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Review

Magnetic Microreactors with Immobilized Enzymes—From Assemblage to Contemporary Applications

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Abstract: Microfluidics, as the technology for continuous flow processing in microscale, is being increasingly elaborated on in enzyme biotechnology and biocatalysis. Enzymatic microreactors are a precious tool for the investigation of catalytic properties and optimization of reaction parameters in a thriving and high-yielding way. The utilization of magnetic forces in the overall microfluidic system has reinforced enzymatic processes, paving the way for novel applications in a variety of research fields. In this review, we hold a discussion on how different magnetic particles combined with the appropriate biocatalyst under the proper system configuration may constitute a powerful microsystem and provide a highly explorable scope.

Keywords: microfluidics; enzymatic microreactors; magnetic particles; nanomaterials; immobilization

1. Introduction

Nowadays, technological progress allows for the fulfillment of human needs, which are consistently growing, leading in turn to novel evolutionary paths. Industrial processes come to serve this purpose by questing high-performance manufacturing tools. On this realm, microscale technology and flow chemistry constitute a promising combination for the development of innovative methods and intensified processes. The global industry relies greatly on bioprocesses and biocatalytic techniques and flow processing has the potential to enrich these fields; by facilitating large-scale production with significantly smaller equipment, a substantial decrease in reaction time and enhancement in space-time yields up to 650-fold in correlation with batch processes [1].

Microfluidics involves the development of methods and devices for the manipulation of bioprocesses on a nano- to microliter scale. The reduction of operating scale allows exploitation of unique micro- or nanoscale physics, offering decisive advantages compared with large-scale reactor systems. Heat and mass transfer can be substantially increased as a result of high surface to volume ratio provided by the microreactor system, resulting in short diffusion paths, while the flow is non-turbulent and highly ordered. In this way, fluidic mixing becomes controllable and laminar flow regimes are fully developed. Spatial and temporal reaction control is also beneficial for reagents prone to decomposition, which can be directly transferred to the next reaction site after generation. The possibility to transport the microreactor system to the place where the substrates are available or the products are required provides an additional degree of freedom, which is of great importance. A strategic advantage is also the potential to perform multiple reactions and explore a broad range of reaction conditions in a fast and affordable way, as minor amounts of reagents and solutions are utilized while waste production is minimized. Other reaction boundaries like substrate toxicity and

catalyst deactivation may also be overcome as a result of the continuous operation mode of such microscale systems because products are removed and substrates are injected constantly [2,3].

Microfluidics has intruded in the vast majority of fields incorporating bioprocesses. Miniaturization and automation of nucleic acid biochemistry in high-throughput formats, along with small-volume analysis, render fundamental improvements in genomic analysis [4]. Furthermore, single-cell and single-molecule measurements are greatly facilitated through compartmentalization that occurs principally in microfluidic systems. This applicability is of apparent importance if we consider reaching a "single-entity" limit for large and complex biological samples, thus preventing loss of significant information, a common phenomenon occurring in assays such as Enzyme-linked Immunosorbent Assay (ELISA), Polymerase chain reaction (PCR), or Western blots. Microfluidic-enabled culture systems are currently being evaluated for prediction of pharmaceutical response in cell lines, eliminating the need for animal models. Performing chemistry in microfluidic reactors generally provides the ability to generate products of exceptional quality in a direct and robust fashion and has found application in the synthesis of nanomaterials, natural products, and a range of small molecule drugs and pharmaceuticals [5]. Last, but not least, biocatalytic processes have significantly been favored by the high-throughput nature of microfluidic reactors, because catalysts and operating conditions can be rapidly and efficiently assessed with the prospect to evolve the ideal catalytic system [6].

Within the framework of this review, we will focus on the incorporation of microfluidic technology in biocatalysis. A brief analysis concerning microreactor systems in biocatalytic processes will be introduced, followed by a presentation of magnetic microreactors and enzyme immobilization on magnetic particles. Finally, selected examples of magnetic microreactor systems employing immobilized enzymes will be demonstrated and assessed.

2. Microfluidics in Enzyme Biotechnology

2.1. Advantages of Microfluidics for Biocatalysis

Continuous flow microreactors bare the potential to upgrade the application of enzymes in terms of integrated processing and analytical control. Enzymatic microreactors may constitute a stepping stone for the optimization of enzyme-related processes. In particular, they can be used for the synthesis of compounds that are difficult to obtain, the selection of appropriate biocatalysis conditions, or for cost–benefit analysis before scaling-up, while enzyme specificity can be investigated for target analytes determination or proteomic studies. Furthermore, enzymatic microreactors are utilized for kinetic studies and questing of enzymatic inhibitors [7]. Such sophisticated systems provide biocatalysts with the chance to work in a favorable environment, suffering limited damages. Namely, controlled substrate concentration, continuous product removal, no mechanical stirring, and highly controlled pressure and temperature conditions pave the way for significant increases in turnover number and frequency [1]. A noteworthy leverage that flow processing offers is the possibility to perform in-line work-ups and purification procedures of the enzymatic reactions products, circumventing limitations faced during batch biotransformations. Accordingly, real-time reaction monitoring has been established by interfacing flow systems with analytical techniques. LC, GC, and mass spectrometry are common techniques integrated for the analysis of the outlet products. Different types of sensors compatible with microfluidic devices have also been developed, for the monitoring of temperature, pH, dissolved oxygen, substrates, and other compounds concentration and seem particularly attractive because of their noninvasive and nondestructive nature [1,3].

