

## “Protein immobilization technology for flow biocatalysis”

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**Highlights:** 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point)

- Enzyme encapsulation in sol-gel matrices, 3D-printing, and all-enzymes hydrogels
- Metal-affinity immobilization of His-Tag enzymes on EziG™ and vortex fluidic devices
- Covalent immobilization of multienzymatic cascade systems in tandem reactors
- Co-immobilization of multienzymatic systems and redox cofactors

**Abstract:** (100 -120 words)

Enzymatic immobilization has been at the forefront of applied biocatalysis as it enables convenient isolation and reuse of the catalyst if the target reaction is conducted in batch, and it has opened up significant opportunities to conduct biocatalysis in continuous mode. Over the last few years, an array of techniques to immobilize enzymes have been developed, spanning from covalent multi-point attachment, to non-covalent electrostatic strategies, to rational architecture to suitably orient the enzyme(s). In addition, new materials have been adapted to support biological catalysts. Here we discuss the advances of the last two years in enzyme immobilization for continuous flow applications.

### Introduction

Continuous flow synthesis of fine chemicals has been rapidly expanding in industrial settings [1]. At the same time, the application of biocatalysis in synthetic chemistry is now considered one of the most promising technologies in the green chemistry context [2], and the potential of merging both technologies, continuous flow synthesis and biocatalysis, has been extensively acknowledged with an important number of publications and several reviews in the area over the past two years [1–3].

This review focusses on the most recent developments in continuous flow biocatalysis with emphasis on novel enzyme immobilization and encapsulation technologies (Figure 1), and their applications in multi-enzymatic systems to address more complex chemical functionalities such as cofactor recycling systems to enable cost-efficient redox biocatalysis, and their implementation.

### Encapsulation and enzyme immobilization in flow biocatalysis

Despite several advantages offered by whole cell biocatalysis [1], and their applicability to continuous flow [4–6], cell free enzyme biocatalysis is generally more versatile for continuous synthetic applications. Although there are examples of soluble enzymes used in continuous flow systems [7], enzyme immobilization technology plays a key role in this field as it allows the catalyst(s) to be retained within the reactor while reagents flow through the system. Enzyme immobilization decreases the required quantity of enzyme, simplifies product work-up and potentially increases enzyme stability. Two main approaches have been developed; enzymes are either immobilized onto the reactor wall

(i.e. wall coated reactor), or on particles (carrier material), which are then packed into a tube (i.e. packed-bed reactor) [8].

Enzyme immobilization techniques can be generally grouped into physical and chemical immobilization methods. Encapsulation is a physical separation of the enzyme molecules from the reaction environment achieved with an organic or an inorganic polymer in a strategic and directional manner. Chemical immobilization involves the interaction of enzymes molecule with a support.

Physical entrapment is generally a milder process for the enzymes, and different strategies have been proposed for continuous flow reactors [9,10]. Poppe and co-workers designed a methodology of sol-gel enzyme entrapment based on bioinformatics and experimental design tools. Lipase B from *Candida antarctica* (CalB) was entrapped in sol-gel matrices, and the resulting biocatalysts were used in the kinetic resolution of secondary alcohols and amines in continuous flow. Remarkably, this “immobilization engineering” approach allowed individual optimization of different organosilane precursor compositions for each substrate. The sol-gel entrapped CalB could be operated in a continuous packed bed reactor for 5 days at 0.6 mL/L flow rate and 60 °C resulting in a space time yield (STY) of 0.81 Kg L<sup>-1</sup> h<sup>-1</sup>, and a specific productivity of the enzyme of 0.44 Kg g<sup>-1</sup> day<sup>-1</sup> reaching very high enantiomeric purity [11].

