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REVIEW

Advances in cofactor immobilization for enhanced continuous-flow biocatalysis

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Abstract

The merging of biocatalysis with continuous-flow chemistry opens up new opportunities for sustainable and efficient chemical synthesis. Cofactor-dependent enzymes are essential for various industrially attractive biocatalytic reactions. However, implementing these enzymes and biocatalytic reactions in industry remains challenging due to the inherent cost of cofactors and the requirement for their external supply in significant quantities. The development of efficient, low cost, simple and versatile methods for cofactor immobilization can address this important obstacle for biocatalysis in flow. This review explores recent progress in cofactor immobilization for biocatalysis by analyzing advantages and current limitations of the available methods that comprise covalent tethering, ionic adsorption, physical entrapment, and hybrid variations thereof. Moreover, this review analyzes all these immobilization techniques specifically for their utilization in continuous-flow chemistry and provides a perspective for future work in this area. This review will serve as a guide for steering the field towards more sustainable and economically viable continuous-flow biocatalysis.

Highlights

- Integration of cofactor immobilization along with enzyme immobilization can greatly enhance the sustainability of biocatalysis in flow.
- Various cofactor immobilization methodologies are available, each of them demonstrating a unique set of advantages and limitations.
- Hybrid co-immobilization methodologies for cofactors and enzymes seem to be promising for the generation of a more universal platform for biocatalysis in flow.

Keywords Continuous-flow biocatalysis · Cofactor immobilization · Enzyme immobilization · Covalent attachment · Ionic adsorption · Entrapment

Introduction

In the dynamic landscape of chemical manufacturing and process industries, the quest for greener, more efficient, and sustainable methodologies remains a paramount objective [1–3]. Biocatalysis, which leverages enzymes to drive chemical transformations, has emerged as an effective tool to contribute toward achieving these goals [4–14]. Recent years have witnessed a surge in interest and application of biocatalysis in conjunction with continuous-flow chemistry, further enhancing its potential across various industrial sectors [15–21]. Continuous-flow grants a high degree of process control and reliability, as well as the possibility for automation and a heightened level of safety [22]. The efficient mixing inherent in continuous-flow systems ensures improved mass transfer rates, leading to enhanced reaction kinetics and higher yields. Besides, when employed for biocatalytic reactions, flow chemistry circumvents possible limitations such as substrate and/or product inhibition, or unfavorable thermodynamic equilibria [22]. To maintain a stable reactor environment and preserve enzymatic activity during continuous-flow biocatalysis, limited leaching of

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enzymes from the system is essential. Enzyme confinement into the reactor can be achieved by either physical immobilization or chemical binding to a carrier, the latter of which via covalent or non-covalent interactions [23]. Therefore, enzyme immobilization ensures sustained catalytic efficiency over extended reaction times and cycles, thereby enabling biocatalyst reusability, ease of separation, and limited sensitivity to process conditions or settings [24].

It is intuitive that cofactor-independent enzymes are the easiest to implement in a continuous flow, especially for industrial applications. However, reactions catalyzed by cofactor-dependent enzymes are among the most attractive. Notable examples are NAD(P)-dependent alcohol dehydrogenases (ADHs) (also called ketoreductases, KREDs) to create chiral alcohols, PLP-dependent transaminases (TAs) for formal reductive amination of prochiral ketones, and diffusible flavin-dependent monooxygenases for e.g., C = C epoxidation [4]. In the absence of a suitable cofactor-recycling system, cofactor-dependent enzymes become inapplicable for industrial application due to the relatively high costs of the cofactors if used in stoichiometric amount. Several cofactor-recycling approaches are currently available for biocatalytic reactions. For example, for the regeneration of NAD(P)H, a coupled-enzyme approach consists of the addition of a formate dehydrogenase (FDH) and formate, or a glucose dehydrogenase (GDH) and D-glucose, or phosphite dehydrogenase and phosphite [25]. On the other hand, a substrate-coupled approach for NADP(H) recycling can be established by adding a sacrificial substrate such as isopropanol that is converted by the same enzyme. The same concept is valid for PMP regeneration in TA-catalyzed reactions. In this case, PLP and an amine donor (e.g., L-alanine) are converted by the same TA into PMP and a co-product (e.g., pyruvate) in the second step of the classical ping-pong bi-bi mechanism [26, 27]. Therefore, the need for continuous addition of cofactors in a high amount and their isolation in down-stream processes represent a major technical challenge in moving biocatalytic reactions into continuous-flow reactors.

A solution to this challenge is the co-immobilization of enzymes and cofactors in flow reactors. This is not a trivial task since the immobilized cofactor must retain high catalytic activity while being accessible to the immobilized enzymes in the confined space of the reactor [28]. This is particularly challenging in reaction environments characterized by high ionic strength that can promote enzyme leaching [29]. The compatibility of the methods used for cofactor and enzyme immobilization is also an indispensable criterion for preserving biocatalytic performance. Cofactor and enzyme must also exhibit high stability within the co-immobilized framework to ensure long-term operational efficiency [30, 31]. Furthermore, the biocatalytic system in flow should ideally exhibit high productivity, total turnover numbers (TTNs), and scalability from laboratory conditions.

This manuscript critically reviews the state-of-the-art for co-immobilization of cofactors and enzymes, mostly focusing on those available for continuous-flow biocatalysis. These methods include covalent tethering to carrier materials, ionic adsorption onto cationic polymers, physical entrapment of both cofactors and enzymes and hybrid variations thereof. For the different immobilization techniques, we will provide a critical analysis of biocatalytic and immobilization efficiencies, discuss their potential for adoption to a diverse set of cofactors and enzymes, and propose prospects for future research and industrial applications.

General approaches for cofactor immobilization

Early examples of cofactor immobilization involved tethering them to large polymers such as polyethylene glycol (PEG). These methods facilitated the successful integration of NAD(P)-dependent enzyme systems into ultrafiltration membrane reactors for continuous-reduction processes, obviating the need for continuous cofactor supply [32–34]. However, economic limitations imposed by ultrafiltration membranes prompted the development of more cost-effective alternatives. Recent advancements in cofactor immobilization comprise covalent attachment, ionic adsorption, entrapment, and hybrid methods (Fig. 1, Table 1).

Innovative approaches have redefined the role of polymers, with them now serving as flexible swinging arms to shuttle cofactors between active sites while remaining anchored to a carrier material. For example, a polypeptide swinging arm was coupled to NAD^+ via an amide bond at the primary amine moiety of its adenine and attached to a resin carrier bead at the C-terminus [35]. Alternatively, short single-stranded DNA fragments can act as flexible swinging linkers. In this context, the cofactor NAD^+ is covalently tethered to an oligonucleotide linker, which itself can be attached to a DNA framework such as a hexagon-type DNA scaffold that allows for topological organization, or a DNA double-crossover tile scaffold [36, 37]. The swinging arm can also be attached to the enzymes; for instance, NAD^+ and ATP were attached to a PEG linker that was positioned between the catalytic enzyme and the cofactor-recycling enzyme [38]. In other covalent attachment techniques, the cofactor is closely or directly attached to the carrier material. Most of these cofactor immobilization approaches are geared towards NAD(P). A prevalent method involves the immobilization of NAD^+ or PLP through covalent binding to epoxide groups on silica nanoparticles, epoxy resin carriers or even sol-gels [39–42]. In alternative methods, the NADH is attached to nano-porous glass supports via a PEG

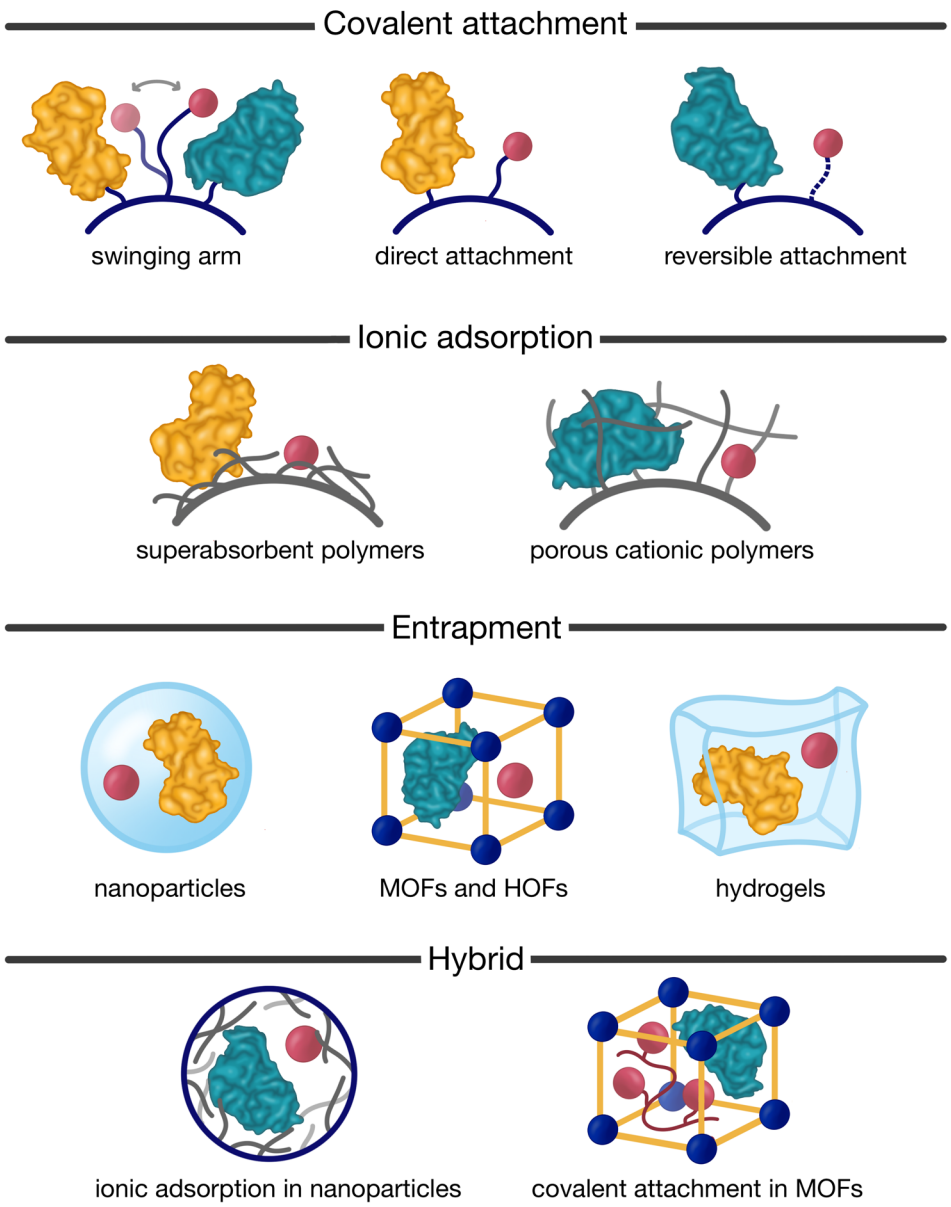


Fig. 1 Schematic depiction of the most common techniques for cofactor immobilization, categorized as covalent attachment, ionic adsorption, entrapment and hybrid systems

