

Biocatalysis and Flow Chemistry: Artificial Cell Factories

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Abstract: Our research focusses on highly sustainable enzymatic methods for the preparation of valuable molecules, spanning from pharmaceuticals, to small chiral intermediates, to flavours and perfumes. Specifically, we aim at developing strategies which will rapidly bridge the gap between academic discovery and industrial implementation. The use of enzymes in industrial processes is becoming more prominent and there is a need to combine the advantages of biocatalysis with high productivity to make it truly attractive. We have been among the pioneers of a new wave of research in the field of flow biocatalysis: whole cells expressing biocatalysts and cell-free systems have been developed by us and others in continuous systems for the preparation of valuable products. Continuous flow biocatalysis is the state of the art in continuous processing and is showing new exceptional properties of enzymes specially for what concerns their efficiency and long-lasting reusability. Here we report on the recent progress in the field by our research group.

Keywords: Biocatalysis · Biotransformations · Continuous flow synthesis · Green chemistry



Prof. Francesca Paradisi is the Chair of Pharmaceutical and Bioorganic Chemistry at the University of Bern since 2019. She holds an MSc and a PhD in Chemistry from the University of Bologna and moved towards biocatalysis during her post doc at University College Dublin with Prof. Engel. She became an academic in 2006 at UCD and moved to the University of Nottingham, UK, in 2016. Biocatalysis as a sustainable

approach to synthesis of valuable products is the focus of her research group. In particular, the group developed a number of enzyme-based processes in continuous flow, reducing the gap between academic discovery and industrial application.

The use of enzymes dates back to middle 1800s and since then, the evolution of enzymatic processes has been applied in several industrial settings, especially those involved in food manufacturing and in the development of bio-detergents. More recently enzymes have been utilized for the production of fine chemicals, agrochemicals, pharmaceuticals, and cosmetics.^[1]

The rapid progress in enzyme engineering, which saw the recognition of Prof. Frances Arnold with the Nobel prize in Chemistry in 2018, has provided exceptional tools which allow the precise design of enzymes so that they are optimal for a given process. Effectively all enzyme classes have been exploited in very diverse processes and applications, and their modification through directed evolution and rational design, combined with efficient screening methods (often employing robotics for high throughput) have been reported, enabling unprecedented chemistry as well as reaction conditions for biological macromolecules.

While the food industry can claim several examples of enzymatic processes,^[2] the application of enzymes in the pharmaceutical industry has been slower, but it is rapidly adjusting and incorporating more enzymatic steps in their strategies. Codexis and Merck teamed up just over a decade ago and jointly developed

one of the first and most remarkable examples of the application of a biocatalyst in industry. The amino transaminase (ATA-117) was heavily engineered by a combination of rational design and directed evolution, generating final variant with 27 mutations, which has been introduced in a key synthetic step for the preparation of the blockbuster drug Sitagliptin.^[3] Selective biocatalysis is now becoming a well-accepted alternative in the synthesis of fine chemicals and progress in enzyme-mediated catalysis now includes not only specialty chemicals, but also polymers, and some bulk chemicals.

In parallel, progress in the understanding of how reactions can be carried out in more efficient manner, has shifted the technical set-up from conventional batch reactors to alternative solutions.^[4] Among them, flow chemistry represents a leap in increasing the safety of a chemical process, as well as its productivity and the overall economic benefits. In the last few years, not surprisingly, new reports on flow processes have grown exponentially.^[5]

More recently, ground-breaking flow-bioreactions have combined the catalytic power of enzymes with the high-flow-chemistry's throughput. The merging of these two modern approaches in a single and multi-enzymatic reaction processes simulates the extremely efficient metabolic pathways of cells.^[6]

Biocatalyst Immobilisation

While both free and immobilised forms of the biocatalysts have been reported for flow systems, those where the catalyst (enzyme) is immobilised are more commonly used. In these conditions, a heterogeneous system is generated where the enzyme retains sufficient flexibility to undergo the conformational changes essential for the catalytic activity to still take place.^[7]

A number of strategies have been described for the immobilisation of whole cell and cell-free enzymes on a variety of supports. Both systems present advantages and disadvantages, but in our research group we favour working with cell-free enzymes as we avoid the possible issues of permeability and stability of the cell wall, as well as the competing cellular metabolism which may reduce the efficiency of the target biotransformation(s).^[8]

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Unlike whole cell systems, cell-free approaches also enable the use of variable amounts of a catalyst so that in a multi-enzyme cascade, each individual step can be optimised for the rate of that specific biotransformation allowing optimal ‘tuning’ of the process. It has been shown that immobilisation of enzymes enhances significantly their stability, though often, especially if the immobilisation is covalent, at the expense of their catalytic efficiency.^[9] In our laboratory, we have adopted a range of techniques for enzyme immobilisation which we have then exploited in packed-bed reactors for continuous biotransformations. One of our most representative examples is the immobilisation of the transaminases from the haloadapted bacterium *Halomonas elongata* (HeWT). This enzyme had shown a remarkable substrate scope and stability when used in solution.^[10] For its immobilisation, we adapted the strategy reported by Guisan and co-workers in 2001, where the poly-His-tag, which is very commonly fused to enzymes and proteins for ease of purification, selectively interacts with an epoxy-resin following metal derivatisation.^[11] These resins are commercially available, and Fig. 1 below shows schematically the chemistry involved to covalently bind an enzyme.

