immobilized Enzymes

3.1 Enzyme Immobilization and Conventional Continuous Reactors: The Need for New Technologies

3.1.1 Enzyme Immobilization and Continuous Reactors

Enzyme immobilization is an approach that enables the confinement of an enzyme within a defined region of the space. The confinement can be carried out at reactor scale either by using membranes that allow retainment of the enzyme or by utilizing the insolubilization of an enzyme via incorporation into a solid matrix [69, 70]. The immobilization of the enzyme into a solid matrix implies a heterogenization of the reaction, since the reaction takes place at the solid–liquid interface and phenomena of mass transfer towards the solid catalytic phase take place. The use of enzyme immobilization in biocatalytic reactors is driven by both technical and functional considerations [71–73]. The technical considerations stem from the idea that the reuse or continuous use of the enzyme catalyst requires the application of a suitable method of enzyme retention or confinement within the reactor. This explains the strong historical link between the development of continuous-flow reactors and

immobilization methodologies [8, 24, 72, 74]. Additionally, fuelled by the advances of the protein immobilization science, the design of immobilized enzymes has integrated the immobilization as a fundamental tool to modulate the final properties of the heterogeneous biocatalysts [71, 73, 75, 76]. Reactor design and enzyme-immobilized design are therefore inextricably interdependent, and their integration must be adequate for both the application of enzymes and the specific reaction characteristics in question.

3.1.2 Format of Conventional Continuous Reactors with Immobilized Enzymes

There are several options for continuous operation using immobilized enzymes (Fig. 6). The primary option is a tubular format consisting of packed-bed reactors (Fig. 6e), where the immobilized enzyme is contained and fixed within the reactor while the substrate stream passes through and the stirred tank (Fig. 6a) where the enzyme is retained in the reactor by an appropriate screen or recovered by ex-situ filtration or centrifugation and recycled back into the reactor (Panels b-d in Fig. 6). An alternative is the expanded or fluidized bed reactor (Fig. 6f), where the enzyme

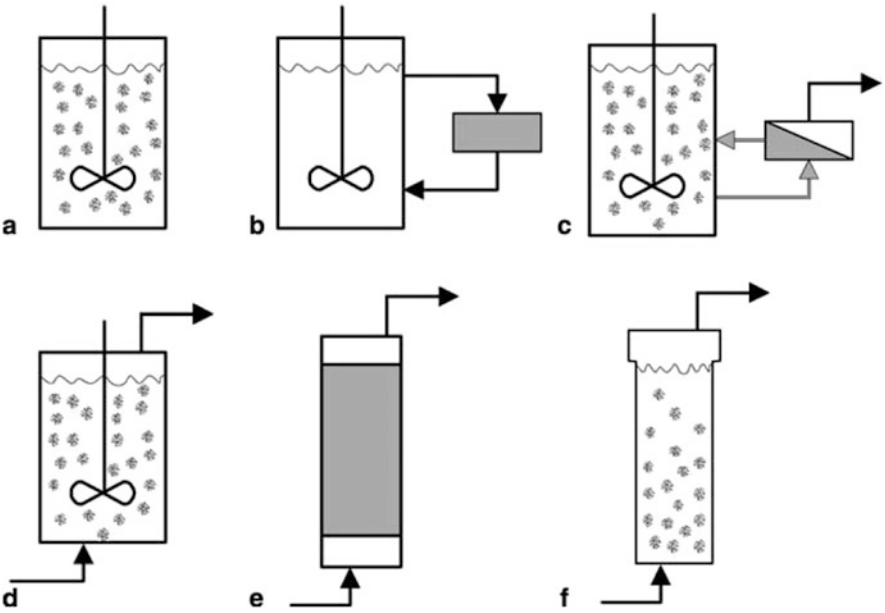


Fig. 6 Different configurations of enzyme-immobilized reactors. Panels (**A**-**C**) show reactor operated in batch mode or semicontinuously. Panels (**D**-**F**) show continuous-flow reactors. (**a**) Batch stirred-tank. (**b**) Recirculation batch stirred-tank. (**c**) Ultrafiltration stirred tank. (**d**): Continuous stirred tank. (**e**): Fixed-bed reactor. (**f**) Fluidized bed reactor. Figure was reproduced with permission from [69]

particles are retained by a hydrodynamic balance between gravity and drag forces promoted by the upflow substrate stream. Both tank and tubular configurations are operated under steady state. Multiple examples at lab-scale and industrial implementation can be found showing successful integration of immobilized enzymes in continuous flow [69, 70, 72, 74].

