



Cite this: *Chem. Commun.*, 2021, 57, 11416

Received 6th August 2021,
Accepted 5th October 2021

DOI: 10.1039/d1cc04315c

rsc.li/chemcomm

Immobilisation and flow chemistry: tools for implementing biocatalysis

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The merger of enzyme immobilisation and flow chemistry has attracted the attention of the scientific community during recent years. Immobilisation enhances enzyme stability and enables recycling, flow chemistry allows process intensification. Their combination is desirable for the development of more efficient and environmentally friendly biocatalytic processes. In this feature article, we aim to point out important metrics for successful enzyme immobilisation and for reporting flow biocatalytic processes. Relevant examples of immobilised enzymes used in flow systems in organic, biphasic and aqueous systems are discussed. Finally, we describe recent developments to address the cofactor recycling hurdle.

Introduction

Most of the active pharmaceutical ingredients (API), natural products and fine chemicals are synthesised using (bio)chemical catalysts in large batch reactors. In recent years the utilisation of enzymes has facilitated the design of more environmentally friendly batch processes that fulfil 10 out of the 12 green

chemistry principles.^{1,2} However, mass transfer limitation, the generation of significant amounts of waste and handling of large volumes of toxic reagents are still problems that have to be overcome. Flow chemistry solves most of these challenges. In a continuous reactor the substrates are pumped through the reactor and the product is collected continuously. This set up improves mass transfer thus increasing reaction rates and reducing reaction time. The reduced reactor volume in flow transformations minimizes energy requirements for heating and cooling (green chemistry – principle 6) and it is also of great benefit to the reduction of waste (green chemistry – principle 1).^{3–6} Indeed, there is an increasing interest in microreactor technology for the synthesis of high added-value products and for the development of high throughput methods at industrial

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chiral synthesis of cyanohydrins in batch and continuous flow systems.

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scale and in academic research.⁷ In addition, the reduction in volume in continuous flow processes increases safety by avoiding handling of and thus potential exposure to large volumes of toxic compounds.^{8,9}

Soluble enzymes can be used for biotransformations in flow but reusability is difficult and the downstream processing needs to include a step for enzyme removal and its possible recycling. Immobilisation of enzymes allows straightforward reuse of the catalyst as it remains in the reactor. Moreover, in many cases increased operational stability is observed. This is an important contributor to the further development of flow chemistry.

With this feature article we aim to highlight important parameters to consider for a successful application of immobilised enzymes and for reporting continuous flow reactions. The latest applications in different reaction media will be discussed. In this context special attention will be paid to cofactors and their recycling in flow.

Challenges for biotransformations in flow with immobilised enzymes

Two main challenges have to be addressed in order to perform a successful biotransformation in flow: (i) immobilisation of the enzyme for recycling and straightforward downstream processing and (ii) suppressing the leaching of the enzyme and/or cofactor (if applicable) into the reaction medium during operation.

Overall, an enzyme can be immobilised by adsorption/deposition, ionic binding, covalent attachment to solid carrier materials, chemical cross linking or encapsulation. All of these methods have advantages and disadvantages that have to be evaluated case by case.^{10–15}

As mentioned above, enzyme and/or cofactor leaching are essential aspects that need to be addressed in flow systems.

Enzymes themselves or organic cofactors that remain within the enzyme active site and are fully regenerated during the catalytic cycle such as pyridoxal 5'-phosphate (PLP) or thiamin diphosphate pose a relatively small problem. Conversely, organic cofactors that are transiently fixed to the enzyme (*i.e.* nicotinamide cofactors) need to be regenerated to their given oxidative state before re-entering the enzyme. Thus, the development of an efficient cofactor regeneration system that gives freedom to the cofactor to leave the active site without losing it from the reactor is essential to allow the economic feasibility of the process for industrial applications. Also, the system must be flexible, allowing the implementation of reactions in cascade with a rapid exchange of substrates and avoiding chemical modifications of the cofactor.¹⁶ Here we do not discuss metal containing enzymes among the cofactor containing enzymes. All the aspects discussed for organic cofactors (and metal containing organic cofactors) equally apply to these enzymes. In this feature for instance *Granulicella tundricola* hydroxynitrile lyase (*GtHNL*) is a Mn^{2+} cofactor containing enzyme.

A number of successful cofactor recycling systems in flow have been reported, for instance by immobilising onto different carriers.^{17–22} The performance of immobilised enzymes and the different cofactor regeneration systems in flow will be discussed for organic, biphasic and aqueous conditions.