Table 1 Classification and descriptions of the common approaches for cofactor immobilization

Category	Approach
Covalent attachment	Cofactor attachment to a flexible swinging arm constructed from a polymer linker
	Direct covalent attachment of the cofactor to a solid carrier material
	Reversible covalent binding of cofactors to carrier beads
Ionic adsorption	Ionic adsorption of cofactors onto acrylate-based superabsorbent polymers
	Cofactor immobilization via ionic adsorption onto porous cationic polymers
Entrapment	Co-compartmentalization of enzymes and cofactors in nanoparticles
	Encapsulation of enzymes and cofactors in MOFs or HOFs
	Cofactor retention in synthetic copolymer- or enzyme-based hydrogels
Hybrid	Cationic polymer aided encapsulation of cofactors into nanoparticles or hydrogels
	Entrapment of a covalently tethered cofactors and enzymes into MOFs or nanoparticles

spacer or to polystyrene particles via the pending succinimide group of its comonomer [43, 44]. Additionally, NAD(P)$^+$ or an analogue can also be successfully bound to a carrier material via amine groups, namely for the immobilization onto the resin and Sepharose carrier beads [45–47]. This amine-based attachment strategy also proved effective for a FAD analogue, which was immobilized onto aminoethyl decorated agarose beads and served as an anchor for the immobilization of flavoenzymes [48]. In a recent adaptation of these approaches, NADH and ATP were reversibly attached to agarose microbeads. The ribose moiety of the cofactors served as the receptive site for binding to aryl boronic acid anchors, resulting in strong albeit reversible attachment to the carrier material [49].

While covalent attachment can be cofactor-specific and potentially alter its properties, immobilization strategies based on ionic interactions offer efficient alternatives. Initial methods in this category involved acrylate-based superabsorbent polymers [50, 51]. These solid materials are modified with cationic groups capable of ionically interacting with the negatively charged phosphorylated cofactors. However, superabsorbent polymers could not efficiently retain cofactors in aqueous media, fostering the development of a different class of cationic polymers. These improved, porous cationic polymers also bear positively charged amine groups which can interact with phosphorylated cofactors such as PLP or NAD(P). However, due to their porous nature, they create an association-dissociation mechanism between active sites and the carrier material without releasing the cofactor into the bulk. Examples of such cationic polymers include polyethyleneimine (PEI) and diethylaminoethyl (DEAE), which can be combined with various carrier materials such as agarose beads, chitin, or even cross-linked enzyme aggregates [52–58].

Cofactors can also be immobilized without relying on intermolecular interactions but depending solely on physical entrapment for immobilization. For instance, cofactors were encapsulated in nanoparticles such as Pickering emulsion droplets or virus-like particle nanocages [59, 60]. Enzymes and cofactors were immobilized in nanoparticles constructed of metal–organic frameworks (MOFs) and hydrogen-bonded organic frameworks (HOFs). NAD-dependent enzymes and their cofactor were co-immobilized in hierarchically porous zeolitic imidazolate frameworks and in HOFs constructed from tetraamidinium and tetracarboxylate with the aid of polyelectrolyte [61, 62]. Hydrogels also offer a promising route for cofactor and enzyme co-immobilization, as demonstrated in instances like the entrapment of cofactors and enzymes into a polyvinyl alcohol (PVA) and sodium alginate (SA) copolymer hydrogel [63]. Hydrogels may even incorporate enzymes as an integral part of the matrix, resulting in a self-assembling all-enzyme hydrogel in which the enzymes are linked together to form the porous hydrogel structure or in crude enzyme hydrogels via their gelation with chelating agents [64, 65].

Hybrid methods combining two types of immobilization techniques have gained increasing popularity, seeking to leverage the advantages of both approaches. Presently, these hybrid methods incorporate encapsulation with either ionic adsorption or covalent attachment. When encapsulation is combined with ionic adsorption, nanoparticles are either coated or embedded with cationic polymers to enhance cofactor retention. For instance, PEI was integrated into barium alginate (BA) hydrogel beads to better retain the cofactor PLP [66]. In an alternative method, the interior of hollow nanofibers was coated with the cationic polyelectrolyte poly(allylamine hydrochloride) (PPAH) to establish robust ion-exchange interactions between NAD and the nanofiber [67, 68]. Covalent attachment combined with entrapment involves tethering the cofactor to a large polymer to prevent it from escaping through the nanoparticle's pores. In one approach, enzymes and NAD$^+$ were co-encapsulated in pluronic-based nanocarriers in which NAD$^+$ was covalently bonded to an alginate polymer through an amide bond between the alginate's carboxylic groups and the cofactor's amine groups [69]. In another example, enzymes and polymer-tethered cofactors were immobilized into MOFs. These are nanoparticles of a zeolitic imidazolate framework-8 in which NAD$^+$ is covalently attached to a phenylboronic acid decorated polymer [70].

Despite the quite broad diversity of cofactor immobilization techniques available, most of them have primarily found applications in batch processes. Though, there has been a recent surge of interest in adapting these methodologies for continuous-flow systems, due to the potential benefits such as enhanced productivity, better control over reaction kinetics, and higher process reliability. A discussion of the approaches applicable to continuous-flow conditions will be explored in greater detail in the following sections, providing insights into the innovative strategies and operational considerations required to increase the effectiveness of these methods.

Covalent immobilization of cofactors

Polymer swinging arms for cofactor immobilization

Hartley et al. designed an ingenious immobilization system whereby a flexible swinging arm covalently attaches a cofactor to a synthetic, multidomain protein, allowing for its simultaneous use and regeneration [38]. Although analogous designs exist, this particular system stands out due to its particular suitability for continuous-flow biocatalysis. The general design of this co-immobilization system consists of a biocatalyst unit that contains three modules: a

catalytic module driving the desired synthetic reaction, a cofactor-recycling module efficiently regenerating the cofactor, and an immobilization module that enables site-specific and covalent conjugation to a carrier material. The modules are connected to each other by an unstructured amino acid spacer. The cofactor is attached to the spacer between the catalytic and cofactor-recycling module through a solvent-accessible cysteine residue, which serves as the attachment point for the cofactor's flexible PEG linker (Fig. 2). This modular design is intended to be versatile and applicable for a broad spectrum of synthetic transformations by creating a set of standardized cofactor-recycling and conjugation modules which can be combined with a wide array of synthesis modules. However, the inherent complexity of this immobilization platform may limit its practicality and widespread adoption.

The synthesis of a D-fagomine (**5**) was selected as the model system for this approach. This process involves three biocatalytic steps in series to convert glycerol (**1**) and protected 3-aminopropanal (**4**) into a precursor of the chiral drug. First, glycerol (**1**) is converted to glycerol-3-phosphate (**2**) by regiospecific phosphorylation in an ATP-dependent step. Next, **2** is oxidized to dihydroxyacetone phosphate (**3**) in an NAD-dependent step. Finally, stereoselective aldol addition, dephosphorylation and cyclization yield D-fagomine (**5**) as the final product (Scheme 1). The glycerol phosphorylation module consisted of a glycerol kinase, an acetate kinase and modified ATP, while the NAD-dependent oxidation module comprised a glycerol-3-phosphate dehydrogenase (PDH) and a water-forming NADH oxidase (NOx). For the conjugation module, a serine hydrolase (SH) enzyme was site-specifically bound to a trifluoroketone suicide inhibitor to form a highly stable, soluble monomeric protein. The conjugation module was highly efficient, and the immobilized enzymes retained their initial catalytic activity.

For their application under continuous-flow conditions, the conjugation modules of the biocatalytic units were immobilized on trifluoroketone-activated agarose beads and packed into glass columns. In the reactors, both cofactor-dependent transformations were run to completion with productivity rates of approximately 0.15 mmol h^{-1} over a 24-h period (Table 2). The continuous-flow reactions outperformed those executed in batch, a fact that can be attributed to the continuous product removal, which alleviates product inhibition and enables the shift of the thermodynamic equilibrium. The TTNs were around 17,000 for ATP in the phosphorylation and around 11,000 for the NAD-dependent oxidation reaction. Notably, the eventual decline in the reactor's performance was due to enzyme deactivation rather than cofactor lixiviation, highlighting the robustness of the co-immobilization approach. Nevertheless, for broader and larger scale applications of the method, the enzymes should preferably be engineered for enhanced stability so that even higher cofactor TTNs could be achieved.