The tunability of this immobilisation protocol is outstanding, the metal can be selected to reduce toxicity (if any) on the catalyst, and the contact time between the enzyme and the resin can also be varied before adding the capping agent so that the number of covalent bonds can be reduced or increased (with an effect on the stability and/or distortion of the quaternary structure of the enzyme). As mentioned, the key element in this strategy is the presence of a poly-His-tag, which is routinely used to purify proteins on metal-affinity chromatography. Therefore, a crude cell extract can also be used in this process, combining in a single step both the purification and the consequent selective immobilisation of the target catalyst on the resin. While a range of supports can be used (changing for example the hydrophilic or hydrophobic nature of the matrix) in our laboratory we tend to prefer when possible non-swelling resins, as they are better suited to flow processes which may involve segmented flow (alternating aqueous and organic mobile phases) because the back pressure is minimised and their volume does not change.

Fig 1. Chemistry on epoxy-resin to enable enzyme immobilisation.

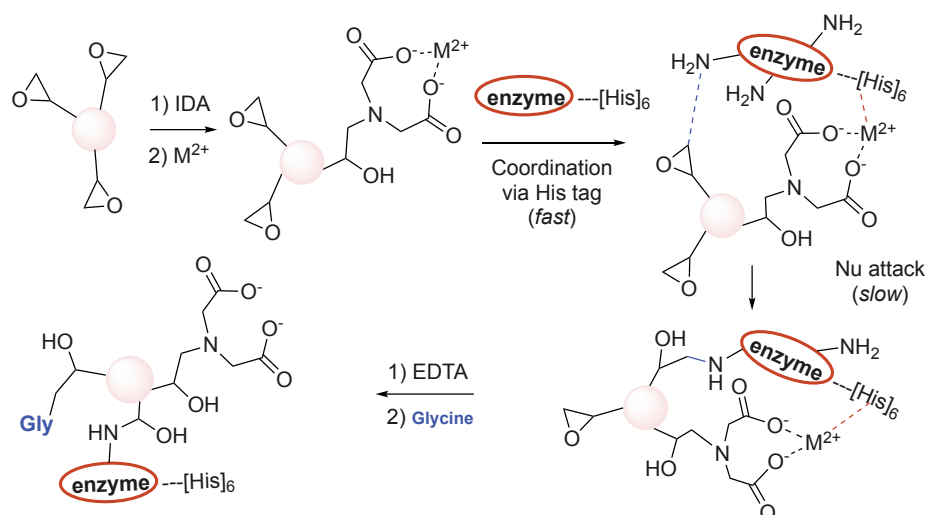
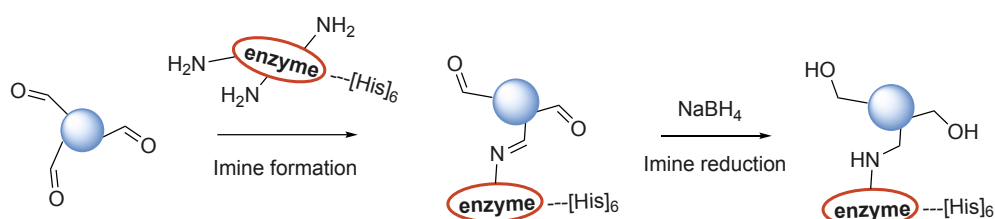


Fig 2. General scheme for the immobilisation of enzymes on aldehyde-functionalised supports.