Metrics

In a recent review, key developments of continuous flow biocatalysis from 2018 to September 2020 were discussed.²³ It was found that the rise in the number of publications about this topic was not coupled to an increase in quality of reporting. Frequently, the productivity of the system as space-time-yield (STY) and the residence time were not given. This indicates that additional efforts must be made by the scientific community in



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A layer of enzyme solution not absorbed onto the carrier during the immobilisation step, might lead to loss of enzyme.²⁶

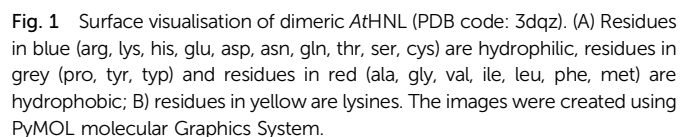
Leaching assay. Once the enzyme has been immobilised, a leaching assay needs to be performed under reaction conditions to evaluate the effectiveness of the immobilisation method and the robustness of the biocatalyst. When biotransformations are performed in organic solvents, desorption of the enzyme is generally avoided due to its insolubility in the reaction medium. A straightforward method to evaluate leaching under reaction conditions is to perform two reactions in parallel, after a certain time the immobilised enzyme is filtered off from one of the reactions. In heterogeneous catalysis, this is well established as “hot-filtration”.²⁷ The arrest of conversion immediately after removal of the immobilised enzyme demonstrates that the enzyme is not leaching from the carrier into the reaction medium under reaction conditions.

Ratio of lysine concentration to carrier functionalities (mol mol⁻¹). Commonly, a covalent immobilisation is achieved by the interaction of lysine residues on the enzyme surface with reactive aldehydes or epoxides of the carrier. For this reason it is essential to determine the number of surface exposed lysine residues in order to choose an appropriate enzyme to support ratio. For an enzyme of known structure the number of surface exposed lysine residues can be determined using a molecular visualisation software (Fig. 1B). With these straightforward calculations the covalent attachment is more likely to be successful.²⁵ The same applies to the carboxylic acid groups of aspartic acid or glutamic acid if they are the functional groups utilised for covalent immobilisation. Similarly, the ratio of a his-tagged enzyme (or any tag) to its counterpart (Fe, Zn, Co, Ni or other) on the carrier should be calculated.

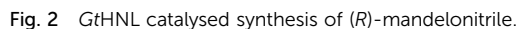
Ratio of carrier pore size to enzyme diameter. Immobilisation performed by adsorption/deposition requires that the enzyme diameter is smaller than the pores of the carrier even in the most unfavourable conformation. For this, transmission electron microscopy (TEM), nitrogen adsorption-desorption isotherms and mercury porosimetry are useful techniques to determine the pore size of the carrier.^{25,28,29} A high ratio of carrier pore size to enzyme diameter leads to high enzyme loading and minimises diffusion limitation.^{13,30,31} The distribution of enzyme molecules on a porous carrier can be modulated by the immobilisation rate. In general, higher immobilisation rates leads to undesired, more heterogeneous distributions.³²

Space-time-yield (STY). This parameter is frequently used to evaluate the productivity of different systems normalised to 1 liter volume ($\text{g h}^{-1} \text{L}^{-1}$). It describes the amount of product formed at a certain flow rate and reaction volume as shown in eqn (1):

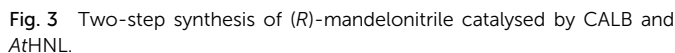
where $[P]$ is the concentration of product leaving the reactor in g L^{-1} , f is the flow rate in L h^{-1} and RV is the reaction volume in L . Since batch and continuous flow system setups have a completely



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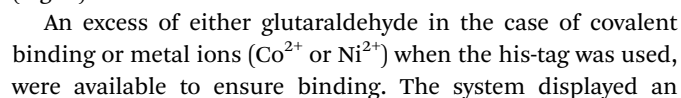
The same *At*HNL was also immobilised *via* the his-tag on the carrier EziG Opal.⁵² This is a controlled porosity glass carrier bearing Fe³⁺ on its surface. The availability of metal ions for the



In this case, a careful examination of the surface characteristics of the enzymes and carriers enabled the successful covalent attachment. As explained above (Fig. 1), the most relevant parameters of *MeHNL* and *HbHNL* such as diameter, hydrophilicity, number of surface exposed lysine residues and their position related to the active site entrance were obtained by analysing the