Rabe and co-workers developed a novel enzyme encapsulation technology through 3D-printing of agarose hydrogel containing a thermostable mutant of ketoisovalerate decarboxylase from *Lactococcus lactus* (KIVD\_mutant) with a syringe-based extrusion printer. The continuous decarboxylation of ketoisovalerate to isobutyraldehyde was then successfully demonstrated by combining four KIDV\_mutant discs in a modular fashion, at a flow rate of 25 µL/min, with a 40% conversion. Despite some leaching after 4 h, the enzyme embedded in the gel showed significant catalytic activity in the presence of organic solvent, and a modular system containing different enzymes encapsulated in different discs was assembled to catalyse a two-step biocatalytic reaction, paving the way for multistep flow biocatalysis with a defined sequence of biocatalysts. Nevertheless, a flow rate of 25 µL/min is low and the productivity could be limited even further as the cascade lengthen [12].

Niemeyer and co-workers reported for the first time a very particular type of encapsulation strategy based on self-assembly of all-enzymes hydrogels (Figure 2). This was successfully applied to the reduction reaction of C<sub>5</sub>-symmetrical 5-nitrononane-2,8-dione (NDK) in a continuous flow microfluidic reactor. This novel technique used reactive peptide partners, SpyCatcher (SC) and SpyTag (ST), which spontaneously form a covalent isopeptide linkage under physiological conditions. The two short peptides were genetically fused to the enzyme catalysing the reaction, an (R)-selective alcohol dehydrogenase from *Lactobacillus brevis* (LbADH-SC), and the cofactor recycling enzyme, glucose 1-dehydrogenase from *Bacillus subtilis* (GDH-ST), respectively. This system was used in a microfluidic device to produce (R, R)-diol from NDK with a flowrate of 10 µL/min. The hydrogel effectively retained the catalysts with a stable conversion of the substrate for more than 6 days and could be used at 200 µL/min with an impressive 45 s reactor residence time [13], significantly improving on previous performance [14].

Another method of affinity immobilization exploits the classic polyhistidine tag (His-tag) routinely fused to proteins for easy purification, to bind the catalyst onto matrices displaying metal ions. Turner *et al.* immobilized a His-tag alcohol oxidase on EziG™ Amber (controlled pore glass coated with an organic polymer and chelated Fe<sup>3+</sup>) reaching an enzyme loading of 10% w w<sup>-1</sup>. At a flow rate of 0.5 mL/min, 30% conversion to hexanal was obtained and it was maintained for at least 120 column volumes [2]. The same immobilization strategy was applied by Mutti and co-workers to immobilize ω-

transaminase from *Arthrobacter* sp. with 100% immobilization yield to develop the continuous flow racemic resolution of rac- $\alpha$ -methyl-benzylamine (rac- $\alpha$ -MBA). 5 g of (S)- $\alpha$ -MBA (>49% conversion, > 99% ee) were produced in 96h, with no detectable loss of catalytic activity and with a calculated TON value of 110,000 with a STY of 335 g L<sup>-1</sup> h<sup>-1</sup> [15].

Britton and co-workers reported a straightforward approach to immobilize proteins by affinity binding on the walls of an angled borosilicate reactor through the micromixing and vibrational effects created by rapid rotation (vortex fluidic device; VFD). Alkaline phosphatase and phosphodiesterase were purified and immobilized by this strategy and used for the continuous flow transformation of *p*-nitrophenol into *p*-nitrophenoxide at 0.5 mL/min flow rate, obtaining 4% conversion in ca. 10 minutes. This strategy could be useful in multi-step flow biocatalysis when compartmentalization and specific ordering of enzymes are required [16].

Although metal-affinity binding immobilization has been used in many bio-flow applications, [2] a lack of enzyme stability, in the absence of rigidification throughout the enzyme structure, is expected. Enzyme stabilization reached by rigidification is especially significant when the binding is covalent [17]. In continuous biocatalysis, where the aim is to achieve high accumulated STY normalised to amount of enzyme, the achieved operational stability of the enzyme upon immobilization is instrumental, and covalent immobilization strategies offer significant advantages [18–21].