However, neither the chemistry nor the support is universally ideal, and for every new enzyme we carry out a range of trials to identify the best support and immobilisation chemistry for that specific biocatalyst. For example, an acyl transferase from *Mycobacterium smegmatis* (MsAcT) showed better recovered activity and stability on hydrophilic activated glyoxyl agarose beads via imine formation and reduction (Fig. 2).^[12]

While more challenging, more than one enzyme can also be immobilised on the same bead. This is particularly useful when the catalyst is cofactor-dependent and a recycling system is needed for the sustainability of the system. We have previously used mix-bed approaches, where an enzyme pair was immobilised on separate resins which were then mixed in the correct proportions in the bioreactor,^[13] however, tailored chemical modification of the surface of the bead will permit sequential immobilisation steps were different type of interactions can be exploited (for example covalent and ionic). In one of our most recent works, we have successfully co-immobilised a lysine dehydrogenase with a pyrroline-5-carboxylate reductase for the synthesis of pipercolic acid. As the enzymes share the cofactor, it was also possible to co-immobilise the NAD⁺ with an additional step (Fig. 3).^[14]

A significant amount of research goes into successful optimisation of the immobilisation strategy, and we have now collected a wealth of information which could be compiled in a searchable database to guide future immobilisations. We are currently working on devising such databases where we look at structural features of the enzymes and how they correlate with immobilisation performance based on the chemistry and the support selected. We believe this will be very useful as additional data are generated by us as well as colleagues working in this field.

Biocatalysis in Continuous Flow

The compartmentalisation of the immobilised enzyme into a reactor (packed-bed reactor) permits a high amount of catalyst to be accumulated in a small space where a substrate is fed through in a controlled manner.

In addition, mass transfer has been shown to be much more efficient in a flow system with respect to batch conditions, allow-

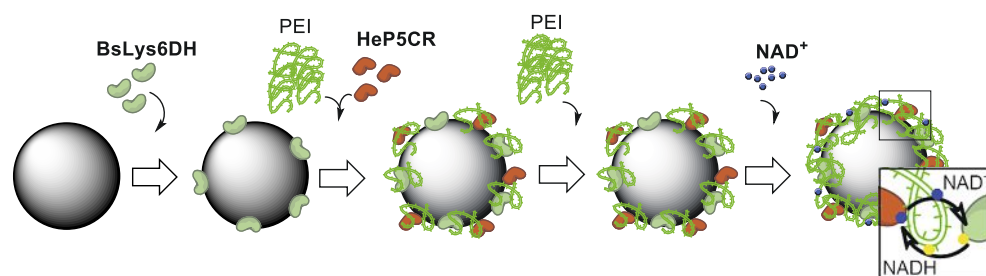


Fig 3. General scheme for the co-immobilisation of enzymes and cofactors.

ing a faster bioconversion which routinely outperforms one-pot set ups, validated also for standard chemical reactions carried out in continuous flow.^[15] Our first example of biocatalysis in continuous flow was the synthesis of amines mediated by *HeWT* which was assembled to include an in-line purification step to enable the recovery of the final product (see the scheme depicted in Fig. 4). With this system we achieved excellent yields in very short reaction times (with respect to batch mode) with a system which was designed with sustainability in mind.^[16]

The reaction could also be done in the reverse direction generating a series of aldehydes from amines.^[17]

The requirement of a cofactor, which several enzymes have (such as all redox systems for example), adds an extra layer of complication as a recycling system normally needs to be implemented as well as a method to avoid loss of such cofactor(s) in the downstream waste. While in PLP-dependent transaminase reactions the cofactor (PLP) is bound in the active site of the enzyme, this is routinely added to the reaction mix in case of leaching during the catalytic cycle. In collaboration with Dr. Lopez-Gallego (San Sebastian, Spain), we have also worked on the co-immobilisation of PLP and *HeWT* which has led to the assembly of fully self-sufficient systems which performed very well in continuous flow.^[18,19]

As our knowledge in the field progressed, we increased the complexity of the cascade, adding a second module in the assembly. Here, the amine to aldehyde/ketone catalysis was followed by a second reactor to perform the reduction to alcohol. This work

included an extensive range of substrates, combining *in situ* cofactor regeneration (in some cases including a coupled enzymatic system) with in-line work-up, but possibly the most advanced element was the achievement of a fully sustainable system where the waste waters were partially purified and recycled (Fig. 5).^[13]

More recently, to demonstrate the translational power of the flow technology, we moved away from the analytical scale and developed a continuous multi-gram synthesis of melatonin (to date exclusively produced synthetically), *via* direct acylation of 5-methoxy-tryptamine mediated by *MsAcT*. A small 2 mL reactor packed with less than 2 mg of immobilized enzyme could handle starting material at 0.5 M concentration (95g/L) with a productivity up to 36g/day (5 min residence time). Again, both the aqueous phase and the solvent could be recovered and reused (Fig. 6).^[12]

The same system was used also for the scaled-up production of flavour esters, again with excellent yields and reaction times.^[20]

Outlook

The progress in our understanding of enzymatic systems and their potential, together with a worldwide target to increase sustainability and reduce waste and emissions, has established a new *status quo* where biocatalysis is becoming a strong ally in industrial processing, with an array of alternative solutions now available to both chemists and engineers. The use of flow biocatalysis is increasing, generating enormous interest in academia and industry because it can dramatically reduce costs of chemical transformations with a very small footprint when it comes to equipment.