Covalent attachment to carrier materials

Zhang et al. proposed another elegant, yet straightforward, cofactor immobilization method [40]. This approach involves the covalent attachment of an enzyme-cofactor complex to an epoxy resin carrier, specifically utilizing the catalytically active complex between TA and

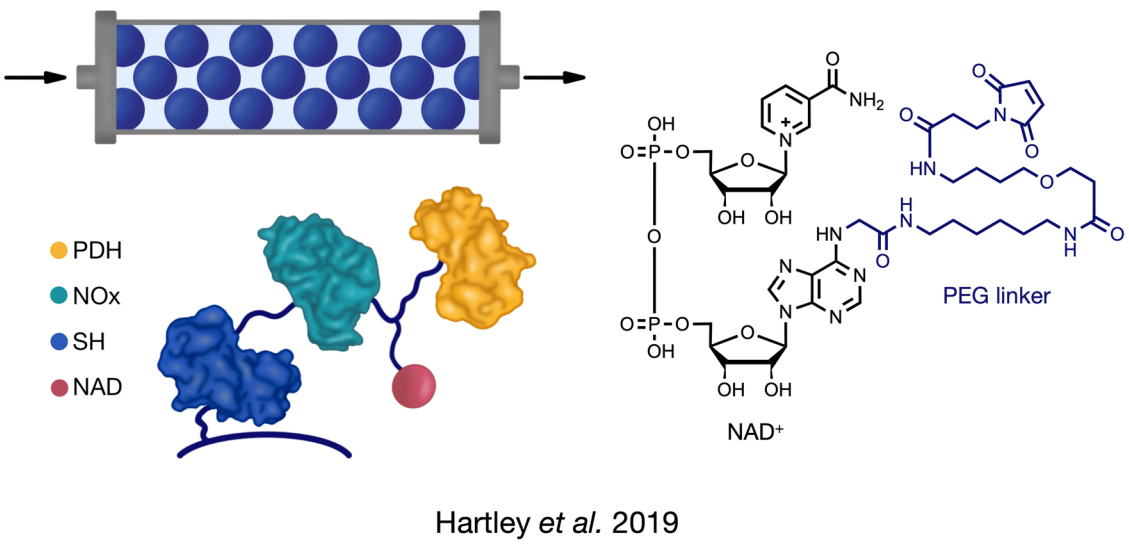
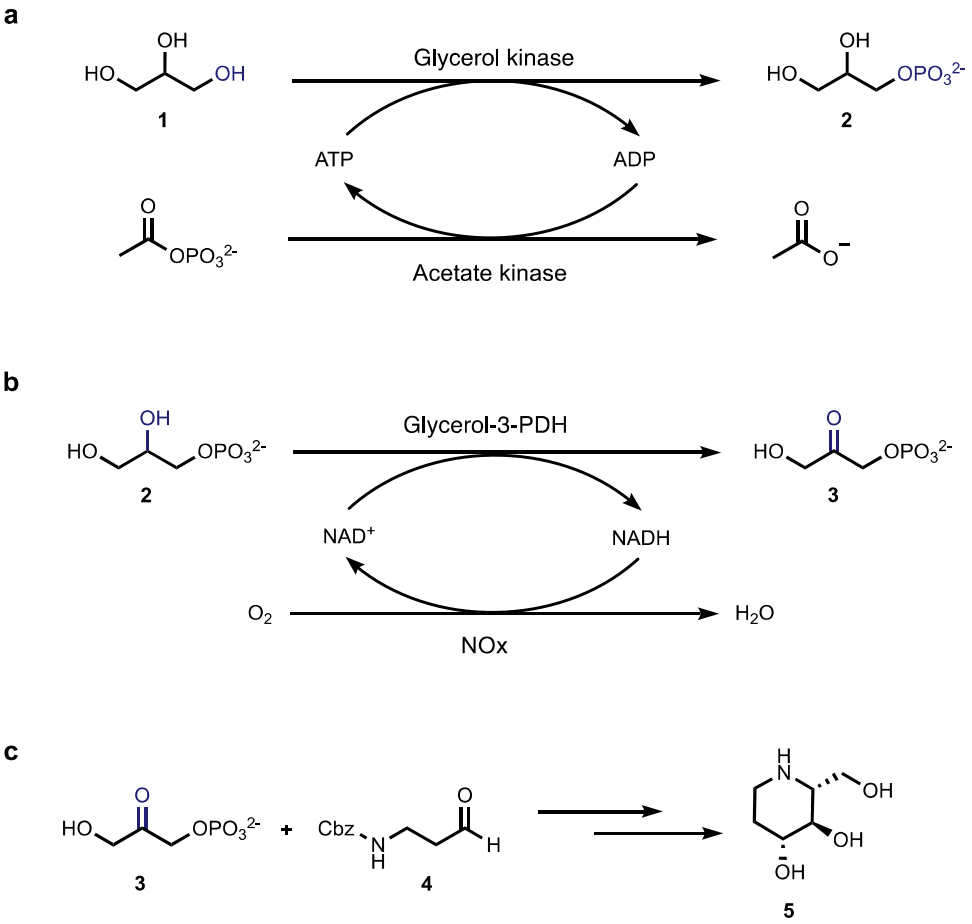


Fig. 2 Schematic representation of Hartley et al.'s swinging arm design, featuring the catalytic phosphate dehydrogenase (PDH), NADH oxidase (NOx) for NAD^+ recycling and the serine hydrolase (SH) unit for conjugation. NAD^+ is tethered between the PDH and NOx through the PEG-based swinging arm



Scheme 1 Reaction scheme of the ATP-dependent (**a**) and NAD-dependent (**b**) steps and the final transformation (**c**) to D-fagomine (**5**), as performed by Hartley et al.'s with the swinging arm immobilization approach

Table 2 Metrics for continuous-flow biocatalytic reactions performed using enzymes and cofactor covalently immobilized

Immobilization	Biotransformation	Productivity[a] (mmol h^{-1})	Flow rate (mL min^{-1})	Conversion (%)	Reactor volume (mL)	Reaction time (h)	TTN cofactor
Flexible swinging arm for the covalent attachment of ATP [38]	Glycerol phosphorylation and ATP regeneration	0.15	0.25	>99	21.2	24	16,848
Flexible swinging arm for the covalent attachment of NAD^+ [38]	Oxidation of glycerol-3-phophate coupled to NAD recycling	0.14	0.25	83–98	28.3	24	10,839
Covalent attachment of PLP and a TA onto epoxy resin carrier beads [40]	Asymmetric synthesis of (*R*)-sitagliptin	1.13	0.27	90–99	45	525	3,000[b]
Reversible covalent attachment of NADH with co-immobilized ADH and GDH [49]	Asymmetric synthesis of L-alanine	0.011	0.025	>99 [a]	0.25	21	20

[a]Estimated average productivity of the process within the duration of the continuous operation

[b]At substrate and ionic buffer concentrations beneficial for the conversion rate

PLP (Fig. 3). Within this complex, the critical interaction is between the PLP's aldehyde group and the catalytic lysine residue of enzyme's active site. The epoxy groups on the carrier material, which bear a C12 linker, serve as the covalent attachment point for the binding of both the amine moieties of the protein and the phosphate group of PLP to the carrier. The immobilization of PLP enhanced the enzyme's structural stability under

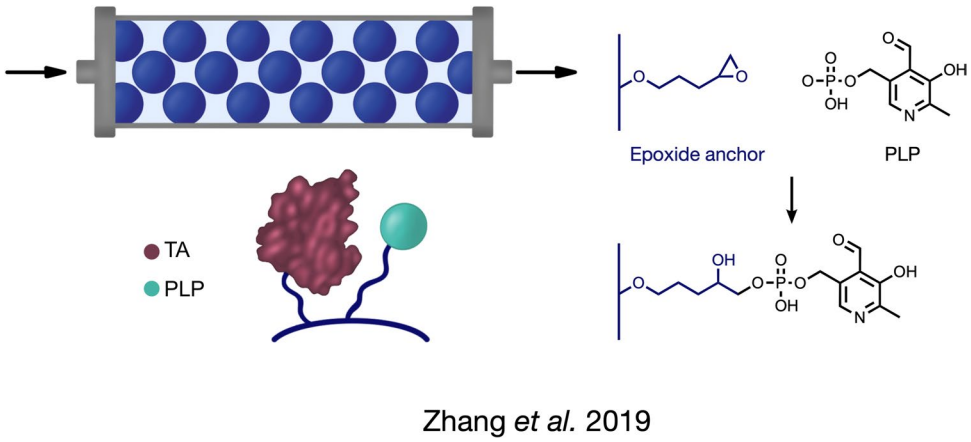


Fig. 3 Depiction of Zhang et al.'s methodology for co-immobilization of PLP and transaminase (TA)

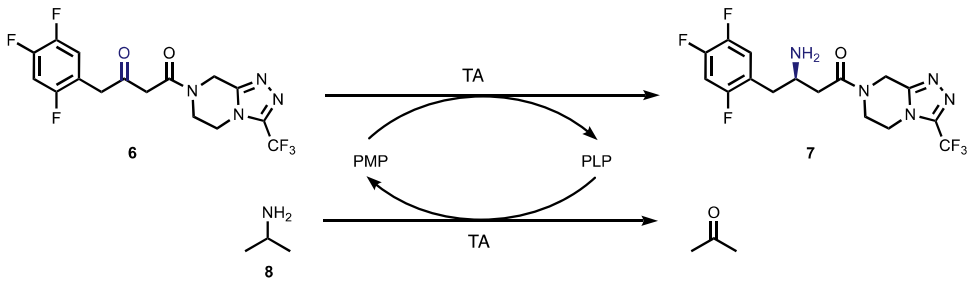
operational conditions and during prolonged storage as well as optimized the interaction efficiency between PLP and the enzyme. This optimization is evidenced by the improved thermal stability (maintained activity at temperatures above 45 °C) and increased half-life of the enzyme (from 4.7 days for the free enzyme to 37.7 days for the immobilized enzymes).

The methodology was applied to the continuous synthesis of (*R*)-sitagliptin (**7**) from the prositagliptin ketone (**6**) utilizing isopropyl amine (**8**) as the amine donor for PLP regeneration (Scheme 2). The continuous-flow system was realized by filling a recirculating packed bed reactor (PBR) with the immobilized TA and PLP epoxy resin beads. However, the specifics of the recirculating process remain undisclosed in the original publication. Over an operational period of more than 22 days, (*R*)-sitagliptin was synthesized with a conversion rate consistently exceeding 90% (Table 2). The minor decline in conversion rate throughout the extended operation time and the limited constraints in reaction conditions underscore the robustness of this approach. Although the authors did not comment on that in the original publication, we point out that the TTN (ca. 3,000 over the 22-day operational period) remains moderate. This fact could be attributed to a reduced cofactor activity that may result from the covalent binding and suboptimal positioning of PLP with respect to the enzyme's active site.