Enzyme immobilization has been extensively reported as a robust biotechnological tool. An immobilized biocatalyst undergoes a wide assortment of benefits, including the following: (a) stabilization against chemical and environmental attacks, a cost-saving feature for large-scale industrial applications; (b) improved substrate accessibility, because aggregation of the hydrophilic protein particles is avoided; (c) improved enzyme performance under extreme reaction conditions and resistance to denaturation; (d) obtainment of the higher product yield per utilized enzyme amount;

(e) reuse of the biocatalyst without any complicated purification procedure; and (f) prolonged enzyme lifetime [7–9]. When it comes to microreactors, an immobilized biocatalyst functioning in the inner of a microcapillary tube has the chance to yield the maximal reactor productivity per its volume and time unit. This fact derives from the high value of enzyme-to-substrate ratio resulting from the local excess of biocatalyst molecules relative to substrate concentration, as well as the large value of the surface-to-volume ratio. A subsequent reduction of the diffusion-related limitations improves the ability of the substrate to reach the active site of the immobilized enzyme [7].

2.2. Fabrication of Enzymatic Microreactors

A microcapillary reactor is an engineered fluidic device that uses a microchannel as the reaction space. Microreactors are made of different materials, usually glass, silicon, or polymeric materials, and are often prepared using GC or LC parts [1]. Glass or silicon-based platforms offer the benefit of hydrophilicity, creating a favorable microenvironment for the biomolecules. They can also be easily modified using well-known organosilanes chemistry (e.g., (3-aminopropyl)triethoxysilane or APTES) and exhibit proper thermal conductivity, baring light transmission properties suitable for detection based on optical effects (fluorescence or light absorption). Concerning polymeric materials, poly(methyl methacrylate) (PMMA) and polydimethylsiloxane (PDMS) are commonly used. PMMA is a biocompatible, mechanically strong, cheap, and transparent material, enabling the spectrophotometric detection and continuous monitoring of the reaction products. PDMS also consists of a low-cost material, which is non-toxic, gas permeable, and light transparent in a wide range of wavelengths. Both of these polymers require surface modification for the improvement of wetting properties (hydrophobic nature) and docking of biomolecules [7,10].

Microfluidic device fabrication has leaped forward with the introduction of three-dimensional (3D) printing techniques. This technology provides a facile way to manufacture devices several micrometers to centimeters in size, in one step, with high-throughput and no need for special trained skills. Reliability and reproducibility may be guaranteed with computer-aided design software (CAD). As conventional microreactor fabrication has long utilized glass and polymeric materials, they also constitute principal 3D printing utilization [11]. It is also noteworthy that 3D printing technology bares the potential to generate sterilizable products, thus expanding the scope of feasible biological applications. Three-dimensional printed microreactors have been reported to consist of biocompatible and photo-curable resins, while heat and organic solvent resistant resins are also currently incorporated in this upcoming technology [12,13].

When it comes to biocatalyst configuration, flow reactors were originally used with free enzymes, but currently, immobilized enzyme reactors (IMERs) have dominated in this research field. The typical microstructured enzyme reactor is a multiphase reactor, in which a solid phase is contacted with the usually aqueous liquid phase. A second, water immiscible liquid phase and/or a gas phase may also be present [3]. There are three major arrangements for immobilized reactors that are commonly used: (i) open-tubular enzymatic microreactors; (ii) monolithic enzymatic microreactors; and (iii) packed-bed enzymatic microreactors.

In open-tubular enzymatic microreactors, the biocatalyst is immobilized in the inner wall of the capillary tube. In such a case, the biomolecule loading capacity is relatively low because of the small available surface area. Therefore, there is a long path for substrate diffusion and the enzymatic conversion can be of a low rate. For this reason, such an IMER needs to be carefully designed in order to be as small as possible to reduce the distance of diffusion and increase surface-to-volume ratio. Another key design feature is the introduction of an additional layer in the inner of the capillary. In this way, minimal flow resistance is achieved, while the substrate molecules barely interact with the support (inner surface), minimizing the retention time in the microreactor [7].

Open-tubular IMERs are usually fabricated using ionic binding, covalent binding, cross-linking, or bioaffinity methods for enzyme immobilization [14]. The inner walls of the capillary can be modified properly so as to introduce reactive groups for the enzyme immobilization. For such

modifications on fused silica capillaries, the silane coupling chemistry is often utilized, by treatment with (3-aminopropyl)trimethoxysilane (APTMS) or (3-aminopropyl)triethoxysilane (APTES). The inner surface of polymer-fabricated capillaries has been treated with polyethylenimine (PEI), which, apart from the large number of amino groups, provides a protective microenvironment for the enzyme. In a following step, these modified surfaces can be treated with glutaraldehyde, a common compound used for the covalent coupling of enzymes to support surfaces. Modification of capillary inner surfaces with polyelectrolytes has also been employed for enzyme immobilization through electrostatic interactions. Attention needs to be paid in the enzyme pI in order to provide a proper charge under given pH conditions, properly adapted to its activity requirements as well [7].