Paradisi and co-workers exploited a covalent enzyme immobilization strategy to immobilize the (S)-selective transaminase from *Halomonas elongata* (HEWT) and developed the first example of cell-free biocatalytic synthesis of different amines in a continuous flow packed-bed reactor [22]. Here, the His-tag-driven affinity binding to the metal-functionalized epoxy-support, is followed by the reaction of the enzyme amine groups with available epoxy groups, promoting covalent immobilization yielding 30-40% recovered activity. This packed-bed flow reactor was applied to the synthesis of cinnamylamine from cinnamylaldehyde (90% conversion, 2 min residence time) with an 86% isolated yield after an in-line purification step. The same packed-bed flow reactor was also used in the reverse reaction to prepare aldehydes with >80% isolation yields [23]. To minimise catalyst distortion, the team fused a sacrificial protein to the target enzyme to form covalent bonds with the support while shielding the biocatalyst and preserving its catalytic activity up to 90% [24,25].

### **Multienzymatic systems in flow biocatalysis**

To meet the demanding diversity of chemical functionalities in industry, the use of biocatalytic cascade systems in synthetic applications has been proposed as a suitable and efficient approach [26]. In continuous flow synthetic biocatalysis, the co-immobilization of multienzymatic systems is therefore required. To date still only few examples have been reported, with various degrees of success.

The EziG™ Amber support was successfully used by Turner and co-workers for the co-immobilization of two enzymes to develop the biocatalytic hydrogen-borrowing cascade for the asymmetric amination of racemic alcohols, in a continuous flow reactor. In addition, they also reported on an engineered variant of secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (TeSADH) and chimeric amine dehydrogenase (ChiAmDH) for the continuous amination of 1-(4-fluorophenyl)propan-2-ol (10 mM) to 4-fluoroamphetamine. This system, with a flow of 0.02 mL/min, yielded 13 g L<sup>-1</sup> day<sup>-1</sup>. Remarkably, only 0.1 equivalents of the redox cofactor NAD<sup>+</sup> were added [2].

Paradisi and co-workers developed an original closed-loop platform of packed-bed flow reactors for the synthesis of high-value primary and secondary alcohols from a variety of commercial amines based

on enzyme covalent immobilization. HEWT and a suitable reductase were covalently immobilized via the His-tag directed strategy described above, to set up two packed-bed reactors. Of industrial relevance, the conversion of dopamine to hydroxytyrosol was achieved with 45 min residence time using a biphasic stream toluene/buffer and yielding 75% isolated product. Similarly, tryptophol and histaminol were synthesised from the corresponding biogenic amines yielding 70% and 68% isolated product, respectively [27] (Figure 3a).

While not the main focus of this review, it is worth mentioning that to address diversity of chemical functionalities in continuous flow systems, the combination of flow biocatalysis with chemo-catalysed steps offers excellent opportunities, provided that the enzyme immobilization technology is compatible with the conditions required in the chemical reaction step, such as organic solvents. Bornscheuer and co-workers developed a two-step one-pot-chemo-enzymatic cascade involving palladium-catalysed Suzuki-Miyaura cross coupling and an engineered variant of transaminase for the continuous flow synthesis of 1-(5-phenylpyridin-3-yl)ethanamine achieving a 43% conversion [28] (Figure 3b). Moreover, Poppe and co-workers achieved a fully continuous dynamic kinetic resolution (DKR) of racemic 1-phenylethylamine (96% yield, >99% e.e.) with a mixed packed-bed reactor combining the robust sol-gel immobilized lipase CalB for DKR, and palladium on aminopropyl-grafted silica for mild racemization [29].