3.1.3 Conventional Continuous Reactors: Limitations and Need for New Technologies

Given the long tradition of continuous operation in the field of biocatalysis, researchers have accumulated significant knowledge about continuous enzyme reactors involving both soluble and immobilized enzymes over the last several decades [70–72, 74]. In recent years, however, the number of enzymes and transformations explored at lab-scale has expanded significantly, and a strong trend towards continuous operation rather than traditional batchwise (bio)chemical transformation has been widely recognized within the literature [4, 5, 8, 24, 25]. Many of these new transformations can be transferred to continuous operation using immobilized enzyme flow reactors [8, 24, 25]. However, there has also been a renewed wave of development and application of new continuous-flow reactors, which is being fuelled by three factors:

- Remaining unsolved problems of traditional immobilized-enzyme reactors, as described below.
- Increasing demands of biocatalysts and enzyme-catalyzed reactions (see Sect. 3.2).
- New technological possibilities offered by development in analytics and microfluidic technology.

Conventional enzyme-immobilized reactors share the limitations and casuistics of free enzyme reactions. Nonetheless, there are specific problems encountered in this type of reactors:

- Reactor designs relying on immobilized enzymes must commonly deal with the mass transfer limitations. Even when only a liquid phase reaction medium is used, there is still an external mass transport from the liquid phase to the solid phase component, as well as the additional internal diffusion step when the solid catalyst is porous [69, 77, 78].
- Continuous stirred tank reactors (CSTR) display poor kinetic performance at high conversions under most kinetic regimes since these reactors operate at the final conversion condition at steady state. The type of carrier material that can be integrated is also limited by considerations of mechanical stability, considering stirring physics and particle size realities. In addition, scalability can become problematic when the controlling phenomena change across scales [69, 77, 78].
- The fixed-bed reactor is restricted to certain types of carrier materials that provide suitable low back pressure. Furthermore, control of operational condition is

difficult along the entire length of the reactor as well as the integration of online monitoring. There is a defined operation window where suitable radial dispersion and absence of axial dispersion take place. Additionally, the residence time is related with the mass flow through the reactor and fluid flow which will influence suitable dispersion, mass, and heat transfer.

3.2 *Modern Heterogeneous Biocatalysis and Emerging Demands: The Context of Microfluidic Technology*

Modern biocatalysis processes have placed new demands on reactor engineering [3, 15–23] and include

- The format and stability of the immobilized catalyst must be adapted to continuous operation.
- Enzyme immobilization into the reactor must incorporate enough activity in cases of biocatalytic reactions with extremely low specific activity.
- Where biocatalytic reactions occur quickly, the reactor must guarantee an extremely efficient contact between the fluid reaction mixture and the solid catalyst.
- New systems require the contact of two fluid phases containing the substrates/products. The reactor design should be focused not only on the reaction kinetic but also on the phenomenology of the mass transfer between fluid–fluid–solid (catalyst) phases.
- Co-immobilization of multi-enzyme catalysts and chemo-enzyme catalysts frequently poses challenges.
- While modern industrial chemistry aims at the implementation of efficient processes, the intensification, characterization, and optimization of immobilized enzymes under operation conditions is extremely complex.

When current technology is confronted with new demands, enormous windows of opportunity arise. Microfluidic technology has arisen as an essential opportunity for implementation of heterogeneous biocatalytic reactions in shifting biocatalytic reactions from batch to continuous mode of operation, and concomitantly, towards intensified bioprocesses.

3.3 *Immobilized Enzymes in Microfluidic Reactors: Challenges and Practical Implementation*

3.3.1 Enzyme Immobilization into Microfluidic Reactors

The fundamental aspect of the design of immobilized-enzyme reactors is the enzyme for retention during continuous operation. The configuration of these reactors can