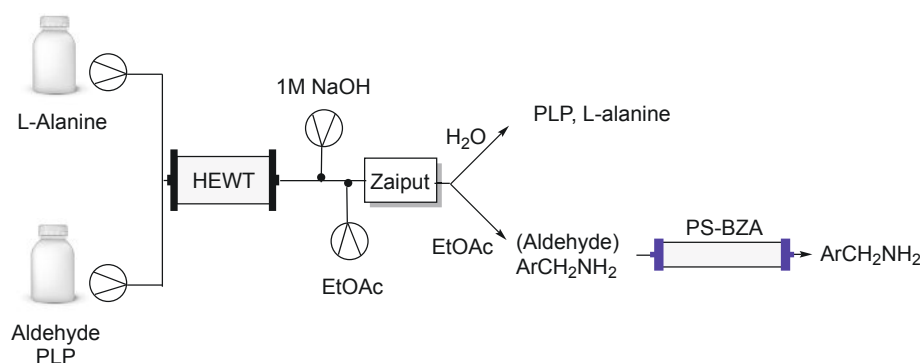


Fig 4. Assembly of a flow reactor for the synthesis of amines from aldehydes (Solution A: amino acceptor and PLP cofactor; Solution B: alanine solution). NaOH and EtOAc inlets downstream enable extraction. Zaiput membrane separator system splits aqueous and organic layers. PS-BZA column traps the eventually unreacted aldehyde.

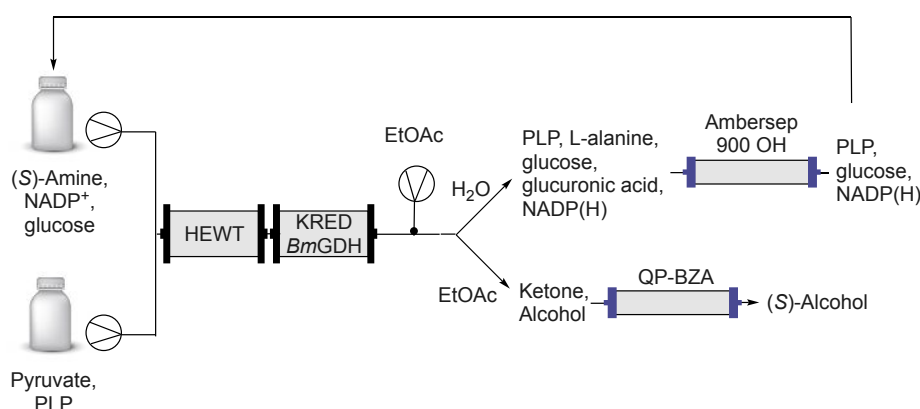
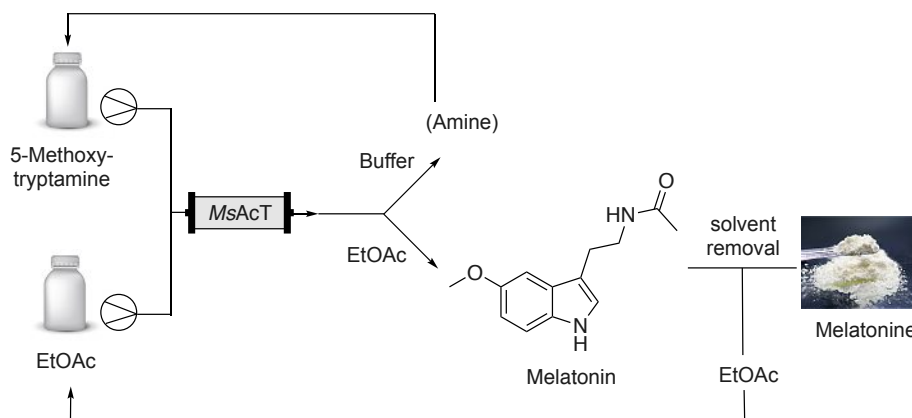


Fig 5. Schematic assembly of the flow reaction for the synthesis of chiral alcohols from chiral amines with recycle and reuse of the waste waters.

Fig 6. Schematic assembly of the flow reaction for the synthesis of melatonin.



Several projects are underway in our laboratory where different enzymes are being combined specifically to synthesise molecules of interest on larger scale. It has to be acknowledged, however, that flow is not always applicable, it must be kept in mind that this is an option, often a very good option, but not a must. Likewise, we are not limiting our interests to pure biocatalytic approaches, in fact working towards the development of biocompatible systems combining multi-step syntheses of traditional organic chemistry and enzyme-mediated reactions, will only strengthen and accelerate the uptake of biocatalysis in industry.

Received: July 9, 2020

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