Reversible covalent binding

Diamanti et al. reported on a novel technique for the covalent binding of cofactors onto carrier material for continuous-flow biocatalysis (Fig. 4) [49]. This method consists of the coordination of the cofactor's ribose hydroxyl moieties to boronic acid functionalities, thereby offering flexibility and precluding undesirable cofactor leaching. The co-immobilization is performed through the hetero-functionalization of agarose microbeads with cobalt ions and aryl boronic acids, which immobilize His-tagged enzymes and adenylated cofactors, respectively. Therefore, ribose-containing cofactors such as NAD(P), FAD and ATP can all theoretically be immobilized with this technique. Notably, cofactor leaching can be avoided even at high ionic strength conditions that are preferable for several enzymatic reactions [49].

The co-immobilization of several NAD(P)-dependent enzymes with NADH successfully prevented cofactor leaching under either low or high ionic strength conditions (up to 1 M NaCl). In batch mode experiments, an ATP-dependent and various NAD(P)-dependent biotransformations were tested. In comparison to batch mode, the immobilized systems consistently showcased superior performance, especially resulting in enhanced specific activity, accelerated reaction rates, and reduced cofactor loading. Noteworthy was the combination of two co-immobilized systems for the amination of aldehydes, the former being a system of a TA and PLP immobilized on the cationic polymer PAH for the transamination of the aldehyde and



Scheme 2 Asymmetric biocatalytic transamination of (*R*)-sitagliptin (**7**) from the prositagliptin ketone (**6**) as performed by the covalent co-immobilized system of Zhang et al.. PLP was recycled using isopropyl amine (**8**) as amine donor

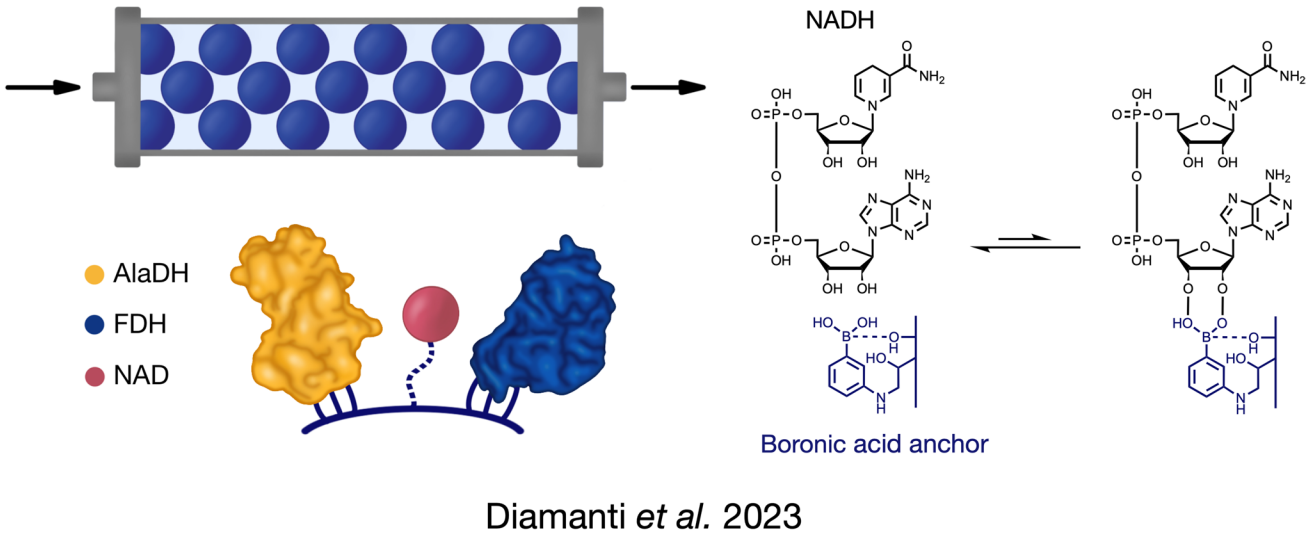
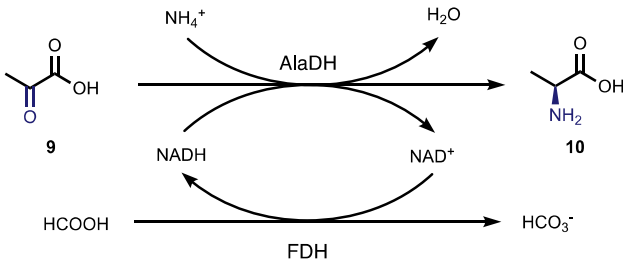


Fig. 4 Schematic depiction of the co-immobilization of NAD, L-alanine dehydrogenase (AlaDH) and formate dehydrogenase (FDH) via Diamanti et al.'s methodology. A detailed view of the reversible covalent attachment mechanism is also depicted



Scheme 3 Asymmetric synthesis of L-alanine (**10**) from pyruvate (**9**) using the reversible covalent immobilization system by Diamanti et al. Recycling of the NADH cofactor is achieved by an FDH-based recycling system

the latter one being a boronic acid immobilized NAD-dependent system with L-alanine dehydrogenase (AlaDH) and FDH. Ammonium formate was the additional reagent to regenerate the L-alanine amine donor for the transamination. This marks the first report of combining two types of immobilized cofactors within a single reaction cascade.

To evaluate the operational stability of enzymes and cofactors immobilized through this approach, co-immobilized AlaDH, NADH and a FDH were packed into a plug-flow reactor for the continuous-flow synthesis of L-alanine (**10**) (Scheme 3). The process reached a peak yield of 99% under low ionic strength and low substrate concentrations (7.5 mM pyruvate (**9**) and 50 mM ammonium formate), but dropped to 23% under higher strength conditions (75 mM pyruvate (**9**) and 500 mM ammonium formate). However, after 21 h of operation at a 25 µL min^{-1} flow rate, the TTNs for NADH remained below 25 for both sets of conditions. These TTNs for the cofactor are significantly lower than those achieved with the same biocatalytic reaction but operated in discontinuous batch mode. The decline in catalytic performance over the time was attributed to intrinsic enzyme deactivation, rather than cofactor or enzyme leaching, indicating a need for further investigations to enhance long-term stability.

In summary, in the pursuit of advancing continuous-flow biocatalysis, researchers have explored various covalent attachment methods for cofactor immobilization. Hartley et al. devised an intricate, yet promising, system for co-immobilization of various cofactors and enzymes through modular design. While demonstrating impressive performance, its long-term operation requires the stabilization of enzymes. Conversely, Zhang et al.'s approach, while simpler, highlights concern about cofactor activity and specificity for certain enzymes, thus currently limiting a wider applicability. The method by Diamanti et al.'s addresses cofactor leaching by binding cofactors' ribose molecules, but it suffers from enzyme deactivation that reduces TTNs. Together, these methods contribute to the advancement of covalent cofactor attachment for application in continuous-flow biocatalysis, emphasizing that specificity and enzyme stability are the focal points for future developments.

Ionic adsorption of cofactors onto polymer-coated beads

Ionic adsorption onto PEI-coated carriers

Velasco-Lozano et al. reported on the application of improved porous cationic polymers for the dynamic ionic

adsorption of cofactors within continuous-flow biocatalysis [52]. Their research involved the immobilization of phosphorylated cofactors such as NAD^+, PLP and FAD on agarose beads coated with the cationic polymer PEI. This approach was successfully implemented for a NAD-dependent system comprising the immobilized cofactor, an alcohol dehydrogenase (ADH), and an FDH for cofactor regeneration (Fig. 5). This system was designed to perform an asymmetric reduction with ADH, and a subsequent cofactor recycling with FDH. It is important to note that synthetic application was not demonstrated for FAD, while a PLP-dependent system was not transitioned from batch to continuous flow due to the deactivation of the immobilized TA in earlier experiments. Benítez-Mateos et al. later addressed this challenge in a study that led to the development of an effective method for the co-immobilization of PLP and TAs and enabling the continuous operation of both deamination and amination reactions [53]. Instead of randomly immobilizing the TAs directly onto aldehydes-activated agarose microbeads, site-oriented attachment through the enzyme's His-tags proved most effective (Fig. 5). In both methods, the PEI-coating results in a polymeric bed with positively charged primary, secondary, and tertiary amines capable of forming salt bridges and reversible imine bonds (Schiff's bases) with the cofactor's phosphate groups.

While these approaches were highly effective for PLP immobilization, NAD^+ and FAD exhibited significant leaching from the carrier material already when exposed to low ionic strength buffer (10 mM sodium phosphate). The sensitivity of this approach to external ionic interaction was further exemplified by the leaching of the NAD when the system operated with an excess of sodium formate (100 mM, 10 equivalents). Notably, immobilized enzymes were similarly active with both exogenous and co-immobilized cofactor, indicating that immobilization negligibly affected the cofactor's availability and activity. When transitioning to continuous-flow conditions, the reactors were packed with PEI-agarose microbeads containing the co-immobilized NAD- or PLP-dependent system. In the case of the NAD-dependent system by Velasco-Lozano et al., the asymmetric reduction was continuously operated for 107 h with an optimal flow rate of 50 µL min^{-1} and a productivity of 0.013 mmol h^{-1}; longer-term operation of the reactor caused a gradual decline in the conversion (Table 3). The origin of this decay was not specified, but it is most likely rooted in enzyme deactivation as limited cofactor lixiviation was observed during operation. The PLP-dependent system of Benítez-Mateos et al. showed excellent stability for a deamination reaction, allowing for flow rates as high as 1.45 mL min^{-1}, which is the highest value reported in this review (Table 3). Due to its promising characteristics, this method was investigated with a total of three TAs, all of them showing excellent enzymatic activity in combination with the immobilized cofactor. However, while the reaction with the first enzyme maintained maximum conversion, the