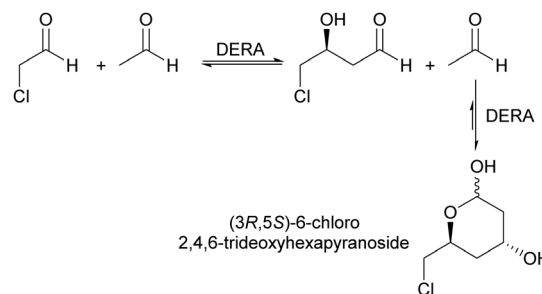
A comparison of batch and flow systems under biphasic conditions revealed the potential of the flow approach.⁵⁷ Lipase from *Thermomyces lanuginosus* (TLL) was covalently attached to Immobead 100 and used as catalyst (Fig. 5). In batch, 53% conversion and enantioselectivity $E = 52$ were achieved after 23 hours of reaction time. Alternatively, when the reaction was performed in flow in a packed bed reactor (250×4 mm) at a total flow rate of 0.5 mL min^{-1} (0.25 mL min^{-1} of substrate in heptane + 0.25 mL min^{-1} 0.1 M Glycine-NaOH buffer pH 9), 17% of conversion with slightly better enantioselectivity $E = 58$ were reached after only 5.5 minutes of residence time. In regard of productivity, the flow reactor displayed a STY of $28.2 \text{ mmol h}^{-1} \text{ L}^{-1}$ whereas the batch reactor yielded only



Recently an application of *MsAcT* for commercially relevant materials was reported.⁶² The successful immobilisation of *MsAcT* onto agarose (Fig. 7.) enabled improved STY in flow. Here, the goal was the synthesis of esters utilising exclusively natural substrates (obtained from nature or by biotechnological approaches). Thus, the natural but less reactive ethyl acetate was used as acyl donor instead of non-natural vinyl acetate. A drawback of performing the transesterification of alcohols and ethyl esters is the negative impact of ethanol on *MsAcT*.

Overall, several successful examples of biotransformations in flow using biphasic systems have been reported. The enhanced mass transfer commonly observed in flow, including segmented flow, helps to circumvent the diffusion limitation of biphasic batch reactions and enables higher substrate loadings as compared to aqueous systems.

As demonstrated above for organic solvents and biphasic mixtures immobilisation and continuous flow are two



The diagram illustrates a microfluidic system for the synthesis of ester products. At the top, a T-junction mixes an alcohol (R-OH) and an ester (R'-COOEt). The alcohol is shown as a mixture of five different compounds: 3-phenylprop-2-en-1-ol, 1-octanol, 2-methyl-6-octene-1-ol, 3-methylbutan-1-ol, and 2-phenylethan-1-ol. The ester is shown as R'-COOEt, where R' = H, Me, Et, Pr, i-Pr. The mixture then flows through a segmented flow section, indicated by a vertical tube with black circles representing the segmented flow. The tube is labeled "MsAcT on agarose". The final products are shown at the bottom, consisting of five different ester compounds: 3-phenylpropyl ester, octyl ester, 2-methyl-6-octyl ester, 3-methylbutyl ester, and 2-phenylethyl ester, along with ethanol (HO-CH₂-CH₃).

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Fig. 8 Continuous *Ec*DERA-C47M catalysed aldol reaction for the synthesis of (3*R*,5*S*)-6-chloro-2,4,6-trideoxyhexapyranoside in aqueous medium.

important techniques to consider for improved enzyme stability for aqueous systems, too. Recently, DERA was utilised in a continuous flow approach in aqueous medium for the coupling of acetaldehyde and its chloro-derivative (Fig. 8).⁷²

For this, lyophilised whole cells of *E. coli* BL21(DE3) expressing *E. coli* DERA-C47M, a variant more stable towards acetaldehyde,⁶⁸ were immobilised inside an alginate matrix by encapsulation and fibrous material obtained from the fruit of the Egyptian Luffa plant, commonly known as the luffa bathroom sponge, was used as support to increase the surface area. From the green chemistry perspective, alginate and luffa sponge are excellent materials for biocatalysis. They are non-toxic, renewable and biodegradable. An enzyme loading of 700 mg led to 80% of conversion of chloroacetaldehyde after *circa* 100 min at a flow rate of 0.1 mL min⁻¹ and the enzyme was stable for more than 5 hours of continuous reaction. No enzyme leaching occurred. The productivity of the system was reported as 4.5 g of product per day but unfortunately different enzyme loadings and substrate concentrations were used for the continuous and batch systems making a reliable comparison of the two systems impossible. This once again emphasised the importance of reporting all metrics. On the other hand the DERA reactor is part of a plug-and-play system in which reactors with different catalysts are combined. The power of this is demonstrated in the next example.