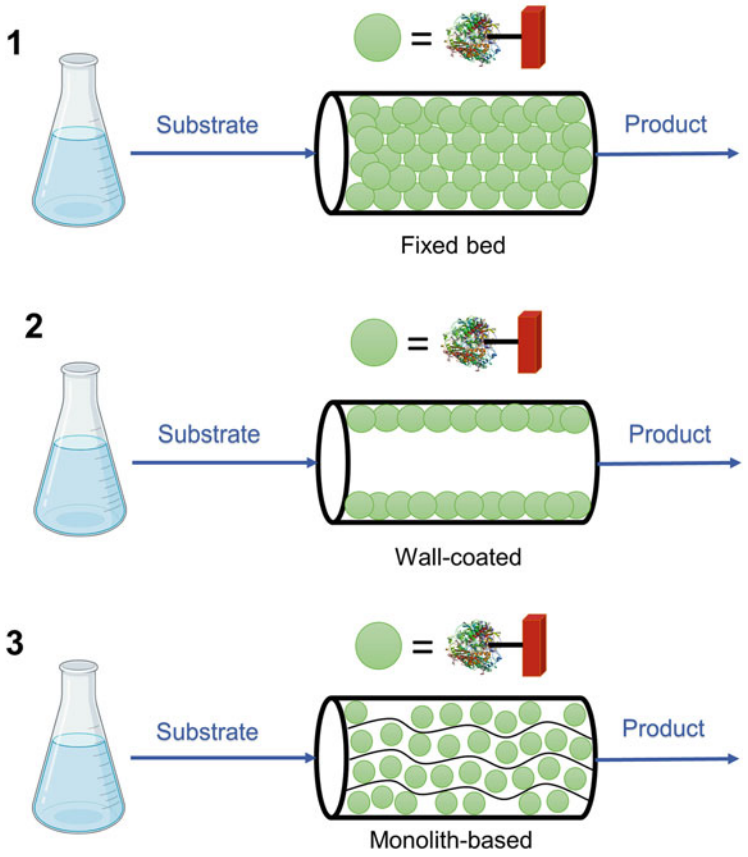


Fig. 7 Examples of enzyme-immobilized microreactors. (1) Wall-coated enzyme-immobilized microreactor where enzyme is directly integrated on the inner wall of the microchannels or supported on (nano)materials coating the inner walls. (2) Fixed-bed enzyme microreactor, where the enzyme is pre-immobilized into pre-existing carrier material that is packed. (3) Monolith enzyme microreactor, where the enzyme is immobilized onto the surface of the pores/channels constituting the monolithic structure

vary depending on the combination of reactor format and the enzyme immobilization method (Fig. 7).

The different reactor configurations can be encountered depending on how the enzymes are immobilized within the reactor space: packed microbead, wall-coated, and monolithic reactor, Fig. 7. The packed microbead reactor resembles traditional packed-bed reactors (PBRs). The enzymes are pre-immobilized into solid carriers, which are then further integrated in the form of a fixed- bed. The wall-coated configuration represents a reactor where the enzyme is surface-immobilized on the inner wall of microfluidic tubes creating a catalytic layer (with the reaction medium circulating through the tube). Lastly, in the monolithic reactor the microchannels are formed into a material network of meso- and macro-porosity. The monoliths material

can be either inorganic, organic, or biobased and natural hydrogels or made out of enzyme-based hydrogels. Enzymes can be immobilized onto these solid supports in several ways [71, 73, 75, 76, 79] and in flow reactors, specifically [8, 24, 25]. Nonetheless, the immobilization of enzymes within microfluidic reactors does pose some of the challenges commonly encountered in macroscale-based reactors [8, 9, 13, 24]. However, some specific considerations must be taken into account, such as:

- Immobilization must either be implemented off-site on previously synthetized materials, or directly onto the internal surface of the reactor.
- Materials used for microreactor fabrication must be compatible with the methodologies of enzyme immobilization or else they can create an unfavorable microenvironment that is not adverse for proper enzymatic function.
- For complex multistep reactions spatial compartmentalization and spatial orientation is essential to optimize the kinetic of the multistep reaction.
- For high-throughput screening and reactor characterization, reversible immobilization is preferable.
- At the microscale, phenomena as aggregation or channel clogging must be taken into account and are commonly encountered.

In addition to these technical requirements, enzyme immobilization must also address one critical question: how much biocatalytic activity per unit reactor volume is required for optimal performance? This is determined via consideration of two fundamental factors: the quantity and the quality of the immobilized enzyme. The quantity depends on the surface area and the volume available for the incorporation of the enzyme into the microreactor; the quality depends on the protein structure following immobilization, which is in turn dependent on the chemistry of the immobilization process that is utilized [71, 73, 75].

3.3.2 High Quality Enzyme Immobilization in Microfluidic Reactors

Among the different strategies to modulate protein-material chemical binding, covalent immobilization by aldehyde chemistry (glutaraldehyde) was initially implemented due to its relative simplicity [7, 8, 24, 25]. Unfortunately, this process lacks granular control of the protein-surface interaction by the glutaraldehyde chemistry. The science of enzyme immobilization has progressed significantly over the years, and researchers now have a rich toolbox of material activation and immobilization chemistries at their disposal to achieve high activity and stability.