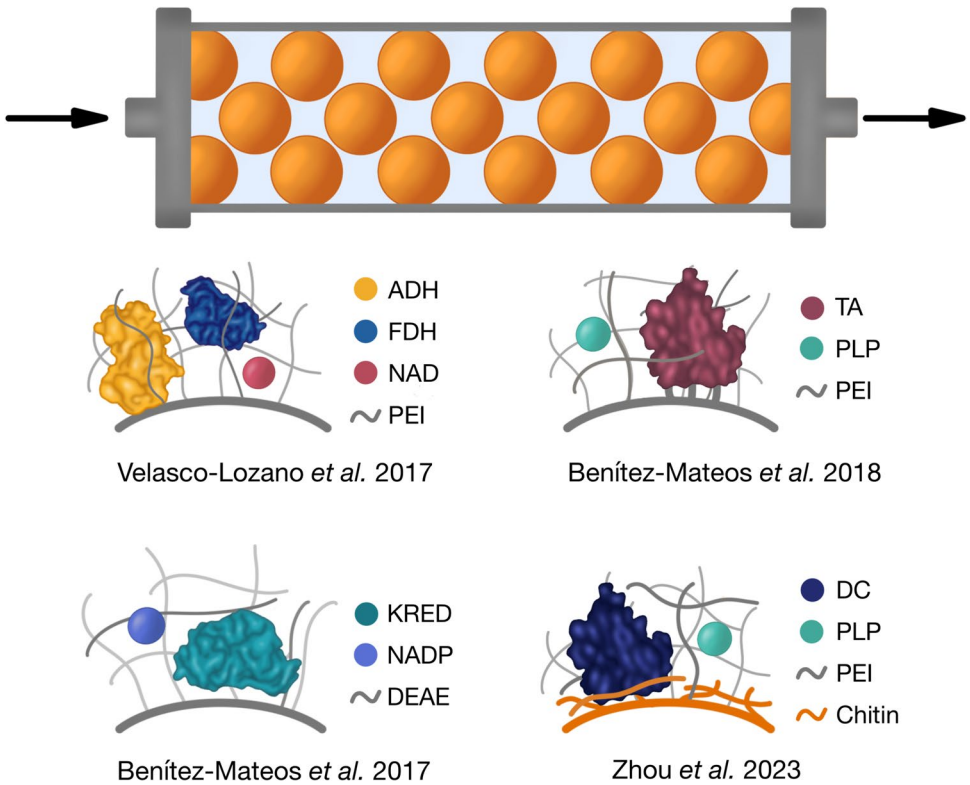


Fig. 5 Schematic depiction of ionic adsorption techniques for continuous-flow biocatalysis: Velasco-Lozano et al.'s NAD-dependent system with immobilized alcohol dehydrogenase (ADH), formate dehydrogenase (FDH) and NAD; Benítez-Mateos et al.'s co-immobilized transaminase (TA) and PLP onto PEI-coated carriers; immobilized ketoreductase (KRED) and NADP onto DEAE-coated carriers; and Zhou et al.'s PEI-coated chitin-chitosan microspheres with immobilized decarboxylase (DC) and PLP

Table 3 Metrics for continuous-flow biocatalytic reactions performed using enzymes and cofactors immobilized by ionic adsorption

Immobilization	Biotransformation	Productivity[a] (mmol h^{-1})	Flow rate (mL min^{-1})	Conversion (%)	Reactor volume (mL)	Reaction time (h)	TTN cofactor
Co-immobilization of NAD^+, an ADH and FDH onto PEI-coated agarose beads [52]	(*S*)-selective asymmetric reduction of acetophenone	0.013	0.050	79–99	0.25	107	85
Immobilization of PLP onto PEI-coated agarose beads and a TA via His-tags [53]	Deamination of (*S*)-*α*-methylbenzylamine	2.18	1.45	>99	3.42	0.83	233[b]
Immobilization of PLP onto PEI-coated agarose beads and TA via His-tags [53]	Amination of *trans*-cinnamaldehyde	0.35	0.725	60–99	3.42	1.67	67[b]
Co-immobilization of NADPH and a KRED onto DEAE-coated agarose beads [54]	Asymmetric reduction of trifluoro-acetaldehyde	0.024	0.050	70–90	0.25	120	1,076
Adsorption of PLP and a DC onto PEI-coated chitin-chitosan microspheres [55]	Decarboxylation of L-lysine to form cadaverine	0.440	0.4	85–99	5.0	16.7	-[c]

[a]Estimated average productivity of the process within the duration of the continuous operation

[b]Calculated based on the final product yield and cofactor loading

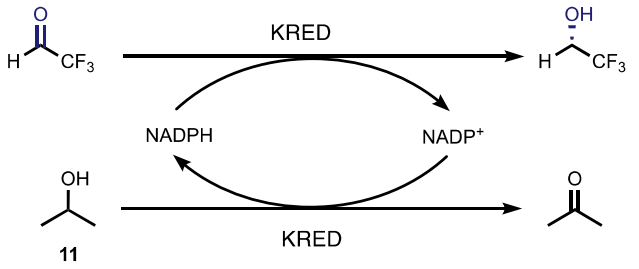
[c]Cofactor loading unknown

use of the other two enzymes resulted in an extensive decay of conversion rate throughout the 50 min of operational period. These observations emphasize the importance of maintaining enzyme stability during continuous-flow operations. This is of particular importance for enzymes that are sensitive to immobilization such as TAs, for which the loss of the homodimer structure leads to non-catalytically active proteins [53, 71]. In conclusion, the continuous synthesis of amines using co-immobilized PLP was carried out solely using the initial and most stable TA. While this system still displayed some stability advantage over external cofactor supplementation, it proved less stable than the system utilized for deamination reactions, requiring double the residence time (or a halved flow rate: 0.75 mL min^{-1}). The steady decline in conversion observed during short-term operation can be attributed to the excess of amine donor, which may provoke cofactor lixiviation due to interaction with its amine and carboxylic groups. Lixiviation of PLP might additionally prompt enzyme deactivation as the presence of PLP significantly stabilizes the enzyme; therefore, PLP lixiviation contributes to the decrease in the system's catalytic activity.

Padrosa et al. also investigated PEI-coated agarose beads for cofactor immobilization within NAD-dependent multienzyme cascade, but this attempt yielded only limited success [72]. Achieving robust performance for their system using this immobilization strategy proved to be impossible. In fact, the high ionic strength necessary to maintain the enzymatic activity of the carboxylate reductase inadvertently leads to cofactor leaching. This work highlights the persistent challenge that prevents the broad applicability of this immobilization methodology: cofactor lixiviation under high ionic strength, particularly for cofactors such as NAD which have a lower affinity for adsorption onto the cationic polymer.

Immobilization through adsorption onto DEAE-coated carriers

The adaptability of ionic adsorption for cofactor immobilization is further exemplified by the possibility to switch the cationic polymer in use. In a study by Benítez-Mateos et al., the carrier material was coated with DEAE instead of PEI to enhance the co-immobilization of NADPH and a commercially available KRED (i.e., another name and acronym to indicate an ADH active on ketones) [54]. The immobilization of this enzyme is especially attractive as it does not only tolerate a wide substrate scope, but also displays exquisite (*R*)-selectivity. Additionally, it can self-regenerate the NADPH through the oxidation of isopropyl alcohol (**11**) as a sacrificial co-substrate (Scheme 4). This fact eliminates the need for an auxiliary enzyme for cofactor recycling. In the case of this KRED, porous agarose beads coated with DEAE outperformed the use of PEI-coated beads because the former provides the necessary conformational flexibility for optimal enzyme performance without inducing adverse structural rearrangements or affecting enzyme anchoring to the carrier material (Fig. 5). Both the KRED and NADP



Scheme 4 Asymmetric reduction performed in continuous-flow and catalyzed by co-immobilized KRED in presence of NADP as reported by Benítez-Mateos et al. The NADPH cofactor is self-regenerated by the KRED through isopropyl alcohol (**11**) oxidation

presented excellent retention in low ionic strength buffer (10 mM Tris–HCl) when co-immobilized on the DEAE-bed. However, similar to systems utilizing PEI, leaching occurred in the presence of high ionic strength (starting at 100 mM NaCl), reiterating the challenge that persists for this immobilization approach. This limitation can however be advantageous in cases where low ionic strength conditions are preferred during the biocatalytic reaction, such as for KRED, allowing for targeted release and reloading when the enzymatic activity becomes insufficient.

The asymmetric reductions of a range of carbonyl compounds were compared by using either the cofactor co-immobilized system or the system with the NADP free-in-solution. Both systems afforded quantitative yields in batch, suggesting negligible influence of cofactor immobilization on the enzymatic activity. The high product yields and cofactor reusability were attributed to the excellent activity of KRED to self-regenerate NADPH by using the isopropyl alcohol under extremely low cofactor concentrations. The extension of this system to continuous-flow conditions for the asymmetric reduction of an aryl ketone in a PBR achieved an 80% conversion throughout the 120 h of operation (Table 3). At a flow rate of 50 µL min^{-1}, a productivity of 0.024 mmol h^{-1} was achieved. Remarkably, neither enzyme deactivation nor cofactor leaching occurred during the continuous operation, as evidenced by the consistent specific activity of the enzyme and a substantial TTN for NADP. Notably, this immobilization approach reduced the necessary amount of cofactor to only 5% compared to the original process without cofactor immobilization.

For the asymmetric reduction of a ketone as part of a chemoenzymatic route towards the synthesis of cinacalcet, Marx et al. explored this specific method for the co-immobilization of a KRED and NADPH [73]. While promising results were achieved in batch mode, the continuous-flow operation showed difficulties that resulted in negligible conversions. In this case, the immobilized KRED was inefficient in the recycling the co-immobilized NADPH in continuous flow synthesis. Therefore, for a successful transfer of this synthesis from batch to continuous flow, the addition of a cofactor recycling system based on the couple-enzyme approach might be necessary.