Dihydroxyacetone phosphate (DHAP) dependent aldolases require a much more complex reaction system than DERA, as the unstable DHAP needs to be generated *in situ*. This multi-step procedure of phosphorylation, aldol reaction and dephosphorylation lends itself ideally to the plug-and-play approach. While the modules for phosphate chemistry can remain the same, different aldolases can be plugged in.

The successful continuous flow synthesis of different carbohydrate analogues by immobilised *Shigella flexneri* acid phosphatase (*Sf*PhoN) and two aldolases (RAMA, rabbit muscle aldolase or RhuA from *Thermotoga maritima*) demonstrates this (Fig. 9).⁷³ The three step cascade reaction starts with the *Sf*PhoN to phosphorylate dihydroxyacetone (DHA). The resulting DHAP then, is converted by the desired aldolase, here either RAMA or RhuA, with different aldehydes and finally in the third step *Sf*PhoN dephosphorylates the aldol product yielding the desired carbohydrate analogue.

SfPhoN was immobilised on methacrylate polymeric beads whereas the immobilisation of RAMA and RhuA was performed on different epoxy carriers. The stability of soluble and immobilised RAMA was evaluated after 24 hour cycles in batch under reaction conditions. Soluble RAMA was unstable with a 50% decrease of conversion after 3 cycles and the enzyme was completely inactive after 5 cycles. Immobilisation demonstrated to be a suitable technique to improve enzyme stability. The best results were observed when RAMA was immobilised on Sepabeads EC-EP or Relyzyme EP403 (rigid methacrylic polymeric beads). The enzyme was fully active after 6 cycles. Remarkably, immobilisation completely suppressed the retroaldol reaction. This might be explained by internal diffusion limitation or a modification of the equilibrium of the reaction. RhuA was also immobilised on epoxy carriers. Complete binding and high activity were observed when RhuA was immobilised on Sepabeads EC-HA. Having established suitable carriers for immobilisation, the cascade reaction was performed in flow with packed bed reactors (Fig. 9). The synthesis of various aldol products in good yield was possible, however higher conversion was observed with RAMA. 68% conversion was observed for the coupling of DHAP to propanal during the first day but this dropped to 51% after 5 days. Higher conversion (80%) was observed for 4-pentenal during the first day, unfortunately the conversion decreased to 7% after 5 days. Finally, 70% of conversion was observed for *N*-alloc-3-aminopropanal, an important starting material for the synthesis of D-fagomine (antidiabetic piperidine iminosugar drug) during the first day with a decrease to 10% after 5 days. Due to the covalent immobilisation methods chosen no leaching occurred.⁷³

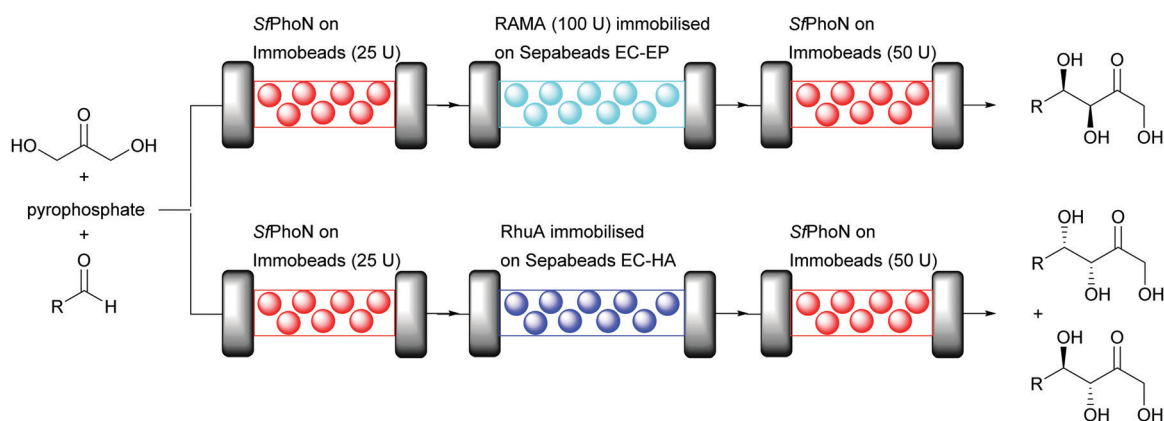


Fig. 9 Aldol cascade synthesis catalysed by *SfPhon* and either RAMA or RhuA in a plug-and-play system in aqueous medium.