Covalent immobilization on aldehyde- or epoxy activated carriers has been implemented in flow reactors [8, 24, 69]. Unfortunately, this immobilization process creates an irreversible binding between the micro-structured element and the protein, disabling reuse of the microreactor system. The functionalization chemistry can also be difficult to be implemented in microreactors. To overcome this problem, reversible binding resting on ionic interactions has been explored [8, 38, 80, 81]. To strengthen the binding and direct the immobilization, however, enzymes may need to be genetically fused to both binding modules and peptide tags. Different strategies

of directed immobilization by peptide modules have been implemented; through this strategy, both purification and immobilization are accomplished in just one-step [82–84]. Reversible immobilization based on protein-based cationic modules or His-tags have also been implemented in both wall-coated reactors and PBRs [8, 82, 83, 85–87]. Contrarily, directed irreversible immobilization can guarantee stable binding without enzyme leaching, although recyclability of the reactor and material might be problematic. Orthogonal or self-immobilizing techniques using Spy, Halo, and streptavidin protein motifs and formylglycine-generating enzymes have been used in microfluidic bed reactors or in wall-coated reactors [8, 88–91].

3.3.3 Enzyme Immobilization in High Quantity in Microfluidic Reactors

Increases in the amount of protein in question may necessitate an efficient use of the surface available for protein binding. The total surface available depends on the reactor format, and the relevant surface area is calculated by reference to the internal surface of the packed material in PBRs, the surface area generated during the monoliths manufacturing, and the inner area of microfluidic tubular reactors [8, 9, 92–102]. Recent examples of the continuous-flow reactors rest in the translation from batch reactors to PBRs using medium mesoporous or macroporous particles of a diverse nature – such as cross-linked agarose, cross-linked polyacrylic polymers, and silica [8, 24]. The combination of medium-high protein loadings (10–100 mg g^{-1}) and dense packing into PBRs typically leads to a high catalyst concentration [8, 24]. In the monolithic reactors, the enzyme is immobilized into an inner porous surface which is created during the synthesis process, which aims to obtain a uniform monolayer via controlled immobilization, or by the controlled formation of thin films [8]. Enhancing the practical use of intensified enzymatic reactors is also now being assisted by advancements in reactor engineering, which include new reactor concepts and fabrication technologies. 3D printed reactors [32, 34] and groove-typed channel microreactors have been tailored to increase the loading capacity [14].

Across all three reactor formats, the amount of catalyst and the format of the material need to be adequate within the interplay with fluid dynamics of the reactor and the suitable residence time [77, 103, 104]. The design of an immobilized enzyme must therefore always consider:

- Incorporating enough enzymes to reach a high space-time yield.
- The format of the immobilized enzyme and reactor dimensions, which must enable the operation of suitable mass flow to achieve a high conversion under suitable fluid flow conditions.
- Unfavorable fluid dynamics which can provoke presence of mass transfer resistances or the creation of preferential channels through the fixed-bed, thereby decreasing the expected conversion and the efficiency of the immobilized enzyme.

- Low back pressure, since the use of small particles packed in microreactors can cause high pressure to drop along the fixed-bed.
- The reactor dimensions (length and diameter), which must be designed according to the superficial velocity along the reactor to operate under suitable regime of excellent radial dispersion and absence of axial dispersion. Otherwise, the reactor operation can deviate from the ideal plug-flow configuration, leading to a corresponding decline in conversion.

In short, enzyme immobilization, reactor design, and operational parameters must all be well integrated into a holistic design [9, 105].

4 Exploitation of Microfluidic Enzyme-Immobilized Reactors

4.1 Promises and Advantages of Microfluidics in Enzyme-Immobilized Reactors

The possibilities and promises that microfluidic reactors offer can be briefly summarized as follows:

- *Improvement of the development of continuous bioprocesses*. The contribution of miniaturization during the development phase stems from both the velocity of the generation of information at low consumption of resources and enhanced controlled evaluation of process conditions [9, 36, 105–107].
- *Reaction intensification by exploitation of microfluidic features*. Microscale effects on transport arise due to short diffusional distances and high surface-to-volume ratio in the channels. These can contribute to the acceleration of the reaction, when compared to a reactor format that is more tightly limited by the mass-transfer across boundaries [9, 28, 29, 36, 105].
- *Generation of new operation windows*. The confinement of reactants under flow in microchannels under submillimeter scale can offer more precise process controls (i.e., regular flow pattern, fast response, and uniform temperature distribution) as well as reliable operations under novel process windows [108–112].
- *Contribution to the development of modular, small efficient production plants*. Recent trends in both pharma and fine chemicals production towards continuous manufacturing with full integration of unit operations are associated with the modularity of micro- and meso-reaction platforms [7, 9, 13, 113–117].