### **Cofactor recycling in flow biocatalysis**

Oxidoreductases catalyse many important reaction steps in synthetic applications. However, the exogenous addition of redox cofactors imposes economic restrictions on process viability on an industrial scale. The coupling of cofactor recycling systems, mostly by auxiliary enzymes, has been proposed as an efficient approach also in flow applications [1,2,27,30]. López Gallego and co-workers further expanded the enzymatic cofactor recycling technology by co-immobilization of a multienzymatic system, together with the required cofactor, and developed the first example of a self-sufficient heterogeneous biocatalyst for the continuous asymmetric reduction of prochiral ketones in aqueous media. To overcome the mass-transfer limitation, the cofactor is immobilized by ionic adsorption on positively charged porous materials allowing association/dissociation equilibrium within the porous space and hence, facilitating diffusion to the active sites of the covalently co-immobilized enzymes (Figure 3c). Co-immobilization of alcohol dehydrogenase, formate dehydrogenase, and NAD<sup>+</sup> was used to set up a packed-bed flow reactor for the continuous reduction of 2,2,2-trifluoroacetophenone to S-(trifluoromethyl)benzyl alcohol without addition of exogenous cofactor. At 50  $\mu\text{L}/\text{min}$  flow rate, 100% conversion was achieved with a volumetric productivity value of 250  $\mu\text{M}_{\text{L-norvaline}}/\text{min}$ , an ee value >99%. Remarkably, immobilized NAD<sup>+</sup> was efficiently recycled, accumulating a TTN value of 85 after 107 hours without significant leaching [31]. This system was expanded to the co-immobilization and co-localization of a commercial ketoreductase (KRED) and NADP<sup>+</sup> for the continuous synthesis to S-(trifluoromethyl)benzyl alcohol from 2,2,2-trifluoroacetophenone [32], and for the co-immobilization of pyridoxal phosphate (PLP) with HEWT transaminase to generate a self-sufficient reactor for the continuous flow synthesis of high value biogenic aldehydes [33].

### **Scale up implementation and process intensification**

Despite recent development on synthetic biocatalytic processes, their implementation on industrial scale demands more thorough economical assessments. Industrial-scale biocatalytic reactions, for example, require significant substrate concentrations, ideally >100 g L<sup>-1</sup>, otherwise the reactor

capacity is wasted by basically “stirring water”, as pointed out by an excellent review by Hollmann and co-workers [34]. An important aspect of flow processes is their scalability [3].

A biocatalytic reaction on a multi-gram scale for the continuous flow synthesis of melatonin (and analogues) in an ultra-efficient closed-loop strategy was developed by Paradisi and co-workers using a covalently immobilized acetyltransferase from *Mycobacterium smegmatis* (MsAcT). A packed-bed flow reactor was used for the continuous synthesis of *N*-(2-phenylethyl)acetamide from 1M 2-phenylethylamine ( $120 \text{ g L}^{-1}$ ) in a biphasic flow system (5:15 aqueous phase/EtOAc), with 5 min residence time, allowing *N*-acetylation with 90% conversion and an unprecedented amide production of  $56 \text{ g day}^{-1}$  using only 1.6 mg of MsAcT (Figure 3d) [35].

Another key consideration in the application of flow biocatalysis on industrial scale is process intensification. Bolívar and co-workers developed a powerful engineering tool for process intensification in oxygen-dependent biocatalytic reactions by the implementation of a “high pressure flow approach”. This strategy allowed to increase the dissolved oxygen concentration in a single liquid aqueous phase 170-fold (43 mM) under pressurized conditions (43 bar), thus eliminating the well-known restriction of gas-liquid oxygen transfer in this type of biocatalytic reactions. D-amino-acid:oxygen oxidoreductase from *Trigonopsis variabilis* (DAAO) and catalase from *Bordetella pertussis* (BpCAT) were immobilized by a versatile non-covalent affinity binding recently used in continuous microflow applications [36–39]. These enzymes were genetically fused to the module  $Z_{\text{basic2}}$ , and co-immobilized on Rel-sulfonate matrix (methacrylic resin displaying anionic groups), to set up a packed-bed flow reactor which was operated at 34 bar and 25 °C. This pressurized packed-bed flow reactor was used for the continuous deamination of D-methionine (100 mM) reaching 80 mM product concentration; > 100,000 TON; and  $190 \text{ g L}^{-1} \text{ h}^{-1}$  (25 mM/min) STY. This technology offers huge potential to increase the STY of oxygen-dependent flow biocatalysis, thus extending its process windows, and providing opportunities for process intensification in this type of biocatalytic reactions [40].