Aqueous systems are the most widely used reaction media in biotransformations. Here enzymes normally display their highest activity. However, the poor solubility of apolar substrates and unwanted side reactions are the main drawbacks for a wider industrial use. Special attention must be taken to avoid leaching of immobilised enzymes into the reaction media. Here this was achieved by covalent linking or the use of whole cells that do not leach easily.

Addressing the cofactor hurdle

Today, enzymes are used as biocatalysts with good success at an industrial scale. Conversely, cofactor recycling remains a hurdle for industrial scale application of cofactor dependent enzymes. The main differentiation in the type of cofactor is:

- The cofactor is fully regenerated during the catalytic cycle and does not leave the active site; for example PLP or thiamin but also metal cofactors.

- The cofactor transiently binds to the enzyme, it has to leave the active site for regeneration. It requires a cofactor regeneration system very often involving a second enzyme and cofactor mobility is essential; NAD(P)H/NAD(P)⁺ are the prime examples.

The simplest system to approach the cofactor recycling hurdle is the use and immobilisation of whole cells as they have the metabolic pathways to regenerate their cofactors. Then, the problem is limited to avoid the leaching of the cofactor to the reaction medium. The use of organic solvents as reaction medium seems to be a good choice because of the often poor solubility of the cofactor in organic solvents.

Cofactors that are fully regenerated during the catalytic cycle

In line with the above the continuous flow synthesis of chiral amines by using a packed bed reactor and water saturated MTBE as reaction medium was reported.⁷⁴ For this, *E. coli* cells containing both ω -transaminases (ω -TA) and PLP were immobilised on methacrylate beads, most probably *via* hydrogen bonding between the peptidoglycan layer of the *E. coli* cell wall (containing amide, alcohol and ether functional groups) and the polymeric carrier. PLP is fully regenerated during the catalytic cycle of the enzyme within the enzyme, therefore a regeneration system is not required. The conversion of several non-natural ketones (from 67% to 94%) with excellent enantioselectivity (>99%) and residence times between 30 and 60 min without leaching of *E. coli* cells, ω -TA and PLP was achieved (Fig. 10). Importantly, no quenching or purification was required and the system was operated for 10 days for the synthesis of mexiletine, a drug used to treat abnormal heart rhythms, chronic pain, and some causes of muscle stiffness.

An essential breakthrough for cofactor application in flow was their attachment to ionic carriers.⁷⁵ Any ionic material that can act as counter ion to phosphate moieties present in most cofactors will transiently bind the ionic cofactors. When a buffer of low ionic strength is used as reaction medium the cofactor will remain on the carrier and will be available for the enzyme, even when it needs to be recycled outside the enzyme



Fig. 10 Stereoselective amine synthesis catalysed by immobilised *E. coli* cells and PLP on methacrylate beads.

active site. This principle was demonstrated with Flavin (FAD), PLP and also NAD⁺ (details discussed below).²¹ The enzymes were attached to porous carriers and polyethyleneimine (PEI), which is an amine, was the counter ion. For PLP and TA this was fully developed.⁷⁶ Purified ω -TA from *Halomonas elongata* (HeTA) and PLP were successfully co-immobilised on porous methacrylate carriers for the continuous synthesis of optically pure amines in aqueous conditions. The beads were functionalised with different reactive groups (cobalt-chelates, epoxides and positively charged amines such as PEI) to allow the electrostatic interaction with his-tagged ω -TAs and PLP. The continuous enantioselective deamination of α -methylbenzyl amine gave >90% conversion for up to 50 column volumes at 1.45 mL min⁻¹ without leaching of the cofactor to the reaction medium. The synthesis reaction of cinnamylamine required a doubling of the reaction time. After an initial decrease in activity it remained stable at 60% for at least 20 column volumes; equiv. to 40 min (Fig. 11). The initial loss of activity might be due to some PLP leaching, induced by the amine donor.