Ionic adsorption of cofactors using chitin-PEI carriers

Utilizing cost-effective, easily available, and renewable carrier materials for immobilization opens exciting opportunities for biocatalysis. Chitin, a natural polymer, stands out for its rich and affordable supply, easy modification, and biocompatibility. Furthermore, chitin and its derivative chitosan are ideal for interacting with cofactors due to the presence of primary and secondary amines as well as alcohol groups. Chitin's ability to selectively adsorb enzymes fused with chitin-binding domains further enhances its potential as a co-immobilization platform. In an initial study, Wei et al. laid the foundation for this approach by demonstrating the potential of a deacetylated chitin powder for the co-immobilization of PLP-dependent L-lysine decarboxylase (DC) and PLP [74]. However, the limited specific surface area of chitin hindered enzyme adsorption, and the unpredictable morphology restricted its application in packed columns for continuous-flow biocatalysis. To circumvent this, Zhou et al. developed an improved system using chitin-chitosan microspheres for the co-immobilization of PLP and three PLP-dependent enzymes: L-lysine DC, L-dopa DC, and a TA (Fig. 5) [55]. A key consideration in this approach is the degree of deacetylation of the chitin. A high degree exposes more positively charged primary amines, facilitating PLP adsorption but diminishes the binding affinity of the chitin-binding domain fused to the enzymes.

Deacetylated chitin-chitosan microspheres demonstrated excellent immobilization properties for PLP and chimeric enzymes having fused chitin-binding domains. However, coating the microspheres with the cationic polymer PEI proved to further enhance the maximum PLP loading, possibly due to the additional amine groups. This PEI coating on the microspheres improved the performance of the co-immobilized system for all three enzymes. However, even with this enhancement, all enzymes exhibited a decrease in conversion after several batch cycles. The conversion decrease can be attributed to a decline in enzymatic activity for L-dopa DC and TA. In contrast, the reduced activity for L-lysine DC was likely due to PLP leaching, as the yield was unaffected when PLP was re-added exogenously. The synthesis of cadaverine (**13**) with the co-immobilized L-lysine DC and PLP was transferred to continuous-flow conditions (Scheme 5). Throughout the 500 min of operation, the system with PEI-coated microspheres maintained around 85% of its initial activity while operating at an adequate flow rate (Table 3). Remarkably, under continuous-flow conditions, the significant drop in conversion observed after several cycles of batch synthesis vanished. Furthermore, akin to previous

immobilization approaches, the chitin-chitosan microsphere carriers could be reused after enzyme deactivation. In fact, high performance was maintained after several aliquots of enzymes and cofactors were reloaded on the carrier in subsequent cycles of operation.

The predominant approach in continuous-flow biocatalysis for cofactor immobilization involves ionic adsorption, where cofactors adhere via ionic interactions to porous cationic polymers like PEI and DEAE. While this method shows promise in aqueous settings, its susceptibility to external ionic interactions, particularly with NAD and FAD, restricts its usage to reactions solely operating at low ionic strength conditions. Despite these challenges, the method exhibits notable versatility due to its compatibility with various phosphorylated cofactors and cofactor-dependent enzymes. The flexibility offered by the option to utilize various cationic polymers further enhances its appeal. However, challenges in replicating these approaches raise doubts about their universal applicability. Therefore, continuous refinement and optimization of these ionic adsorption techniques are imperative for their successful integration into practical and sustainable biocatalytic processes.

Entrapment of cofactors and enzymes

Encapsulation in nanostructures

Despite various methods available for the co-encapsulation of enzymes and cofactors into nanoparticles such as bacteriophages, pluronic-based nanoparticles and even MOFs or HOFs, Pickering emulsion droplets are the sole nanoparticle-based immobilization technique to have been applied in continuous-flow. Pickering emulsions employ nanoparticles as emulsifying agents rather than traditional surfactants and have emerged as a promising platform for biphasic catalysis. The resulting Pickering emulsion droplets have demonstrated exceptional stability, resistance to coalescence, and can be efficiently packed into a column reactor for continuous-flow reactions [75]. Leveraging on these unique properties, Wei et al. have devised a co-immobilization strategy for continuous-flow biocatalysis by harnessing the distinctive interfacial adsorption and confinement effects inherent to Pickering emulsion droplets [59]. In this approach, an organic-aqueous bi-phasic system containing enzymes and cofactors is transformed into a water-in-oil Pickering emulsion (Fig. 6). This process generates numerous water droplets within which both enzymes and cofactors are co-compartmentalized. The resulting droplets can subsequently be loaded into a column reactor, effectively creating a fixed-bed reactor tailored for continuous-flow biocatalysis. The interspaces among these droplets allow the substrate-containing oil phase to flow around the droplet interfaces, providing an ideal environment for enzymatic reactions to occur. Importantly, during continuous-flow reactions, the enzymes persistently reside at the droplet interface due to their strong affinity for adsorption at this location. Simultaneously, water-soluble cofactors remain confined, but are capable of moving freely within the droplets. The performance of the system relies heavily on the enzyme interfacial adsorption. Besides the high retention and stability of both cofactor and enzyme when immobilized, the method's most notable advantage lies in its simplicity, requiring only a one-step emulsification procedure for cofactor loading.

The co-compartmentalization approach was initially tested for the asymmetric reduction of an acetophenone derivative using an NADP-dependent ADH, which catalyzes the reaction depicted in Scheme 4. Over an operational period of 300 h, a conversion rate exceeding 90% was consistently maintained for this system (Table 4). However, this achievement was accompanied by a low flow rate of 1.0 mL h^{-1}. It is important to note that the slight decrease in conversion was attributed primarily to enzyme deactivation rather than cofactor deactivation or leaching. Remarkably, the TTN for the NADP cofactor reached an impressive 59,204, marking it as the highest value reported in this review. Moreover, even after long-term operation, the droplets remained virtually unchanged in terms of morphology and size when compared to droplets prior to the reaction.

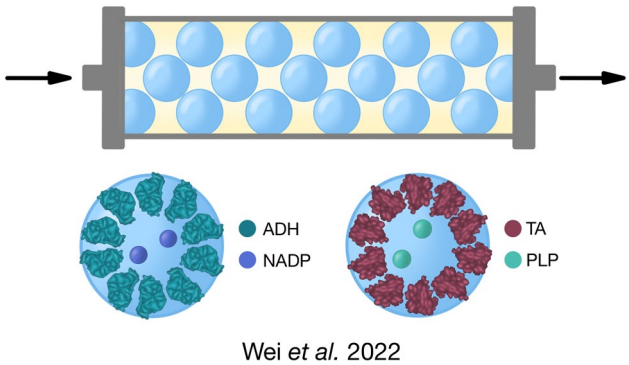
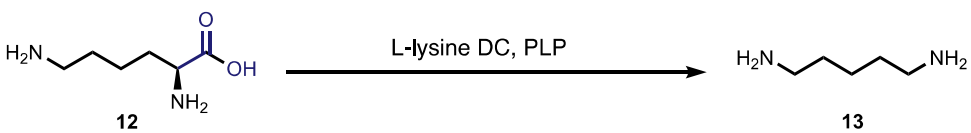


Fig. 6 Pickering emulsion droplets for continuous-flow biocatalysis as developed by Wei et al. An alcohol dehydrogenase (ADH) with NADP and a transaminase (TA) with PLP were encapsulated in the droplets

Scheme 5 The synthesis of cadaverine (**13**) through decarboxylation of L-lysine (**12**) by L-lysine DC and PLP



Additionally, the versatility of this co-immobilization method was exemplified in the synthesis of a diverse set of high-value chiral alcohols. It also proved to be effective in conducting multiple amination reactions using a co-immobilized system of PLP and TA, in a similar way as depicted in Scheme 2, although phenylethylamine was employed as the amine donor. In all cases, conversion rates consistently exceeded 80% during continuous operation enduring at least 100 h.

Immobilization into hydrogels

A non-invasive and seemingly simple technique for the immobilization of biocatalysts that is widely used for continuous-flow biocatalysis entails their incorporation within a hydrogel matrix. These porous structures, crafted by interconnecting polymers, such as PVA or proteins, provide an accommodating environment for enzyme immobilization while also retaining cofactors within the hydrogel's interstices. A particularly ingenious hydrogel co-immobilization method deploys enzymes themselves as the building blocks for gel formation, relying on covalent isopeptide bonds between genetically encoded reactive partners fused to the enzymes [76]. Peschke et al. have exploited the specific application of such a self-assembling all-enzyme hydrogel for an asymmetric reduction in continuous-flow (Fig. 7) [64]. Two homotetrameric enzymes were selected for the gelation, namely a highly (*R*)-selective ADH and a NADPH-regenerating GDH. These enzymes underwent cross-linking between a SpyTag, which is a genetically fused 13 amino acid peptide chain bearing a terminal lysine residue, and a SpyCatcher that is a genetically fused small protein bearing a terminal aspartic acid residue. A His-tag was also added to the SpyCatcher of the recombinant enzyme. In practice, by mixing SpyTag-ADH with SpyCatcher-GDH, the terminal residues of the SpyTag and SpyCatcher rapidly react with each other to form covalent isopeptide bonds, thereby creating cross-links between the two homotetrameric enzymes. This process is simple and remarkably efficient since cross-linking occurs rapidly after the enzymes are added into the aqueous buffer.