Monolithic supports have been used for enzyme immobilization as they offer interesting properties, like better accessibility of the active site for substrates, stability in most solvents, and versatility of available surface functional groups. Macro- and mesopores ensure the high specific surface area, a feature that makes monolithic microreactors perform greatly, with short diffusion paths and fast mass transfer. The characteristics of immobilized biocatalysts are affected by pore size, porosity, and surface chemistry. Although monolithic IMERs are pretty stable, there is a relatively high degree of complexity for their fabrication. The support materials can be inorganic (e.g., sol-gels), organic polymers (e.g., acrylates or acrylamides), or organic–inorganic hybrid materials. In one approach, the enzyme is immobilized on the support material off-line and the enzyme-material mixture is injected in a next step in the microreactor system. Another approach utilizes monolithic-fabrication techniques, such as radical polymerization, polycondesation or freeze-drying, to modify the inner of the microchannel with the support material, followed by the enzyme immobilization in the modified microreactor [7,14].

Packed-bed enzymatic microreactors are preferred in many cases when compared with open-tubular or monolithic microreactors, because they offer the highest surface-to-volume ratio and sample capacity, while their packing procedure is relatively easy to handle [14]. In this scenario, different particles of micro- or nano-dimensions are employed for enzyme immobilization, using all the methods mentioned above for open-tubular IMERs. The immobilization procedure is carried out in a separate vial before injection into the microreactor system. This fact offers the privilege to perform several assays off-line, related with the enzyme-support interactions, enzyme performance, and enzyme catalytic properties. It is noteworthy that in a study by Boehm et al., it was proven that a packed-bed microreactor yields considerably higher substrate capture and subsequent conversion to product than an open-tubular microreactor, especially when it comes to high flow rates [15]. This hypothesis was also amplified by the lower amount of active enzyme after immobilization on the inner microcapillary walls compared with the chemically less complex immobilization on microspheres. This type of microreactor possesses all the diverse properties of the immobilization support used, which is translated in several advantageous features. For example, different nanoscale supports have been found to reduce diffusion limitations, enhance biocatalytic efficiency, and increasing the enzyme loading given the superior surface area per mass unit. Nanomaterials are wisely designed to enable long-term storage and recycling stability of the immobilized biocatalyst, while their unique physical and chemical properties create a favorable microenvironment for optimal enzymatic efficiencies [16,17].

Once fabricated, the microfluidic reactor has to be attached to the overall microfluidic system via suitable tubes. In general, a microfluidic system involves a fluid supply, a reaction chamber (microreactor) and an outflow collection system. In most cases, all the necessary fluids are provided through appropriate pumps (usually peristaltic or automated syringe pumps). Several analytic methods can also be integrated in the outlet of the microfluidic system in order to achieve in-line analysis. Alternatively, the outflowing product can be analyzed off-line using any of the known analytical techniques.

3. Magnetic Microreactors

3.1. Overview

Application of a magnetic field in bioreactor technology has long been adopted as an approach to improve the performance of batch-scale fluidized bed reactors. Magnetic particles (microparticles, nanoparticles, or beads) have been used either as an additive to the biocatalyst support material, or as an immobilization support themselves. Application of an external magnetic field to control the behavior of the biocatalyst inside the reactor was found to improve the reactor's stability with the absence of strong shear forces and relatively constant pressure, making the reactor system an ideal host for delicate biocatalysts such as enzymes and whole cells. These features have made magnetic reactors useful tools for intensified processes, while the need for downstream separation processes for biocatalyst recovery has been eliminated. Studies employing immobilized enzyme reactors have demonstrated high reaction rates attributed to the vigorous stirring and low agglomeration achieved with the appropriate magnetic field application. Good mixing facilitates a greater surface area exploitation for substrate molecules, making the whole process far more efficient. Studies have also shown elevated stability percentage and several cycles of reusability in comparison with conventional fluidized beds [18,19].

Downscaling this technology with the manufacture of magnetic immobilized enzyme microreactors has revolutionized this scope of research. In microsystems, laminar flow dominates and mixing occurs via diffusion, thus overcoming two principal issues faced in batch scale magnetic reactors. Moreover, greater control over reaction conditions and rapid temperature changes are possible, leading to greater yields of products and higher purities. Small volumes required and facile heat exchange and mass transfer are also crucial features that distinguish microsystems from conventional apparatus [20,21].

3.2. Enzyme Immobilization on Magnetic Particles

Magnetic particles range in size from several nanometers to hundreds of micrometers. These particles consist of a magnetic material (iron, cobalt, nickel, and metal oxides) and a chemical component with functionality [22]. Additionally, microparticles made of nonmagnetic substances (e.g., silica) can be rendered magnetic by coating the silica with ferrous material (e.g., Fe_3O_4) [10]. Magnetic particles are commercially available with a wide variety of different chemical functionalities [23]. When it comes to nanometer scale, excellent properties are observed, such as high surface-area-to-volume ratio, biocompatibility, good mechanical strength, and superparamagnetism [24]. Depending on the synthetic method used, the size, shape, stability, and dispersity of magnetic nanoparticles (MNPs) can be controlled. The synthesized MNPs tend to aggregate because of their high surface energy caused by the large specific surface area. Moreover, iron oxide NPs can be easily oxidized in air, resulting in loss of magnetism and dispersibility [25]. Surface modification of MNPs prevents their aggregation and oxidation. Silica coating is a typical method for the modification of MNPs, in which silica shells are formed on the surfaces of magnetic cores. Silica shells improve the hydrophilicity and biocompatibility, while they provide MNPs with functional groups for further modification with reagents of interest [26]. Metal-organic frameworks (MOFs) have also been used in the construction of magnetic microreactors. MOFs refer to crystalline microporous networks formed by the assembly of metal ions and organic linkers, and bare interesting properties, such as high surface area and readily tunable structures, pore sizes, and compositions [27]. Magnetic micro- and nanoparticles are applied to a wide range of fields including ferrofluids, magnetic resonance imaging, magnetic separation, immobilization of enzymes, biological detection, magnetic catalysis, and water treatment [28].