An intriguing covalent immobilisation of the FAD containing phenylacetone monooxygenase (PAMO) *via* its cofactor was described. The cofactor was attached to agarose *via* a tether and then the apo enzyme from *Thermobifida fusca* was added.⁷⁷ The immobilised enzyme displayed similar activity as compared to its free form but higher thermostability after 1 h of incubation at 60 °C. However, a recyclability study showed low enzyme stability with a decrease of *circa* 40% after 3 cycles.

Cofactors that do not require a recycling system and remain in the active site can readily be used in flow systems. Both organic solvents that suppress solubility or aqueous systems with ionic carriers at low buffer concentrations prevent leaching of enzyme and cofactor.

Cofactors that require recycling systems

The application of pure enzymes and cofactors reduces side reactions and it is therefore also preferred in systems which



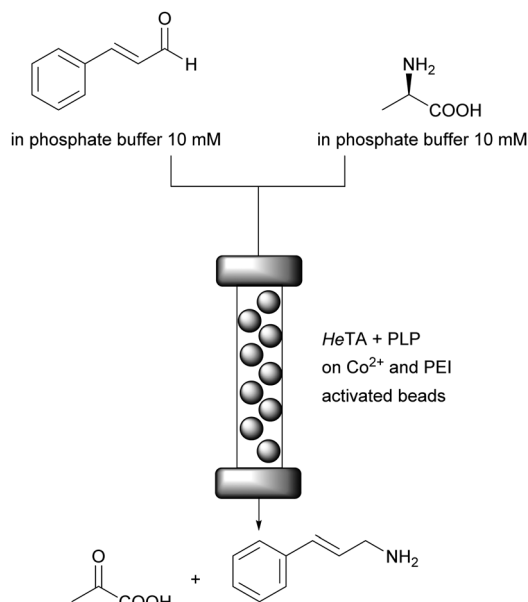


Fig. 11 Synthesis of cinnamylamine in continuous flow. Enzyme and PLP are immobilised *via* ionic interactions. The HeTA *via* Co^{2+} on the carrier and a his-tag, the PLP *via* PEI attached to the carrier.

apply cofactors that need to be recycled. The above mentioned immobilisation *via* ionic interactions was of equal success here.²¹ Commercial porous carriers were coated with PEI to allow the co-immobilisation of enzymes and phosphorylated cofactors such as NAD^+ . The cofactor adsorption is dynamic and allows to establish an association–dissociation equilibrium without leaving the porous carrier. It thus is available for the enzyme performing the desired reaction, here alcohol dehydrogenase from *Thermus thermophilus* (*Tt*ADH2) and the enzyme required for cofactor recycling, here formate dehydrogenase from *Candida boidinii* (*Cb*FDH). The two enzymes and the cofactor were co-immobilised on an anionic exchanger and tested in the continuous asymmetric reduction of 2,2,2-trifluoro-1-phenylethan-1-one (Scheme 2A). Full conversion with a productivity of $250 \mu\text{M min}^{-1}$ and a TTN of 85 for immobilised NAD^+ after 107 hours on stream in continuous flow with less than 10% NAD^+ loss were achieved.

The system was further improved by applying a commercial ADH that can accept isopropanol as co-substrate. This makes the second enzyme redundant and the cofactor does not have to leave the active site.²² Enzyme and NADPH were co-immobilised on porous agarose beads coated with PEI. The system displayed STYs between 97 and $112 \text{ g L}^{-1} \text{ day}^{-1}$ for a range of ketones and the immobilised cofactor reached a TTN of 1076 for 120 hours. During this time, neither the enzyme nor the cofactor were inactivated or leached (Scheme 2B).

This can directly be compared to a recent,¹⁶ successful NADPH cofactor regeneration system for the synthesis of chiral alcohols based on a membrane liquid/liquid extractor for continuous flow. The cofactor remained in the aqueous layer and was recycled (Fig. 12).

The organic phase was added after the reaction mixture passed through the immobilised enzyme. This regeneration



Scheme 2 (A) Asymmetric reduction of 2,2,2-trifluoro-1-phenylethan-1-one catalysed by *Tt*ADH2 with external cofactor recycling by *Cb*FDH; (B) asymmetric reduction of ketones with internal cofactor recycling.

system without any chemical modification of the cofactor enabled the reduction of four different ketones with STYs from $14 \text{ g L}^{-1} \text{ h}^{-1}$ to $117 \text{ g L}^{-1} \text{ h}^{-1}$, cofactor turnover numbers ranging from 128 to $2023 \text{ mol mol}^{-1}$ and excellent enantioselectivity ($>99\%$). The reliability and robustness of the system was demonstrated with the continuous synthesis of ethyl (*S*)-4-chloro-3-hydroxybutanoate over 32 hours without any loss in performance displaying a STY of $121 \text{ g L}^{-1} \text{ h}^{-1}$. A longer run (123 h) exhibited an astonishing cofactor turnover number of $12855 \text{ mol mol}^{-1}$ which represents a step forward compared to previous reports.^{17–19}

Amine dehydrogenases (AmDH) enable the synthesis of chiral amines from cheap ammonium salts as amine donors.