In the following section, we are offering some examples that illustrate how these benefits have at least to some extent already been realized – while also highlighting the considerable challenges that still remain relating to the implementation of immobilized enzymes in microfluidic systems.

4.2 *Intensification of Solid–Liquid Reactions in Microfluidic Reactors*

In solid–liquid reactions, the enzyme is considered the solid phase (i.e., the solid phase is the inner wall of wall-coated reactors, the surface of pores of monoliths or the internal surface of porous particles) while the reaction medium is the liquid phase. The liquid phase is directed by laminar flow through the channels, and the transport is affected via molecular diffusion. In this context, the first question that must be addressed is whether the internal area is sufficiently large to provide enough overall reaction rate?

4.2.1 Miniaturization in Flow Wall-Coated Reactors and Fast Reactions

In principle – given the relatively small dimension of the diameter channel – it might be expected that high enzyme concentration and high volumetric activities can be reached with high surface-to-volume ratio at the microfluidic scale, thereby enabling the operation under a kinetic control regime in the absence of diffusion limitations [118]. To analyze this effect, timescale analysis has been performed in wall-coated reactors. Timescale analysis is based on the comparison of the characteristic times of the respective phenomena being examined [118]. With this aim, the magnitude of the reaction time, diffusion time, and residence time are all calculated and then compared with draw different windows (Fig. 8). Such timescale analysis enables the identification of key variables in design (i.e., diameter tube) and immobilization (i.e., enzyme loading and specific activity) as they impact the operation itself (i.e., flow rate). The maximum space-time-yield scales directly with the enzyme activity immobilized on the available wall surface, which itself is reciprocally related to the diameter channel. Consequently, a reduction of the channel dimension below

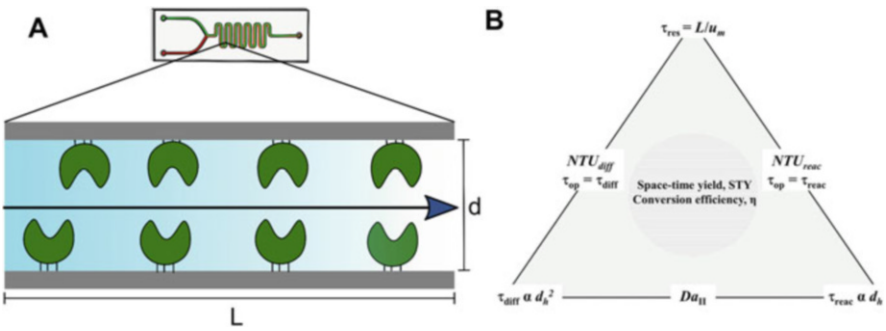


Fig. 8 Schematic representation of an enzyme-immobilized microfluidic reactor (**a**). Panel (**b**) shows reaction engineering analysis of the wall-coated immobilized enzyme microreactor. The operational window for space-time yield (*STY*) and conversion efficiency is determined by the interplay of the characteristic times of reaction (τ_{reac}), diffusion (τ_{diff}) and reactor operation (mean residence time, τ_{res}). For details see [86, 118]. Figure was adapted with permission from [86, 118]

100 μm boosts reaction rate above 50 mM min^{-1} for enzymes with high catalytic turnover (>50 s^{-1}). As the transport time decreases reciprocally to the diameter of the channel, the miniaturization not only boosts the reaction rate, but also enhances the transport enabling the reactor in an operation regime of kinetic control. The subsequent interplay between reaction characteristics, microchannel geometry, and reactor operation allows the identification of further operation windows (i.e., residence time) to achieve high conversion. This has been proved for both phosphorylation and glycosylation reactions [80, 118].

These features can be widely exploited to determine the intrinsic kinetic parameters of immobilized enzymes within a reactor [119], or to optimize both conversion and space-time yield aided by timescale analysis and mathematical modelling [85, 118]. The way an operation in microfluidic wall-coated reactor performs under a regime of a perfect radial mixture with low axial dispersion has been both experimentally and mathematically studied in some detail [85, 120]. Moreover, in this example, the model successfully predicted the performance of two consecutively connected microreactors coated enzyme – and could potentially be used to design and optimize efficient and sustainable processes of chiral amine synthesis catalyzed with surface-immobilized enzymes [121]. Capillary reactors have been also very effective to make effective use of their high surface to volume ratio [122].