Systems containing micrometer- or submicrometer-sized magnetic particles, properly functionalized, have been widely applied as carriers for binding proteins, enzymes, and drugs [29]. Magnetic carriers provide large specific surfaces and can be separated rapidly and reliably by applying external magnetic forces [23]. In this way, the separation process is rendered gentle to biomolecules, avoiding the shear forces caused by centrifugation [22]. The magnetic nature of these particles provides the additional

advantage of recovery of expensive proteins and simplification of the purification of complex enzyme mixtures. Another useful utilization of the magnetic property is mixing, an essential facility for enzymatic reactions, as mixing patterns can be generated with the application of oscillating magnetic fields [10].

A diversity of enzyme immobilization procedures for continuous-flow operation systems have been reported, differing in specificity, efficiency, simplicity, and purpose [30]. There are approaches that require genetic manipulation, like the use of genetically encoded tags, which results in an exact site-specific immobilization. Other approaches lead to random distribution and orientation of the enzymes on the support, like "click chemistry" strategies [23].

Physical adsorption is a simple process resulting from the non-specific binding of enzymes on the particles surface because of protein–support interactions. Physical adsorption mostly depends on hydrophobic and electrostatic interactions between the enzyme and the support, while hydrogen bonds and van der Waals forces may also occur [31]. On this realm, direct coupling for enzymes or bioactive molecules onto magnetic particles has been utilized as a result of the presence of hydroxyl groups on the magnetic supports. This method favors the overall process because these magnetic particles are not coated with functionalization materials, conserving a smaller size, thus increasing the ratio of surface-area-to-volume, allowing a greater response to any magnetic field [29].

Covalent binding leads to highly stable biocatalytic systems. The formation of a covalent bond between the support and the enzyme usually requires the presence of a chemical reagent that acts as a cross-linker between terminal functional groups on the surface of particles and the amino-acid side chains on the protein surface. For example, our team has recently reported the covalent immobilization of β-glucosidase on hybrid nanomaterials of graphene oxide-iron magnetic nanoparticles functionalized with APTES and/or oleylamine [32]. The immobilization was carried out using glutaraldehyde as the cross-linker, and the resulted biocatalysts exhibited up to 4.4-fold higher half-time constants than free enzyme, while they were able to be reused up to 12 reaction cycles, retaining up to 40% of their initial activity.

Cross-linked enzyme aggregates (CLEAs) are obtained by precipitation of the enzyme from aqueous media followed by the cross-linking of the physical aggregates using a bifunctional reagent, such as glutaraldehyde [32]. It is a simple and cheap procedure with high immobilization yields that leads to the production of very stable biocatalysts. Lately, magnetic nanoparticles have been introduced to the formation of CLEAs to enhance the catalytic properties of the immobilized biocatalysts. The cellulase enzyme has been reported to form a CLEA–MNP composite with increased hydrolytic activity and the potential to stand in extreme environments [33]. The easy separation of MNPs from media solutions and stability of the synthesized complex during frequent use were proposed as key advantages for the promotion of cellulase industrial applications.

4. Selected Examples

Some noteworthy examples of enzymatic microreactors utilizing a magnetic field are demonstrated in Table 1. A brief analysis of these studies is unfolded in the following paragraphs.

4.1. Exploring the Optimal System Configuration

Several studies focus on optimizing the system configuration in order to exploit magnetic forces in the best possible way. A widely used magnetic system utilizes electric coils in a Helmholtz arrangement. In their research, Hübner et al. used this arrangement for their compartmented microfluidic bioreactor system incorporating a 3D-printed capillary holder (Table 1, entry 8). The microreactor consisted of a reaction area of 25 mm in length and 12 μL reaction volume, loaded with horseradish peroxidase enzyme (HRP), which was immobilized on polyvinyl alcohol-magnetite (M-PVA) spherical beads. The system was provided with an alternating electromagnetic field (alternating current, AC-field) and additionally equipped with cavities to carry cubic permanent magnets to achieve particle retention during processing and easy separation after the reaction is complete. Magnetically-induced