## Conclusions

An overview of the progress in the field of enzyme immobilization to facilitate continuous synthesis via (multi)biocatalytic steps of the last two years has been presented. It is unquestionable that this field is rapidly expanding, and it is also very clear how there is no universal immobilization technique. Systems with increased complexity are also being developed, with new challenges becoming obvious (compatibility of the different enzymatic steps, provisions for co-factor requirements, waste management, etc.) and innovative solutions are enabling excellent alternatives to traditional synthetic approaches. The merging of flow-chemistry with biocatalysis is showing how common issues observed in batch mode (feed-back inhibition, inefficient catalysis, intermediate degradation, etc.) are no longer observed in continuous processing. A clear effort in bridging the gap between academia and industry is also ongoing, with several examples highlighting the scale that can be achieved through biotransformation in continuous. The horizon for flow-biocatalysis is bright and it is expected that this field will continue to grow. Industrial uptake of this technology has not yet been significant, but it is likely only a matter of time.

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## Figures captions

Figure 1. General overview of the review focusing on the advances of the last two years in enzyme immobilization and encapsulation technologies for continuous flow applications in synthetic biocatalysis.

Figure 2. A schematic description of the encapsulation strategy based on self-assembly of all-enzymes hydrogels based on the interaction of reactive peptide partners, SpyCatcher (SC) and SpyTag (ST). The two short peptides were genetically fused to the enzymes catalysing the reaction, an (R)-selective alcohol dehydrogenase from *Lactobacillus brevis* (LbADH-SC; red), and the cofactor recycling enzyme, glucose 1-dehydrogenase from *Bacillus subtilis* (GDH-ST; blue).

Figure 3. A schematic description of different immobilization technologies. (A) A two-step immobilization strategy by affinity binding of a His-tagged enzyme to the metal-functionalized epoxy-support, followed by the reaction of the lysine reactive groups with nearby epoxy groups, generating covalent bonds, and its application in a closed-loop platform of packed-bed reactors for a multienzymatic biocatalytic cascade. (B) An affinity immobilization method exploiting a His-tagged enzyme to bind onto matrices displaying metal ions, and its application in a two-step one-pot-chemo-enzymatic cascade involving Pd-catalysed Suzuki-Miyaura cross coupling. (C) An ionic adsorption immobilization strategy which binds the negatively charged NAD<sup>+</sup> cofactor to the positively charged polyethylenimine (PEI) coating the enzymes covalently immobilized to the support, and its use for in-situ cofactor recycling. (D) A covalent immobilization strategy on glyoxyl activated support applied to a multi-gram scale biocatalytic synthesis in continuous flow.