4.2.2 Miniaturization in Flow and Reaction Intensification

For slow reactions (i.e., with a catalytic constant below 5 s^{-1}), the high inner area combined with a short characteristic dimension at the microscale could be insufficient, since the enzyme activity confined into the reactor is not enough to provide a high enough reaction rate. In such cases, surface coating with nanomaterials (i.e., nanoparticles, nanosprings, nanotubes, etc.) [123–126] and polymers [127] increases the enzyme loadings, thereby enhancing the reactor performance. The use of porous particles to coat the inner wall of microchannels also increases enzyme loading working in conditions of short diffusional paths. This has also been shown for phosphorylation and glycosylation reactions. In those examples, reaction rate is reduced from several days to several hours, with a space-time yield of 500 mmol L^{-1} h^{-1} at product titers of ∼200 mM [123, 124]. Procedures based on the integration of material sciences, advanced reactor printing, protein chemistry, and protein engineering represent a wonderful opportunity. The enhancement of the catalytic phase and catalyst concentration has been also achieved by using enzyme immobilized onto nanoparticles that are flown through microchannels [128, 129].

Many recent examples of the called flow biocatalysis are built on the packing of porous particles into fixed-bed reactors. Increases in reaction rates when compared with batch processing are related to the high catalyst concentration compatible with a suitable mass transfer. This feature is exploited both to shorten the reaction time of slow reactions and also to ensure an extremely efficient contact between the fluid reaction mixture and the solid catalyst for fast reaction. In many cases this is accompanied by unstable product or unstable reaction intermediates. Intensification

in flow reactors at different dimensions has been recently reviewed in the literature [4, 8, 25]. In general, more extensive studies into reactor backpressure, dispersion, distribution of residence times, and external mass transfer still need to be carefully performed, both from an experimental and a modelling point of view, in order to gain a more fulsome assessment of the reactors and window of operation. These are based on the fact that in a fixed-bed reactor, internal diffusional limitations of the catalyst particle are not alleviated. On the contrary, it can actually aggravate external transport limitations when low superficial velocities are used [69, 78]. Additionally, the design and operation must ensure low backpressure and adequate distribution of the liquid through the fixed-bed. Nevertheless, opportunities also potentially arise from the window of operation at laminar flow at short diameter, characterized by a perfect mixing in the radial dimension but the absence of mixing along the axial dimension [62, 103, 104], an achievement of high volumetric activities, and more precise control of short residence times [130, 131].

Monoliths represent a combination of the large internal area of packed porous particles and the ordered laminar flow directed by the monolith channels. Silica monoliths enable high loading activity and are suitable to work under high flow at low backpressure at high reaction rate [8, 132, 133]. For instance, macrocellular silica monoliths prepared by a sol-gel method based on emulsion templating [134, 135] have been used for the adsorption and covalent grafting of transaminases. Aside from silica, monoliths can be also formed with biopolymers [136] like agarose [137, 138]. Through this approach, several thermostable enzymes have been successfully entrapped, recovering 80–90% activity upon the immobilization process. Alternatively, carrier-free immobilization has been proven to be very effective in achieving high enzyme loadings. The procedure can be based on the chemical cross-linking of proteins [139], or the aggregation can be genetically programmed via protein domains fused to the enzymes, in order to trigger the self-assembly of a 3D gel network within microreactors [88–90]. Studies on the influence of the mass transport (external and internal), residence time distribution, and the efficient use of enzymes are currently under current development [140–142].

4.3 Intensification of Solid–Fluid–Fluid Reactions in Microfluidic Reactors

In solid–fluid–fluid reactions, the enzyme is on solid phase (packed beads, inner surface) and the reaction medium is composed by at least two fluid phases [28, 143–146]. Multiphase flow with free enzymes has been previously discussed above, in Sect. 2 of this chapter. In this section, we focus more narrowly on enzyme-catalyzed reactions where two fluids are present, and the reaction takes place into the solid phase where the enzyme is immobilized involving gas–liquid–solid or liquid–liquid–solid systems.

4.3.1 Liquid–Liquid Reactions with Immobilized Enzymes in Flow

In liquid–liquid–solid reactions, the phenomena limiting the reaction rate and, thereby, the reactor performance is usually the mass transfer across phase boundaries. The main limitation can be focused on the transport between the two fluid phases or between the fluid phases towards the solid catalytic phase. Liquid–liquid–solid reactions are becoming increasingly common in continuous-flow reactions [28, 29]. The increase of the interfacial area in microfluidic reactors enhances these rates of transport reciprocally to the diameter of the flow channels. In fixed-bed reactors, to overcome mass transport limitation the dimension of the particles used and superficial velocity must be accordingly balanced [147, 148].