re-suspension inside the fluidic reaction compartment was also achieved with the M-PVA particles causing the formation of particle-chains along the magnetic field lines. Under the influence of the AC-field, the enzyme showed a decreased activity (1.6 $U/g_{particles}$) compared with the perfectly mixed conditions in a reaction tube (6.4 $U/g_{particles}$). This fact was attributed to the agglomeration of enzyme particles affecting the overall substrate accessibility. Recycling tests showed high conversion rates for several cycles for the AC-field mixed immobilisates. The microsystem was efficiently used for flexible process control, allowing testing of several reaction parameters. Ramana et al. explored an innovative method to capture magnetic microparticles in commercially available liquid- and air-based capillary coolant systems (Table 1, entry 3). The magnetic setup comprised a 3D-printed magnet holder for each system (liquid-based and air-based) and neodymium cube magnets aligned either in the repulsion or attraction position. A fused silica capillary was used as reaction chamber in each case, loaded with superparamagnetic silica microparticles (MMs) with immobilized human flavin-containing monooxygenase 3 (hFMO3). This drug-metabolizing enzyme was used for the catalysis of clozapine as a model substrate. In the liquid-based system, it was found that only the attraction arrangement effectively captured the MMs, while the air-based system captured MMs in both arrangements. With regard to catalytic features, in the repulsion position, where the magnetic strength was lower, decreased yields of reaction products were observed compared with the attraction position. However, the reactor structure is better defined with repulsion leading to a better reproducibility of the catalytic system. An alternative study by Šalić et al. compared two different microreactor configurations: a microreactor equipped with permanent square magnets, and a microreactor influenced by an oscillating magnetic field that enables particles movement in the microreactor (Figure 1), (Table 1, entry 5). In this work, alcohol dehydrogenase (ADH) enzyme was immobilized on maghemite (γ-Fe_2O_3) nanoparticles and applied for the NADH oxidation. As the authors notice, when working with permanent magnets, nanoparticles are attached onto one side of the microchannel so the amount of the enzyme available to substrate is restricted, endowing the development of a microreactor driven by an oscillating magnetic field. Compared with a batch reactor working in the same conditions (k_{cat} 1.46 $\times$ 10^2 s^{-1}), the microreactor provided with an oscillating magnetic field offers higher turnover numbers with minimal enzyme consumption (k_{cat} 5.12 $\times$ 10^2 s^{-1}), a fact attributed to the elimination of gravity and inertia forces in the micrometer scale.

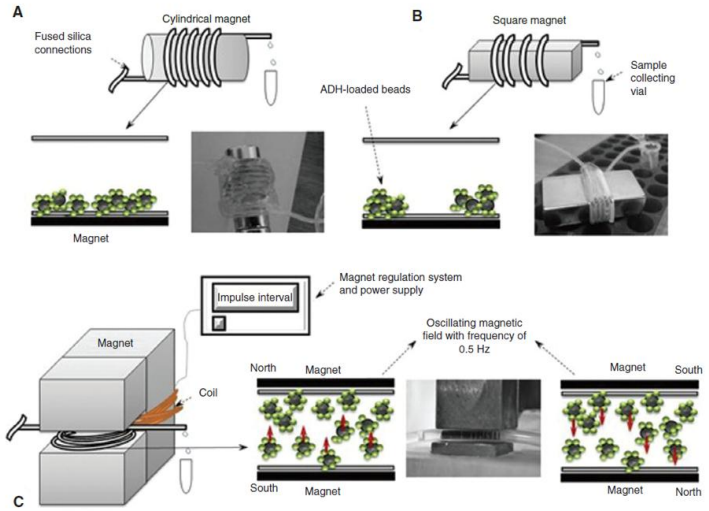

Figure 1. Different microreactor configurations (**A**) with a permanent cylindrical magnet, (**B**) with a permanent square magnet, and (**C**) with an oscillating magnetic field. Reproduced with permission from A. Šalić et al.; NAD^+ oxidation in a microreactor catalysed by ADH immobilized on γ-Fe_2O_3 nanoparticles, published by De Gruyter, 2013.

4.2. Utilizing Multiple-Enzyme Systems

Industrial imperatives have directed research towards biocatalytic pathways through multi-enzyme and cascade enzymatic reactions. The term 'enzymatic cascades' is used to describe (chemo)enzymatic processes that consist of two or more steps for the production of compounds of interest [34]. An in vitro system can be made with the ability to assemble non-natural biocatalytic cascade reactions by mixing and matching enzymes from different sources to generate a desirable end product. Systems biocatalysis has evolved as a novel territory with the aim to design artificial metabolic networks for in vitro biocatalysis [35,36]. Microfluidic systems are already used in this field, as they enable miniaturization and compartmentalization, with small biocatalyst volumes and high spatiotemporal reaction control [15]. A study by J. Shi et al. reported the use of superparamagnetic beads (MBs) for the selective immobilization of the enzymes alcohol dehydrogenase (ADH) and lactate dehydrogenase (LDH), which belong to the family of NAD^+ dependent dehydrogenases (Table 1, entry 1). The enzyme-coated magnetic beads were placed inside a fused silica capillary, in a defined distance, holded by two pairs of identical Nd–Fe–B magnets. A substrate mixture containing acetaldehyde and pyruvate was injected in the capillary, resulting in the formation of two reaction chambers and the simultaneous determination of NADH consumption using a UV-detector placed in the outlet. After optimizing some functional parameters (effect of NADH concentration, amount of MBs, electrophoretic resolution), the system was tested for its reproducibility and enzyme stability, with no significant changes in the activity of enzymes for at least 15 runs. Therefore, a microreactor system was built, with the potential to perform two simultaneous enzyme assays utilizing the same coenzyme in one run. It is noteworthy that the K_m values for the immobilized enzymes were close to the free enzyme forms, while the system was successfully used for the detection of acetaldehyde and pyruvate in real samples. A magnetic microreactor system was also utilized by Peschke et al. for the establishment of a microfluidic enzyme cascade (Figure 2), (Table 1, entry 10). In this research, two stereoselective ketoreductase enzymes, the (R)-selective alcohol dehydrogenase LbADH and the (S)-selective methylglyoxal reductase Gre2p, along with the NADP(H)-regeneration enzyme glucose 1-dehydrogenase (GDH), were immobilized on magnetic beads with the streptavidin binding system. The substrate compound was injected through a computer-controlled automated pump to a multi-microchannel system loaded with enzyme-coated magnetic beads, with each channel containing either the LbADH or the Gre2p enzyme along with the NADP(H) regeneration enzyme in each case, while the outflow was fractionated automatically in 96-well plates and analyzed with chiral HPLC. This way, a convenient configuration was achieved for the control of the stereoselectivity of a two-step biocatalytic transformation. It was shown that the immobilization procedure did not affect the enzymes activity and/or stereoselectivity (operation for 14 days without significant decrease in the enzymes activity or stereoselectivity). The robustness of both the immobilized enzymes and the microfluidic device was proven by long-term stability experiments for each enzyme separately and for the two-enzyme system. Similar experiments were carried out with crude cell extracts immobilized on magnetic beads showing comparable results to the purified enzymes. A study was also carried out concerning different orthogonal tags, demonstrating alternative immobilization methods for enzymes on beads under flow conditions.