For evaluating the performance of the self-assembling all-enzyme hydrogels, a prochiral *meso* diketone (**14**) was chosen as the test substrate (Scheme 6). In principle, this

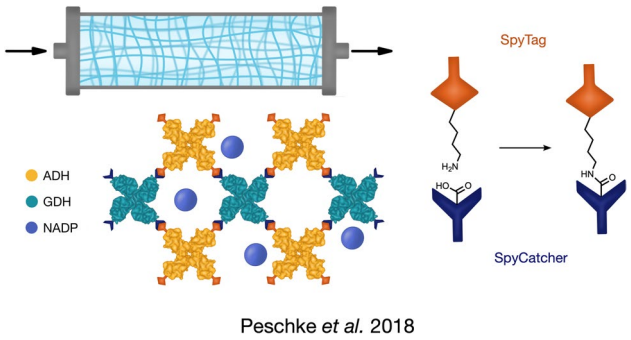


Fig. 7 Peschke et al.'s all-enzyme self-assembly hydrogel with interlocked alcohol dehydrogenase (ADH) and glucose dehydrogenase (GDH). The interlocking mechanism using SpyTags and SpyCatchers is also highlighted

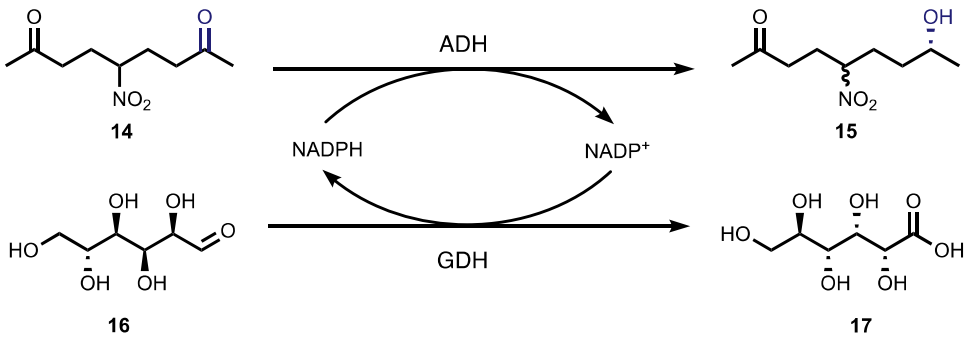
Table 4 Metrics for continuous-flow biocatalytic reactions using enzymes and cofactors immobilized by entrapment approaches

Immobilization	Biotransformation	Productivity[a] (mmol h^{-1})	Flow rate (mL min^{-1})	Conversion (%)	Reactor volume (mL)	Reaction time (h)	TTN cofactor
Pickering emulsion droplets entrapping NADP and an ADH [59]	Enantioselective reduction of an acetophenone	0.09	0.017	90	5.4	300	59,204
Pickering emulsion droplets entrapping PLP and a TA [59]	Enantioselective amination of an acetophenone	0.023	0.008	88–95	9.0	400	1,600[c]
Self-assembling all-enzyme hydrogel of an ADH, GDH and retained NADP [64]	(*R*)-selective asymmetric reduction of 5-nitrononane-2,8-dione	0.002	0.010	60–90	0.15	30	14,000[c]
Crude enzyme- assembled hydrogel to retain NADP and an ADH [65]	Asymmetric reduction of acetophenone	0.04	0.020	50–93	0.09	12	4,800
Copolymer hydrogel for the retention of PLP and a TA [63]	Deamination of (S)-α-methylbenzylamine	0.004	0.002	72–88	1.13	240	18,500[b]

[a]Estimated average productivity of the process within the duration of the continuous operation

[b]Calculated based on the final product yield and cofactor loading

[c]Resulting from conditions optimized for TTN



Scheme 6 Asymmetric reduction of ketones by Peschke et al.'s NADP-dependent co-immobilization system. ADH performs the asymmetric reduction of the substrate, while GDH recycles the NADPH through the oxidation of D-glucose (**16**) into D-gluconic acid (**17**)

substrate can be reduced at either one or both of its carbonyl groups. Initial batch mode operations revealed mass transport limitations, which were relieved when the system was transitioned to continuous-flow condition. While the isolated ADH and GDH predominantly formed the mono-reduced hydroxyketone (**15**), the immobilized ADH-GDH system led to the full reduction of the diketone substrate (**14**) to the diol. This observation suggests that the retention of the intermediate **15** in the hydrogel allows the ADH to accomplish the reduction of the second carbonyl moiety. In other words, one may assume that the confinement of substrate, intermediate, cofactor and enzymes in the hydrogel reduces the apparent K_M value for the reduction of **15** by the ADH. Obviously, this feature can be an advantage or a disadvantage depending on the real target product; therefore, kinetics for the reduction of e.g., *meso*-diketones should be studied case by case. Nevertheless, hydrogels containing entrapped NADP demonstrated impressive performance, continuously converting the diketone for over 30 h, with effective retainment and recycling of the cofactor for over 124 column volumes at a flow rate of 10 µL min^{-1} (Table 4). Notably, the flow rate employed was substantially lower than when NADP was externally added (200 µL min^{-1}), indicating a crucial trade-off between productivity and cofactor retention. As an additional test of the hydrogel's efficiency in continuous-flow biocatalysis, the system was tested for the continuous production of (*R*)-configured alcohols from three other methyl ketone substrates. Encouragingly, all substrates underwent near quantitative conversion over an operational period of 10 h.

Chen et al. introduced an alternative enzyme-rich hydrogel strategy for the co-immobilization of enzymes and cofactors and application in continuous-flow. The use of low-cost crude enzyme preparations to create the hydrogel eliminates the need for possibly costly carrier materials [65]. In the crude enzyme-assembled hydrogels, crude enzymes form a porous architecture through interactions with 3-methyl-1,2,4-triazole (Hmtz) and magnesium ion chelating agents (Fig. 8). The rich presence of magnesium ions coupled with the abundance of hydrogen binding sites provided by Hmtz, facilitate the creation of a hydrogel that can anchor phosphorylated cofactors. Effective retention of the cofactors likely relied on a combination of various ionic and polar interaction sites within the hydrogel matrix. One remarkable aspect of this strategy is the in situ formation of the crude enzyme-assembled hydrogel within the microchannels of a continuous-flow reactor. Furthermore, immobilization of additional aliquots of enzymes and cofactor can be seamlessly integrated into the process, thus compensating for any potential loss of enzymes and cofactors during operation. Additionally, the hydrogel monolith can be effectively and gently eluted from the reactor by flowing an aqueous solution of sodium dodecyl sulfate.

To evaluate the feasibility of this co-immobilization method for biocatalysis, a crude ADH was utilized in combination with NADP. This setup facilitated the asymmetric reduction of a prochiral ketone with isopropyl alcohol (**11**) serving as the hydride donor for NADPH regeneration, akin to Scheme 4. Notably, both the continuous-flow process and the hydrogel state effectively mitigated product inhibition of the enzyme. Furthermore, the ADH displayed enhanced tolerance to the hydride donor isopropyl alcohol (**11**) when in the hydrogel state, which is advantageous when solubilization of hydrophobic products is necessary. It is crucial to highlight that maintaining a high activity in the crude enzyme-assembled hydrogel hinges on creating the appropriate microenvironment. The system's performance was very sensitive to the flow rate, with an optimal rate of 10 µL min^{-1}. Deviation from this rate results in a lower activity, attributed either to enzyme detachment or product-induced inhibitory effects. Over a 12-h operational period, the conversion remained consistently above 74%. Increasing the flow rate enhanced productivity, but at the cost of reduced conversion and an elevated risk of cofactor and enzyme lixiviation. Similarly, elevating the substrate concentration boosted the TTN of NADP although it led to a conversion rate of less than 50% after 12 h (Table 4). During extended operation, lixiviation of the NADP from the system was inevitable. However, a periodic microfluidic retention process was effective in reloading the cofactor into the hydrogel. While this approach substantially reduced cofactor consumption compared to continuous exogenous

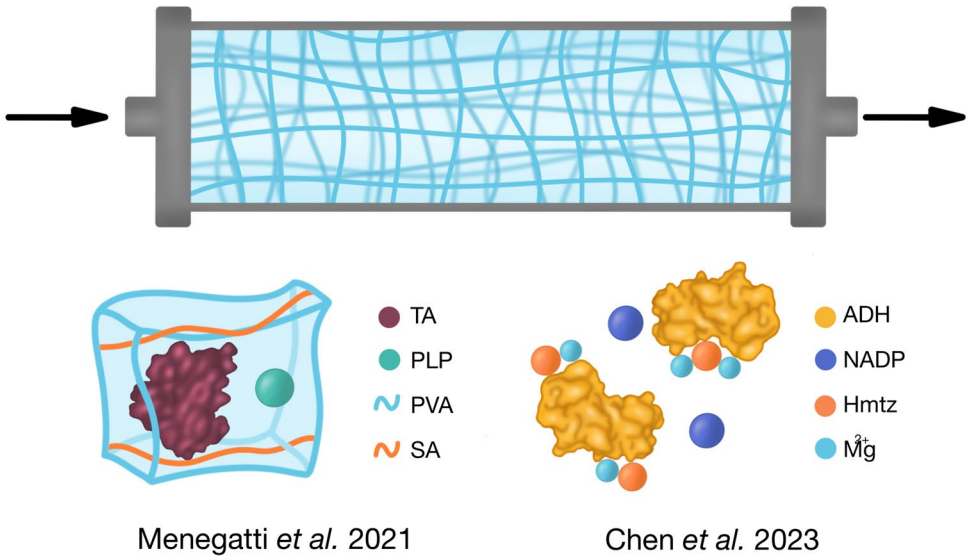


Fig. 8 Overview of the hydrogel cofactor entrapments methods by Menegatti et al. and Chen et al. The former method utilizes a polyvinyl alcohol (PVA) and sodium alginate (SA) copolymer hydrogel to immobilize a transaminase (TA) and PLP, while the latter creates a crude enzyme-assembled hydrogel with chelating agents 3-methyl-1,2,4-triazole (Hmtz) and magnesium ions to entrap an alcohol dehydrogenase (ADH) and NADP

cofactor addition, it also led to a decrease in the quantity of synthesized products.

Menegatti et al. presented an approach that diverges from the enzyme-based hydrogels by utilizing PVA and sodium alginate (SA) to form a copolymer hydrogel (Fig. 8) [63]. Theoretically, the copolymer matrix can accommodate a wide range of enzymes without the need for any modifications. This hydrogel demonstrated enhanced stability for a TA and PLP in terms of pH and temperature tolerance. This enhancement was primarily attributed to the presence of PLP during incubation, as the cofactor stabilizes the quaternary structure of the enzyme and preserves essential interactions between the cofactor and the active site. To assess the hydrogel's performance for continuous-flow biocatalysis, a continuous deamination was conducted as per the reverse reaction of Scheme 2. Unfortunately, the conditions tested necessitated extended residence times to achieve suitable conversion rates. Consequently, the optimal flow rate for long-term operation was set at 2 µL min^{-1} (Table 4). Nevertheless, the system demonstrated remarkable robustness during continuous operation, maintaining a conversion exceeding 72% over a period of 10 days and without PLP lixiviation. This high activity is further emphasized by the TTN of approximately 44,000 for PLP achieved during this reaction time window.