Fig. 12 Synthesis of chiral alcohols catalysed by immobilised *Lb*ADH with cofactor recycling rather than immobilisation.

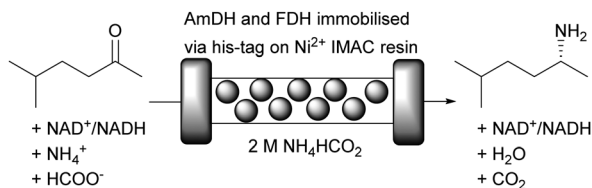


Fig. 13 Reductive amination of 5-methyl-2-hexanone with cofactor regeneration catalysed by immobilised AmDH and immobilised FDH in aqueous ammonium formate buffer. Ratio ketone to NAD⁺ 10:1.

Unfortunately, this reaction is commonly hampered by substrate and product inhibition. Immobilisation and continuous flow helps to address these problems, as was recently shown.²⁰ The asymmetric reductive amination of 5-methyl-2-hexanone was achieved by co-immobilisation of an amine dehydrogenase (AmDH) and FDH onto Nuvia IMAC (immobilised metal affinity chromatography) resin from BioRad (Fig. 13). By immobilising both his-tagged enzymes to Ni^{2+} on the surface of the carrier, the synthesis of (*R*)-5-methyl-2-amino-hexane in a packed bed reactor became possible. This setup displayed a STY of $107 \text{ g L}^{-1} \text{ day}^{-1}$ at 48% of conversion moving up to $443 \text{ g L}^{-1} \text{ day}^{-1}$ at 24% of conversion. Remarkably the flow system remained operational for more than 120 hours.

Earlier the continuous synthesis of 4-fluoroamphetamine under aqueous conditions catalysed by co-immobilised AmDH and FDH was reported.⁷⁸ EziG Amber, a controlled porosity glass carrier with Fe³⁺ on the surface for his-tag binding was used in a packed bed reactor. This system displayed a STY in the same range (300 g L⁻¹ day⁻¹), however a rapid enzyme deactivation was observed after 6 hours. In both examples low cofactor concentrations could be used and it was recycled during the reaction. Nonetheless, the cofactor was not immobilised and thus finally lost.

Recently, the cofactor regeneration problem in flow was beautifully addressed by applying an enzyme engineering approach.¹⁷ A three step cascade was employed for the conversion of glycerol to a precursor of D-fagomine (Fig. 14). In each step one enzyme reaction is performed by multi-enzyme modules.

The modules consist of the catalytically active enzyme, if required the recycling enzyme and an esterase (Est) that reacts with the carrier to covalently immobilise the multi-enzyme modules. Thus, two three-enzyme and one two-enzyme modules are covalently attached to the carriers, each combination in a separate reactor. The two cofactors (ATP and NAD⁺) required were functionalised for the covalent attachment to the multi-enzymes modules with a long PEG-linker at the adenine. This linker allows the movement of the cofactor between the catalytic and cofactor recycling enzymes of the respective module. Subsequently, each cofactor was tethered to the protein linker between the catalytic and the recycling enzyme *via* an accessible thiol group. The key distinction of this set up is that the cofactor was immobilised to a specific amino acid close to catalytic and recycling enzymes whereas in previous reports the linkage was not specific.^{79,80}

First, glycerol was phosphorylated to glycerol-3-phosphate by *Thermococcus kodakarensis* glycerol kinase (*TkGlpK*) and for the ATP regeneration *Mycobacterium smegmatis* acetate kinase (*MsAceK*) was used. Secondly, *E. coli* glycerol-3-phosphate dehydrogenase (*EcG3PD*) performed the oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate (DHAP) and the cofactor NAD^+ was regenerated by an NADH oxidase from *Clostridium aminovalericum* (*CaNOX*). Finally, an aldol reaction catalysed by a monomeric FruA from *Staphylococcus carnosus* produced the desired product, precursor of D-fagomine, with excellent STY ($70 \text{ g L}^{-1} \text{ h}^{-1} \text{ g}^{-1}$) and astonishing high cofactor turnovers (16 848 for ATP and 10 839 for NAD^+).