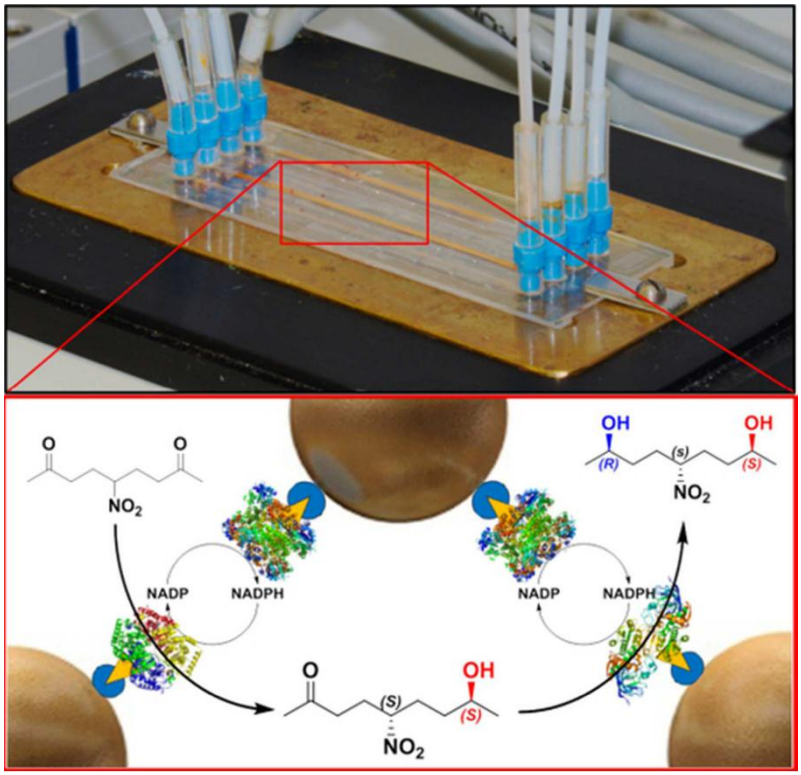

Figure 2. Compartmentalized microreactor with multiple enzymes. Reproduced with permission from Peschke et al.; self-immobilizing fusion enzymes for compartmentalized biocatalysis, published by the American Chemical Society, 2017.

4.3. Emphasizing on the Immobilization Support

Improving the enzyme properties is a main objective of enzyme immobilization and the immobilization system (support, activation method, and immobilization conditions) needs to be carefully designed in order to serve this purpose. Their mechanical strength and recovery potential has made magnetic nanoparticles widely exploited in biosciences [37]. For such applications, magnetic nanoparticles have to bare essential properties, like non-toxicity, chemical stability, size uniformity, stability under physiological conditions, biocompatibility, and high magnetization [38]. However, naked magnetic nanoparticles cannot be used directly for enzyme immobilization and need to be properly modified to prevent aggregation and oxidation, in order to improve their water compatibility and stability and to interact effectively with protein particles [25,26,37,38]. Surface functionalization of magnetic nanoparticles has been performed both with inorganic compounds, like silica, metals, and metal oxides, and organic compounds, like amines, amino–silanes, thiols, and synthetic and natural polymers (PEG, PVA, alginate, dextran, chitosan) [26]. Mandai et al. demonstrated the construction of a microreactor that incorporates magnetic retention of a nanobiocatalyst composed of bacteriogenic iron oxide (BIOX) and lipase enzyme, applied for organic synthesis (Table 1, entry 13). BIOX is a unique tubular assemblage of iron oxide nanoparticles produced by aquatic iron-oxidizing bacteria, with a complex porous surface structure, resulting in relatively large surface area. BIOX by *Leptothrix ochracea* has also been proved as a useful, environmentally benign, and ubiquitous material [39,40], and has been used as a solid support for immobilized enzymes for the kinetic resolution of secondary alcohols [41]. In the work by Mandai et al., magnetized iron oxide derived from BIOX and covered with silicate was used as a solid support for magnetically recoverable and recyclable immobilized lipase [42]. This way, a microreactor was constructed with the use of easily obtainable bio-oriented materials, which was assembled in a simple microflow system with the use of a linear assemblage of neodymium