In conclusion, two main entrapment methods have emerged in continuous-flow biocatalysis: Pickering emulsion droplets and hydrogels. Wei et al. utilized Pickering emulsion droplets that offered a straightforward co-immobilization strategy, albeit with scalability concerns due to lower flow rates needed for droplet stability. Despite this, its versatility is evident through the successful operations of NADP- and PLP-dependent systems. Hydrogel-based approaches, like Peschke et al.'s all-enzyme self-assembling hydrogel and Chen et al.'s crude enzyme-assembled hydrogel, integrate enzymes and cofactors into the matrix, but face challenges in controlling enzymatic reactions and maintaining stability under high flow rates that can possibly limit the scalability. Despite these hurdles, enzymes entrapped into hydrogels exhibit remarkable TTNs for their cofactors. Further optimization must aim at unlocking their full potential for continuous-flow biocatalytic processes.

Hybrid approaches for cofactors immobilization

Hybrid approaches for cofactor immobilization have recently gained popularity and are now being applied in continuous-flow systems. Mi et al. have developed a hybrid approach that combines the entrapment properties of hydrogels with the cofactor adsorption capabilities of the cationic polymer PEI (Fig. 9) [66]. This strategy offers promising opportunities for enhancing continuous-flow biocatalysis as demonstrated through the co-immobilization of L-lysine DC and PLP for the continuous synthesis of cadaverine (Scheme 5). The His-tagged DC was co-immobilized alongside PLP within barium alginate (BA) hydrogel microspheres, aided by PEI. PEI's amine groups form reversible ionic and imine bonds with PLP, thus retaining the cofactor more effectively. Notably, the preparation of the system was simplified to a one-step purification and co-immobilization procedure by using immobilized metal-ion affinity chromatography, which additionally limits the enzyme inactivation during the separation process. During the optimization of the alginate hydrogel microspheres preparation, long-term stability emerged as the main concern due to the potential dissolution of the hydrogel microspheres that was driven by the release

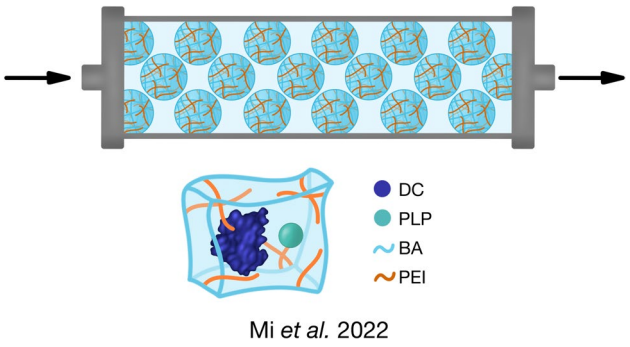


Fig. 9 Schematic depiction of Mi et al.'s hybrid approach for the immobilization of a decarboxylase (DC) and PLP in barium alginate (BA) hydrogel microspheres embedded with PEI

of divalent ions into the media. However, the researchers noted high stability for the barium-based alginate hydrogel, thereby circumventing this issue.

The co-immobilized L-lysine DC and PLP exhibited superior catalytic activity compared to the same reaction but reliant on exogenous cofactor supplementation. Furthermore, this co-immobilization approach enabled biocatalysis at higher substrate concentrations, improved overall system stability including wider temperature and pH ranges, resilience in the presence of organic solvents, and an extended storage period. Intriguingly, the introduction of PEI facilitated the adsorption of PLP as well as contributed to enhancing the reaction rates. This phenomenon was attributed to PEI's capability of stabilizing the enzyme's active conformation and improving the enzyme's proximity to PLP. To run the continuous synthesis of cadaverine (**13**), a microreactor was packed with the BA hydrogel microspheres containing co-immobilized L-lysine DC and PLP. Over an operational period of 6 h, a conversion rate above 80% was maintained (Table 5). The synthesis was performed at a flow rate of 0.07 ml min^{-1} with a substrate loading of 1 M, resulting in a high productivity of 3.8 mmol h^{-1}. The observed decline in conversion was attributed to the potential leaching of either the enzyme or the cofactor, although this feature was not specifically investigated.

The hybrid approach to cofactor immobilization, as demonstrated by Mi et al., undoubtedly holds potential for continuous-flow biocatalysis. Although this innovative strategy appeared promising in co-immobilizing L-lysine DC and PLP for the continuous synthesis of e.g., cadaverine, it is important to acknowledge concerns regarding the long-term stability and the limited range of conditions that are compatible with the BA hydrogel microspheres. While this initial work serves as a proof-of-concept for the hybrid co-immobilization approach, future efforts should focus on enhancing the catalytic stability to ensure improved viability and reliability in continuous-flow biocatalysis.

Conclusions and future perspectives

Enhanced sustainability and environmental friendliness of chemical transformations can be achieved through the development of biocatalytic processes with immobilized cofactors. The elimination of external cofactor supply can greatly reduce operational costs, simplify downstream processing, and improve the overall reactor performance. Over the years, numerous methods have been explored for cofactor immobilization, but adapting them to continuous-flow systems is still a challenge. The methods for cofactor immobilization that are amenable for continuous-flow biocatalysis were found to be covalent attachment, ionic adsorption, entrapment, and hybrid variations thereof. Notably, each of them showed its own advantages and limitations.

Covalent attachment methods offer a non-leaching and robust cofactor immobilization, but often lack versatility for different cofactors and require high stabilities for the enzymes. On the other hand, ionic adsorption is more versatile regarding cofactor compatibility, but the cofactor is susceptible to leaching in the presence of strong external ionic interactions and donor substrates that might be required for cofactor regeneration. Therefore, its applicability is limited to certain situations and requires careful consideration of the specific biocatalytic system. Entrapment methods, such as Pickering emulsion droplets and hydrogels,

Table 5 Metrics for continuous-flow biocatalytic reactions using enzymes and cofactor immobilized by hybrid approaches

Immobilization	Biotransformation	Productivity[a] (mmol h^{-1})	Flow rate (mL min^{-1})	Conversion (%)	Reactor volume (mL)	Reaction time (h)	TTN cofactor
PEI-infused BA hydrogel microspheres for the immobilization of PLP and a DC [66]	Decarboxylation of L-lysine to form cadaverine	3.8	0.07	80–99	10	6	n. c.[b]

[a]Estimated average productivity of the process within the duration of the continuous operation

[b]Not calculated due to unknown cofactor loading

have demonstrated promising stability and moderate conversion rates. Pickering emulsion droplets, in particular, offer high stability and compatibility with long-term operation but suffer from low flow rates to maintain droplet integrity. In contrast, hydrogels provide a more versatile platform with the possibility to eliminate carrier materials; this advantage comes at the expense of limited control over the reaction conditions due to substrate retention and low flow rates, which can also lead to cofactor leaching. Hybrid methods that combine two different immobilization approaches are promising, but also encounter challenges. For instance, alginate-based hydrogel beads have achieved high productivity through very high substrate concentrations, but face challenges related to cofactor and enzyme leaching.

The search for an effective immobilization method for cofactors in continuous-flow biocatalysis remains a complex and ongoing endeavor, as no single approach fits all scenarios due to the diversity of cofactors and reaction conditions. Moreover, the existing methods seem to suffer at a certain extent from a lack of reproducibility from one lab to another one, as the sole documented instances of replicating cofactor immobilization approaches were unsuccessful attempts (specifically, PEI- and DEAE-based ionic adsorption strategies). The current issues in reproducibility may stem from a lack of widespread applicability or well-defined and reported protocols for synthesizing and utilizing the cofactor immobilization systems. The generation of common guidelines within the flow-biocatalysis community for reporting these procedures will surely help to improve and facilitate reproducibility from lab to lab.

Future efforts should also focus on enhancing the stability of nanoparticles and hydrogel microspheres to withstand high flow rates and intense operating conditions. Novel materials and surface modifications may provide solutions to this challenge, enabling a more widespread adoption of entrapment methods, and possibly ionic adsorption approaches in continuous-flow biocatalysis. Further investigation into hybrid nanoparticle systems could additionally lead to the development of versatile immobilization strategies combining the advantages of multiple methods. These systems have the potential to address the limitations of individual approaches and provide tailored solutions for specific biocatalytic processes. Furthermore, the absence of cofactor leaching makes covalent attachment an interesting candidate for future developments in cofactor immobilization. Exploring new techniques, as exemplified by reversible covalent bonding that simplify the immobilization process and promote generality is essential. Identifying common attachment motifs among frequently used cofactors could open up opportunities for more standardized and widely applicable immobilization methods.

Besides the type of immobilization approach, it is also essential to consider the specific cofactors used in biocatalytic processes. Currently, NAD(P) and PLP are among the most commonly immobilized cofactors. However, to develop more universally applicable approaches, it is imperative to explore a broader spectrum of cofactors, especially others also possessing phosphate groups. For instance, cofactors like diffusible FAD [77], PAPS [78], and ATP [79] remain largely unexplored for immobilization, but have a high industrial potential and are expensive if supplied continuously in high amounts. Developing robust immobilization strategies for these cofactors could have a significant impact on the cost-effectiveness of biocatalytic processes. However, it is important to note that cofactor immobilization approaches are closely intertwined with enzyme immobilization techniques and cofactor-recycling systems, the latter of which must be simple and readily available.

While the pursuit for a general platform for co-immobilization of all cofactors and enzymes may remain an elusive goal, a pragmatic approach is to establish stable, robust, and thoroughly described methods for similar cofactors. Thus, minor adjustments might be needed to co-immobilized different enzymes. Consequently, the better integration of cofactor immobilization techniques into various biocatalytic processes would facilitate a widespread adoption of biocatalysis in flow and more broadly contribute to the final goal of sustainable and efficient chemical synthesis. In conclusion, the journey toward effective and versatile cofactor immobilization in continuous-flow biocatalysis is ongoing, but the field is ripe with opportunities for innovation. By addressing current limitations and exploring new frontiers, the biocatalysis community can contribute to the development of more efficient and sustainable enzymatic processes and bring us closer to the goal of eliminating the need for exogenous cofactors supplementation.

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Declarations

Conflict of interest The authors have no conflicts of interests to declare.

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