4.3.2 Gas–Liquid Reactions with Immobilized Enzyme in Microfluidic Reactions

In gas–liquid reactions, gaseous substrate usually requires transport into the aqueous liquid phase, where it reacts upon contact with surface-immobilized enzyme [9, 28, 144]. Gas–liquid–liquid reactions are of critical importance in bioprocessing. Oxidative O_2-dependent biotransformations are of interest for implementation in chemical synthesis, but their application is limited by the supply of oxygen to the active catalytic phase. As noted before, this limitation can be focused on the transport from gas to the liquid phase, or on the transport to the solid catalytic phase. These limitations are further aggravated by the relatively low oxygen solubility in the aqueous liquid phase. There are many examples of the intensification of O_2-dependent enzymatic reactions in continuous flow, but there are very few examples of the application with immobilized enzymes [9, 28, 81, 149–151]. The two main limitation steps of oxygen-dependent reactions can be studied comprehensively in microfluidic reactors.

First, the analysis can be focused on the transport from the dissolved oxygen from the liquid phase to the solid phase. For that purpose, immobilization of D-amino acid oxidase on borosilicate microchannel plates was performed. The immobilized enzyme activity was in the range expected for monolayer coverage of the plain surface with oxidase. Performance of the reactor was studied by employing in-line measurement of dissolved O_2, and off-line determination of the keto-acid product. Reaction-diffusion timescale analysis for different flow conditions showed that the heterogeneously catalyzed reaction was always slower than diffusion of O_2 to the solid surface, even though the immobilized enzyme confined in the microchannel reached a high volumetric activity of 10 mM min^{-1}. That demonstrates how the application of immobilized enzymes in microchannel wall-coated reactors not only boosts the volumetric activity but also enhances the transport rate of a scarce soluble compound to the catalytic phase [86]. In another study, a detailed analysis was performed on oxidation of cholesterol in microchannel reactor and compared reaction performance at microscale to reaction performances in stirred batch reactor and

continuously operated packed bed reactor [48, 49]. The results revealed a ~100-fold decrease in residence time at microscale process operation.

Second, the application of the oxygen-dependent reactions also increases the transport rate from the gas to liquid phase. Intensification of the transport across phases has been broadly demonstrated in process engineering [28]. To demonstrate the application of biocatalytic reactions a fully integrated falling film microreactor that provides controllable counter current gas–liquid phase contacting in a multi-channel micro-structured reaction plate was implemented. Advanced non-invasive optical sensing is applied to measure liquid phase oxygen concentrations in both in- and out-flow as well as directly in the microchannels to show how the reactor can supply up to 100 mM min^{-1} of oxygen to the liquid phase [152].

4.4 Assembly of Enzyme-Immobilized Cascades

The implementation of multistep enzyme catalyzed reactions in microfluidic systems was previously discussed. Enzyme cascades with the compartmentalization of the reactions by enzyme immobilization have been now addressed [14, 153]. Sequential and parallel cascades have already been assembled in enzyme-immobilized microreactors [154] but recently, the synthesis of the antiviral Islatravir was implemented by the immobilization of several engineered enzymes (galactose oxidase and kinases) and implemented in a continuous flow [153]. In another notable example, a wall-coated microfluidic reactor containing a three-enzyme cascade compartmentalized in three microreactor modules was implemented by using directed immobilization [155, 156], displaying precise control of the spatial organization and reaction control. An enzymatic reactor consisting of a packed tube was used to facilitate the in vitro study of this dual enzyme pathway consisting of a transketolase and transaminase. That allowed a quantitative evaluation of the conversion kinetics [87]. Another compartmentalization method arises from the use of magnetic microbeads loaded in microfluidic flow cells. Recently, a microfluidic system was used to optimize the enzymatic production of both levodopa (L-DOPA) and dopamine in both single-step and multistep reaction sequences, which led to a yield of approximately 30% for LDOPA production and 70% for dopamine production [157]. Incompatibility between different reaction steps in cascades in series has also been solved via compartmentalization in connected reactors [158]. Orthogonal cascades have been implemented to overcome some critical problems of the implementation of continuous processes. Continuous-flow applications for biocatalysis face substantial technical obstacles, particularly for enzymes that require cofactors [159–161].

4.5 Generation of Novel Process Windows

It has been proposed that the application of micro-structured reactors could expand the window of operation of the chemical processes [111, 112]. The confinement of reactants under flow in microchannels under submillimeter scale promises not only transport intensification, but also more precise process control (i.e., regular flow pattern, fast response, and uniform temperature distribution) and more reliable operation under novel process windows (i.e., elevated temperatures, high pressure, explosive, toxic conditions). In addition, some physical transport phenomena beneficial for chemical synthesis take place below a certain channel diameter (e.g., regular laminar flow, surface-tension dominated droplet/bubble flow, inhibition of explosion propagation for enhanced safety). Exploiting these effects, microreactors allow the achievement of operation conditions, and reactor performance not achievable in other configurations [26, 27].