magnets. The microreactor showed higher product yield when operated for 14 days for the kinetic resolution of alcohols (2,079,000 total turnover number), indicating a more stable product supply than a corresponding batch system (49,500 total turnover number). Liang et al. introduced GO/Fe_3O_4 magnetic nanocomposites as a tunable enzyme immobilization platform for the construction of an enzymatic microreactor (Table 1, entry 4). This nanocomposite can combine the high absorption capacity of graphene oxide and the manipulation convenience of magnetite nanoparticles constituting a promising material for both robust enzyme immobilization and easy retrieval during processing. Acetylcholinesterase (AChE) enzyme was immobilized on the nanocomposite and filled in a PDMS microreactor retained by two permanent magnets. The microdevice was tested for its performance in the catalysis of dimethoate pesticide so that a detection system could be developed. A low detection limit and high sensitivity was realized and attributed to the excellent biocompatibility and high surface area of the magnetic nanocomposite support. The studies also demonstrated that the microreactor could be used repeatedly with a favorable reproducibility (83.2% residual activity for 10 cycles), while the recovery of dimethoate from real samples was proven to be highly accurate (98% to 103.3%).

Table 1. Summary of studies utilizing magnetic enzyme microreactors.

Entry	Type of Reactor	Immobilization Support	Enzyme	Application	Ref.
1	fused-silica capillary microreactor	superparamagnetic beads	alcohol dehydrogenase (ADH)lactate dehydrogenase (LDH)	determination of acetaldeyde and pyruvate content	[43]
2	fused-silica capillary microreactor	magnetite nanoparticles	laccase from *Trametes versicolor*	online recording dopamine (DA) release in the rat brain	[44]
3	fused-silica capillary microreactor	superparamagnetic silica microparticles	human flavin-containing monooxygenase 3 (hFMO3)	drug metabolism	[45]
4	cross-type PDMS microchip	GO/Fe_3O_4 magnetic nanocomposites	acetylcholinesterase (AChe)	determination of organophosphorus pesticides	[46]
5	glass tubular microreactor	maghemite (γ-Fe_2O_3) nanoparticles	alcohol dehydrogenase (ADH)	NADH oxidation	[47]
6	micro-fluidized PDMS chip	carboxyl-functionalized magnetic beads	trypsin from bovine pancreas	tryptic digestion of transthyretin	[48]
7	fused-silica cpillary microreactor	magnetic SiMAG-carboxyl microparticles	cytochrome P450 2C9	on-line kinetic and inhibition studies of clinically and pharmacologically important CYP2C9	[49]
8	fluorinated ethylene propylene capillary microreactor	polyvinyl alcohol–magnetite composite microparticles	horseradish peroxidase (HRP) Type VI	kinetic and recycling studies of immobilized HRP	[50]
9	fused-silica microchip	superparamagnetic nanoparticles	trypsin from bovine pancreas	protein digestion	[51]
10	four-channel PMMA chip	STV-functionalized superparagnetic microbeads	(R)-selective alcohol dehydrogenase (LbADH) (S)-selective methylglyoxal reductase (Gre2p) glucose 1-dehydrogenase (GDH)	stereoselective multi-step reactions	[52]
11	magnetic oscillation microfluidic chip	magnetic beads	benzoylformate decarboxylase from *Pseudomonas putida* (BFD)	stereoselective biocatalytic synthesis of chiral 2-hydroxy ketones	[53]
12	Fused-silica capillary microreacror	hydroxyl group modified superparamagnetic nanospheres	Glucose oxidase (GOx) from *Aspergillus niger*	quantitative detection of glucose in human serum samples	[54]
13	PTFE microtube reactor	Silicate-covered bacteriogenic iron oxide nanoparticles	lipase from *Burkholderia cepacia* (BCL)	kinetic resolution of secondary alcohols	[42]
14	Magne-Chip with multiple magnetic cells	epoxy-magnetic nanoparticles	phenylalanine ammonia-lyase (PAL)	ammonia elimination	[55]

4.4. Optimizing Process Parameters for Scaling-Up

Transition from laboratory to production scale for biocatalytic process development requires preliminary experimentation for the screening of various parameters. Elaboration of microreactors to downside the traditional equipment can reinforce process optimization for the fast and high-throughput testing of activity, stability, and the overall enzyme performance [3]. Jussen et al. utilized a magnetic microfluidic reactor (μMORE) for accelerated parameter optimization prior to scaling-up (Table 1, entry 11). In this research, the required magnetic field was generated by a combination of permanent magnets and oscillating magnetism, providing both magnetic mixing and retention with the minimal disruption to the enzymatic system. Dye distribution tests with rhodamine B were performed in order to optimize the system geometry and evaluate the magnetic bead retention and mixing. In a next stage, the potential of the μMORE system for process development was tested by immobilizing benzoylformate decarboxylase from *Pseudomonas putida* (BFD) enzyme on commercial magnetic beads for the carboligation of benzaldeyde and acetaldehyde. This model reaction is highly demanding and has been utilized in several batch reactor systems. Following, an optimization of catalytic features concerning pH, temperature and substrate concentration indicated that the data obtained were consistent with batch biotransformations. After refining residence time to reach the highest conversion yields, the μMORE system was compared with a 10 mL enzyme membrane reactor (EMR). It is admirable that the μMORE required only 0.005% of the enzyme amount necessary for the EMR, while the μMORE allows parallelization of up to six reactions with the corresponding lab space that an EMR setup requires. Time-saving experimentation was also realized because 34 experimental days in an EMR account for 9 days with the use of a μMORE system. The researchers conclude on a miniaturized system that holds great promise in the screening of new biocatalysts and the obtaining of optimized process parameters with the minimal consumption of catalysts, substrates, solvents, and total experimental duration.