Figure 1

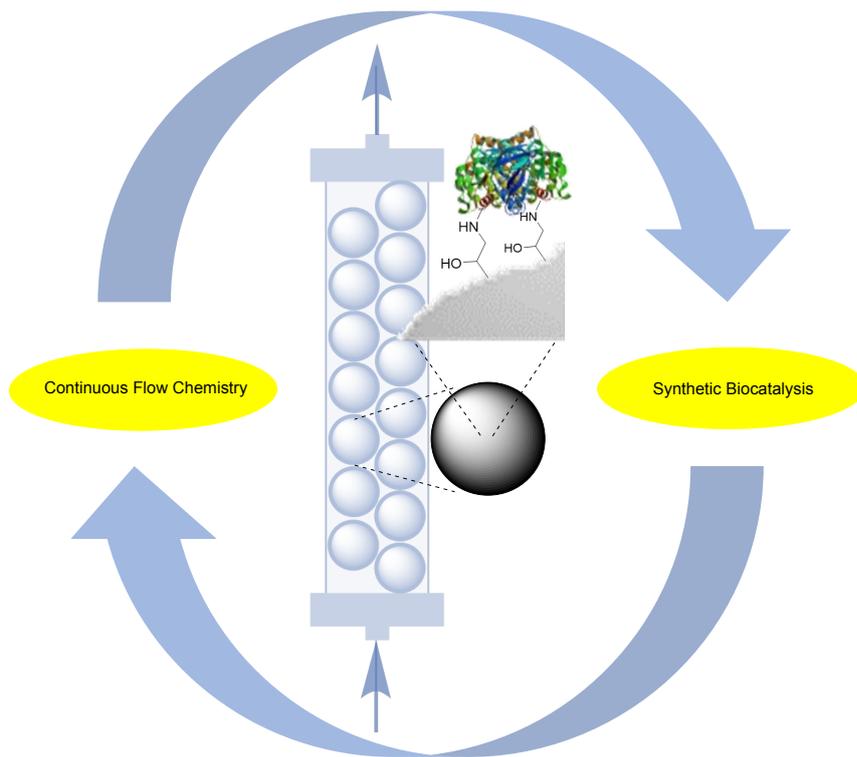


Figure 2

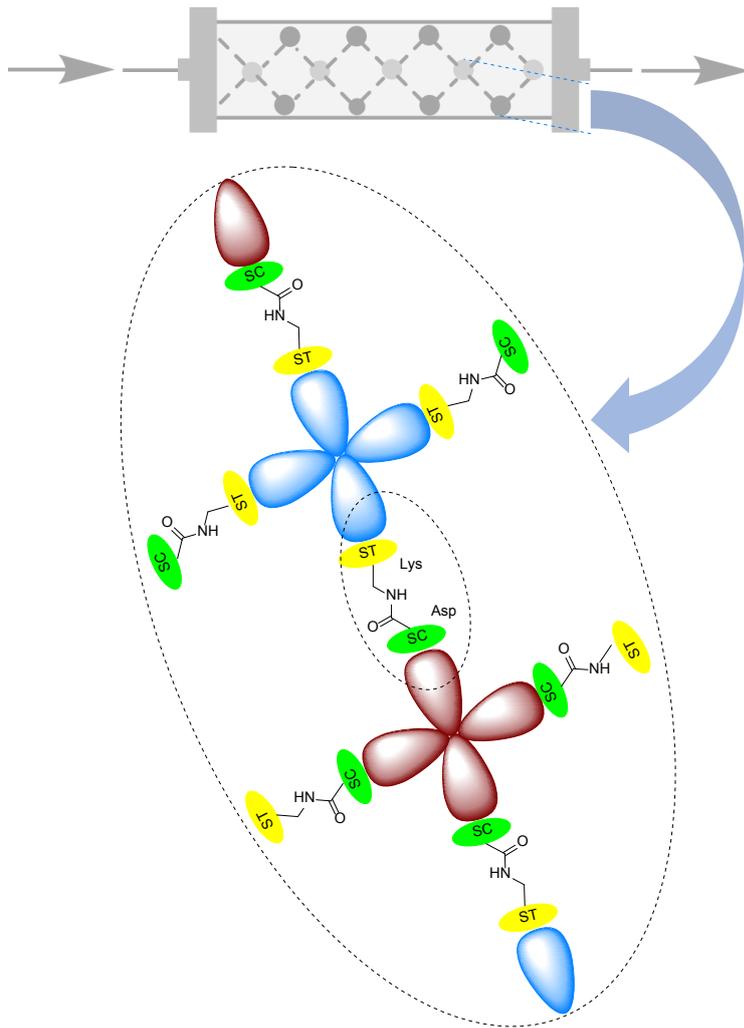
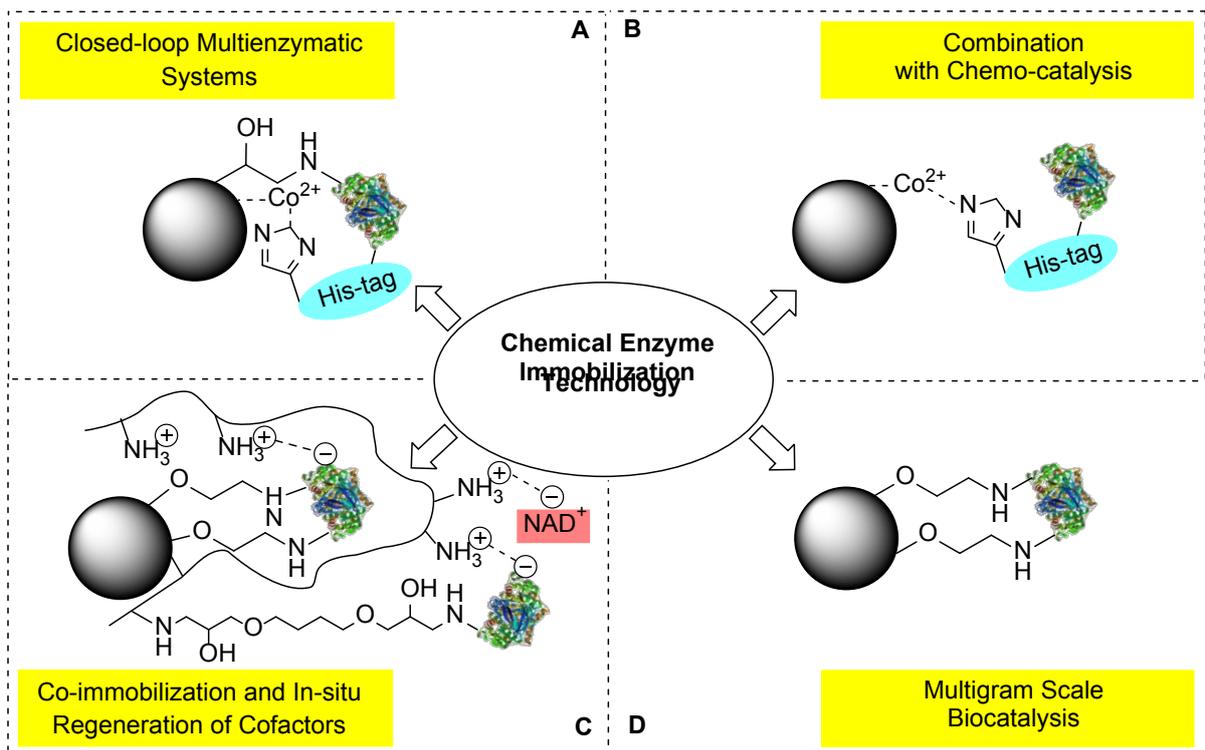