Cofactor dependent enzymes are important biocatalysts for synthesis. When the cofactor is regenerated during the catalytic cycle such as PLP or FAD the main concern is to avoid leaching of the cofactor to the reaction media. The use of organic solvents has been reported as an important tool to circumvent this limitation. When the cofactor is not regenerated during the catalytic cycle, the implementation of a cofactor recycling system is required. For this co-immobilisation of enzymes and cofactor is an attractive approach. However, the efficiency of this recycling system is limited ($TTN_{NADPH} = 1076$ and $TTN_{NAD^+} = 85$).^{21,22} Higher cofactor recycling efficiency has

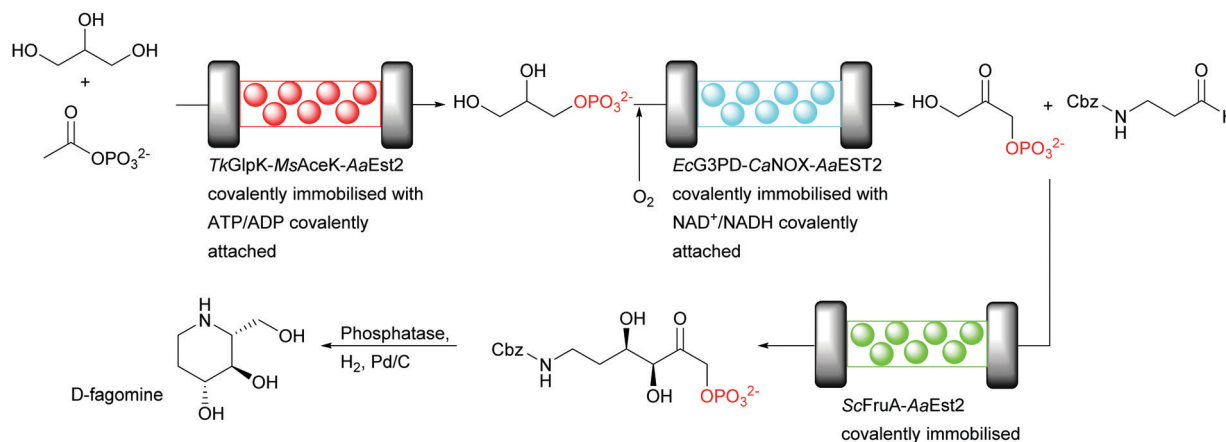


Fig. 14 Cascade synthesis of D-fagomine in continuous flow in aqueous medium. The cofactors were covalently attached to the catalysts *via* a specific thiol group. The modules of two or three enzymes are attached to the carrier *via* a covalent linker between AaEST2 and the carrier.

been reported by using membrane technology ($TTN_{NADPH} = 12855$).¹⁶ Nevertheless, the high cost of ultra or nanofiltration technologies impair their economic viability. Finally, the immobilisation of cofactors of either type using a protein engineering approach demonstrated to be as efficient as the use of membrane technology reaching a $TTN_{ATP} = 16\,848$ and $TTN_{NAD^+} = 10\,839$.¹⁷

Conclusions

Immobilisation and flow chemistry are important tools for the further development of biocatalysis. Process intensification, better control of the processes, reduced reactor volumes and therefore increased safety are advantages commonly reported in organic, biphasic and aqueous systems. To fully appreciate the advantages and to also probe them rigorously solid metrics are essential.

Similarly, large steps have been made to address the cofactor recycling challenge in flow. Co-immobilisation of enzymes and cofactors, membrane based separation and protein engineering techniques have allowed the development of efficient regeneration systems for challenging cascade reactions with cofactor dependent enzymes. Pronounced progress to answer the two challenges, enzyme immobilisation and prevention of leaching of enzyme or cofactor (including metals) during the flow process, have been made. Overall, the implementation of enzyme immobilisation and flow chemistry allow for more efficient, safe and thus green and environmentally friendly processes.

Author contributions

U. H. conceptualised, supervised and edited the manuscript; P.-L. H. and Y. G. edited the manuscript. J. C. wrote and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This research was funded by the Secretary of Higher Education, Science, Technology and Innovation of Ecuador (Senescyt) and Universidad Laica Eloy Alfaro de Manabí (ULEAM).

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