4.5. Other Applications

As mentioned introductory, microfluidic systems have been incorporated in a wide variety of research fields relative to bioprocesses. Schejbal et al. utilized a microreactor in the era of drug development (Table 1, entry 7). Kinetic and inhibition studies of cytochrome P450 were performed after immobilization on magnetic microparticles and integration in a microreactor system for the catalysis of diclofenac as a model substrate. The system required only 0.5 pmol of enzyme for six subsequent analyses, and along with the ease of replacement because of magnetic support, constitutes a promising tool for early stages of drug development.

In the field of biomedicine, sensitive detection systems for online measurements need to be established for the understanding and precaution of various pathological conditions. Lin et al. designed an online analytical system for continuous measurements of dopamine in the brain microdialysate of freely moving rats (Table 1, entry 2). A magnetic microreactor was integrated in the system consisting of laccase enzyme immobilized on magnetite nanoparticles adsorbed onto the inner wall through the effect of an external magnetic field. The microreactor was proven to be highly efficient for the conversion of dopamine and long-term stable for reusability. It was also demonstrated that the immobilized laccase microreactor transited primary compounds into electroinactive products for the avoidance of interference with the dopamine electrochemical signal. The same research group, in another study, demonstrated that a similar configuration can be used for a multi-enzyme detection system, utilizing choline oxidase and catalase enzymes for acetylcholine (Ach) detection *in vivo* [56].

Liu et al. applied a magnetic microreactor in the field of proteolysis (Table 1, entry 9). Trypsin enzyme was immobilized on magnetite nanoparticles and loaded on a microreactor system for the residue-specific proteolysis to generate digested fragments for protein identification. Cytochrome c, bovine serum albumin, and myoglobin were used as model proteins and the digestion through the microreactor produced similar results to the free enzyme form in solution. It is also important that low-abundance proteins can also be detected with this system because a high loading enzyme

concentration is possible and the magnetic nanoparticles provide high surface-area-to-volume ratio. Rat liver extract was also tested in the microreactor system with immobilized trypsin demonstrating efficient digestion of the protein mixture. Bataille et al. developed a microfluidic trypsin microreactor as well (Table 1, entry 6). In this case, enzymatic digestion of the clinically important protein transthyretin was tested with the use of two immobilized microreactors: a magnetic packed-bed and a monolith-based one. The packed-bed microreactor showed a drastic reduction of substrate residence time compared with a batch system. However, the reaction yield was significantly enhanced with the use of the monolithic microreactor. It is noteworthy that the major difference between the two microreactors was the support functionalization (carboxyl for the magnetic particles and amino-functionalized for the monolith support), which could result in distinct enzyme immobilization efficiency, although this fact was not studied further.

Microfluidic systems are currently implemented as new generation biosensors to track and detect biological factors. An optimized diagnostic approach has been enabled because such systems offer high performance, small sizes, and portability, while the costs and diagnostic time are substantially reduced [57]. Sheng et al. demonstrated a microfluidic device for the amperometric determination of glucose, with a convenient and tunable magnetic enzyme microreactor (Table 1, entry 12). The researchers immobilized glucose oxidase (GOx) on magnetic nanoparticles that were packed homogeneously in a microcapillary with the use of external magnets. The reaction product from glucose oxidation, hydrogen peroxide, was electrochemically detected. The detection of glucose was proven to be highly sensitive, while no activity loss was observed for over 50 cycles. It is also important that device-to-device reproducibility was 95.6%, a crucial matter of fact for obtaining consistent results from different devices, that are designed in a specified fashion. The device was also tested in human serum samples without any pretreatment, demonstrating high accuracy.

5. Concluding Remarks and Future Prospects

Microfluidic devices are preferred from an industrial standpoint because of substantial leverages, such as the superior process control and their continuous nature. Immobilized enzyme microreactors take a strong position in current initiatives because they have revolutionized biocatalytic processes. Magnetic materials of microscale dimensions lead to strong magnetic field gradients and enhanced forces. The combination of microfluidic technology, enzyme catalytic properties, and magnetic supports seems to be extremely promising if we consider the magnitude of research effort exerted.

Detailed studies have already been performed with emphasis on different system parameters. Instrumentation and magnetic force generation constitute a fundamental research aspect in order to fully exploit the system capabilities and to open the road towards hyphenated lab-on-a-chip systems.

Microreactors have intruded in the field of synthetic biology and attempts are made to integrate multiple enzyme systems in a single microreactor space. This era of research, despite the major difficulties that are faced because of the intrinsic complexity, is about to bring radical novelties for in vitro biocatalysis, by combining non-natural enzymatic activities in one run.

Material science is also implicated with the microreactor technology, as novel immobilization supports need to be designed. It has victoriously been proven that the proper support material has the potential to reinforce enzyme catalytic properties, leading to microfluidic systems with exceptional yields.

Diverse research fields like drug development, biomedicine, proteomic analysis, and biosensors have also incorporated magnetic enzyme microreactors with long-term perspectives.

In conclusion, there is still much to optimize and several barriers to surpass concerning magnetic enzyme microreactor configurations. Nevertheless, it is obvious that we are looking towards a "technology of the future", with small, but stable steps in almost every aspect of biotechnology.

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