Figure 3



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This work describes a novel enzyme encapsulation technology through 3D-printing of agarose hydrogel containing thermostable enzymes embedded in their structures, using an enzyme-containing bio-ink in a syringe-based extrusion printer. This strategy was used for the fabrication of flow reactor cartridges that were applied in a continuous decarboxylation reaction of ketoisovalerate to isobutyraldehyde.

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First example in the literature of fabrication of all-enzymes hydrogels. This is a very particular type of enzyme encapsulation strategy based on self-assembly of enzymes fused to reactive peptide partners, SpyCatcher and SpyTag, which spontaneously form a covalent isopeptide linkage under physiological conditions. This was successfully applied to the reduction reaction of C<sub>5</sub>-symmetrical 5-nitrononane-2,8-dione in a continuous flow microfluidic reactor.

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Straightforward approach to proteins immobilization by affinity binding on the walls of an angled borosilicate reactor through the micromixing and vibrational effects created by rapid rotation. The relevance of this technology is its speed (ten-minutes) which allows direct purification and immobilization of His-tag fused enzymes, from the cell lysate, and was successfully used for the continuous flow biotransformation of *p*-nitrophenol into *p*-nitrophenoxide.

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Application of a covalent immobilization strategy based on affinity binding to set up an original closed-loop platform made of sequential packed bed reactors. The covalent immobilization strategy allows for extensive enzyme stability in a biphasic stream toluene/buffer used in the synthesis of alcohols from amines, and shows the continuous synthesis of industrially relevant alcohols, such as hydroxytyrosol, with high product yields and in-line purification steps.

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Application of a non-covalent immobilization strategy, based on the affinity binding of the fused Z<sub>basic2</sub> module, to the implementation of a “high pressure flow approach” for the first time in literature. This approach allows to increase the dissolved oxygen concentration in a single liquid aqueous phase under pressurized conditions, and it was successfully used for the continuous biocatalytic reaction of oxidative deamination of D-methionine. This work demonstrates that this technology provides opportunities for process intensification in oxygen-dependent biocatalytic reactions.