One interesting case is the commented oxidative O_2-dependent biotransformations. It has been shown how continuous-flow microreactor technology can expand the process window by increasing the medium pressure range ($\leq$34 bar), enabling biotransformations to be conducted within a single liquid phase at boosted concentrations of the dissolved O_2 (up to 43 mM). Using soluble enzymes in liquid flow, a rate enhancement (up to six-fold) stemming from the effect of elevated O_2 concentrations was observed on the oxidase kinetics. When additional catalase was used to recycle dissolved O_2 from the H_2O_2 released in the oxidase reaction, product formation was doubled compared to the O_2 supplied, in the absence of transfer from a gas phase. A packed-bed reactor containing oxidase and catalase co-immobilized on porous beads was implemented to demonstrate catalyst recyclability and operational stability during continuous high-pressure conversion. Product concentrations of up to 80 mM were obtained at low residence times (1–4 min) [81].

4.6 Scale-Up and Scale-Down Impact on Productivity and Space-Time Yield

Microfluidic reactors enable the achievement of a high space-time yield (g L^{-1} h^{-1}) at efficient use of the enzyme catalyst. One major question in the applications of microfluidic flow reactors is the scalability in terms of increasing (or suiting) the required total productivity (g h^{-1}). Studies performed in wall-coated enzyme-immobilized reactors have shown that a decrease in the characteristic reactor dimension can allow the reduction of the volume while still preserving conversion and total productivity [89]. Although it is widely acknowledged that microreactors can also be used for the actual production in addition to enhanced bioprocess development, studies actually comparing the effect of reactor format on space-time yield and productivity remain relatively scarce [8]. Most reports in the literature mention the usefulness of the microfluidic systems for process screening in enzymatic reactors,

but very few actually proceed to increase the scale in order to benchmark the results obtained [162]. In one recent example, a microfluidic enzymatic reactor for L-DOPA production was up-scaled (780-fold increase) to a milliliter scale system by maintaining similar mass transport properties resulting in the same yield, space-time yield, and biocatalyst yield as its microscale counterpart. The results obtained for yield and biocatalyst yield were like what is reported in the literature for similar systems, however the space-time yield was higher [157]. Calculations on productivity were made available for enzymatic microreactors and cost analysis shows the potential for high-value pharmaceutical synthesis [163, 164]. In another illustrative example, comparison between different laccase/reactor formats revealed that the catechol oxidation was more efficient when the enzyme was immobilized on the surface of microchannels [165]. Scale-out and numbering up are approaches to increase the total production while keeping constant characteristic distance [162, 166].

5 Conclusions

Microfluidic enzyme reactors play an important role in the current trend of transit to continuous bioprocesses and process intensification. Progress is strongly anchored on an interdisciplinary approach where material sciences, protein engineering, enzyme immobilization, process engineering, mathematical modelling and analytical chemistry are combined. However, despite all the progress in this field in recent years, not all promises of these systems have been fulfilled as their chemical synthesis counterparts (Table 1).

There are still challenges to be addressed for an effective use of continuous-flow microreactors, namely: the overall gain in process intensification and economic

Table 1 Demonstrated benefits of flow biocatalysis using continuous-flow microreactors (adapted from [13])

	Promised benefit of flow biocatalysis
Demonstrated	Continuous processing at smaller scales
	Better spatial and temporal control
	High surface-to-volume ratio
	Improved transport in multiphasic systems
	Product removal/product isolation
Partially demonstrated	Faster process development
	Plug-and-play construction of process configuration
	Expanded biocatalytic process windows
Not demonstrated	Safety, health, and environmental advantages
	Mobile process plants
	Energy efficiency
	Cost-effectiveness (e.g., capital expenditures and cost of goods)

feasibility; the long-term robustness and stability of the enzymatic process; recycling of streams, including enzymes and recycling/regeneration of cofactor; enzyme preparation, in particular for immobilized form, and associated costs; and matching reaction and recovery times in cascade reactions. Additionally, general limitations for further uptake in industry include the lack of a more comprehensive monitoring of all process variables and automation, insufficient sample volume for quality control, and integration of downstream processing. The standardization of device and components, as well as, the development of sensor technology would allow the implementation of Process Analytical Technology (PAT), reducing dependency of end-users on specific manufactures, reducing operator-induced variability whilst improving product quality [36]. Until all these are addressed, flow biocatalysis will be implemented on a case-by-case approach and not truly universal.

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